Universidade de Aveiro
Departamento de Química
Ano 2019

Sónia de Jesus Amarante

Otimização da extração de florotaninos e avaliação das propriedades antioxidantes e anti-inflamatórias da alga *Fucus vesiculosus*

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Susana Maria de Almeida Cardoso, investigadora Doutorada do Departamento de Química da Universidade de Aveiro, e da Doutora Rita Maria Pinho Ferreira, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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"*All our dreams can come true… if we have the courage to pursue them*."

– Walt Disney

Palavras-chave

Algas castanhas; florotaninos; extração assistida por microondas de florotaninos; antioxidante; anti-inflamatória.

Resumo

Os florotaninos, uma classe de polifenóis exclusiva das algas castanhas, demonstraram e têm sido associados a numerosas propriedades benéficas para a saúde. Tendo isto em consideração, esta dissertação de mestrado teve como objetivo a otimização do processo de extração de florotaninos da alga *Fucus vesiculosus* através do método de microondas usando um solvente "verde", como o etanol, bem como caracterizar os constituintes dos extratos obtidos e perceber as suas potencialidades biológicas. A otimização do processo de extração por microondas foi realizada através da análise do fator único seguido da análise por Box-Behnken, tendo em vista a recuperação máxima total de florotaninos, tendo esta resultado num valor máximo de 3,16 ± 0,17 mg equivalentes de floroglucinol/g peso seco$_{alga}$. O extrato bruto obtido foi submetido a partição líquido-líquido e as frações resultantes, *n*-hexano (Hex), acetato de etilo (EtOAc) e aquosa (Aq), foram caracterizadas por UHPLC-DAD-ESI/MS. Quer o extrato bruto quer as frações purificadas foram rastreadas em relação às suas capacidades antioxidante através de métodos químicos. Adicionalmente, as frações com melhor capacidade antioxidante, nomeadamente, EtOAc e Hex, foram testadas em macrófagos RAW 267.4 estimulados com lipopolissacarídeo.

A análise química das frações purificadas permitiu identificar alguns florotaninos principalmente na fração EtOAc e Aq. Por outro lado, a fração de Hex era rica em fucoxantina, estando esta também, juntamente com outros pigmentos, presente na fração EtOAc. No global, as frações EtOAc e Hex demostraram ser as mais eficazes relativamente à capacidade antiradical, e em especial, contra o radical de óxido nitrico. Esta duas frações reduziram também significativamente os níveis de nitritos produzidos pelos macrófagos ativados, sem causar decréscimo no seu metabolismo celular. Ainda, ambas provocaram uma redução drásticas na expressão de iNOS e, em particular a fração de EtOAc, induziu um decréscimo da expressão da COX-2. É possivel que estes factos estejam relacionados com a regulação do fator transcricional NF-kB, uma vez que foi observado uma diminuição do teor da subunidade p65 nos lisados celulares dos macrófagos pré-tratados, acompanhado pela inibição da fosforilação do *p*-IkB-α. Em conclusão, os resultados da presente dissertação sugerem que as frações EtOAc e Hex de *F. vesiculosus* possuem atividades antioxidantes e anti-inflamatórias relevantes.

Keywords

Brown seaweeds; phlorotannins; microwave assisted-extraction of phlorotannins; antioxidant; anti-inflammatory.

Abstract

Phlorotannins, a class of polyphenols exclusive to brown seaweeds, have been associated with numerous health-promoting properties. In this, the objective of the master's thesis was to optimize the extration process of phlorotannins from the seaweed *Fucus vesiculosus,* using microwave-assisted extraction methodology and a green solvent, such as ethanol, as well as to characterize the constituents of the obtained extracts and to realize its biological potentialities. The optimization of the microwave extraction process was performed by single-factor analysis followed by Box-Behnken analysis, considering the maximum recovery of phlorotannins, yielding a maximum of 3.16 ± 0.17 mg phloroglucinol equivalents/g dry weight$_{algae}$. The crude extract obtained was partitioned and the resulting fractions, *n*-hexane (Hex), ethyl acetate (EtOAc) and aqueous (Aq), were characterized by UHPLC-DAD-ESI/MS. Both crude extract and purified fractions were screened for antioxidant capacity by *in chemico* assays. Additionally, fractions with better antioxidant capacity, namely EtOAc and Hex, were tested on lipopolysaccharide-stimulated RAW 267.4 macrophages.

The chemical analysis of the purified fractions allowed to identify some phlorotannins, mainly in EtOAc and Aq. On the other hand, the Hex fraction was rich in fucoxanthin, which along with other pigments, was also present in the EtOAc fraction. Overall, the EtOAc and Hex fractions were shown to be most effective regarding the antiradical capacity, and in particular against the nitric oxide radical. Both these fractions reduced significantly the nitrite levels produced by activated macrophages without causing a decrease in their cellular metabolism. In addition they were able to downregulate the inflammation-mediating enzyme iNOS and, particularly EtOAc reduced the levels of COX-2. It is possible that these facts can be related with the transcriptional factor NF-kB, since the expression of p65 subunit was significantly decreased in the cellular lysates of the pre-treated macrophages, followed by the inhibition of *p*-IkB-α phosphorylation. Taken together, the findings from the present dissertation suggest that EtOAc and Hex fractions of *F. vesiculosus* have relevant antioxidant and anti-inflammatory activities.

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ABBREVIATIONS

AAE	Ascorbic acid equivalents
$ABTS^{\bullet+}$	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
Acetyl-CoA	Acetyl-coenzyme A
ANOVA	Analysis of variance
AP-1	Activator protein 1
Aq	Aqueous fraction
BBD	Box-Behnken design
BK	Bradykinin
CAT	Catalase
CNS	Central nervous system
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
DMBA	2,4-dimethoxybenzaldehyde
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNEM	Dulbecco's Modified Eagle Medium
DPPH	1,1-diphenyl 1,2-picrylhydrazyl
DW	Dry weight
EAE	Enzyme-assisted extraction
EtOAc	Ethyl acetate soluble fraction
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GPx	Glutathione peroxidase
GSH	Glutathione
Hex	*n*-Hexane fraction
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
IFN-γ	Interferon-gamma
ILs	Interleukins
IL-1	Interleukin 1
IL-6	Interleukin 6

IL-10	Interleukin 10
IL-12	Interleukin 12
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MAE	Microwave-assisted extraction
MCP-1	Monocyte chemoattractant protein 1
Malonyl-CoA	Malonyl-coenzyme A
MAPK	Mitogen Activated Protein Kinases
NBT	Nitrotetrazolium Blue chloride
NF-kB	Nuclear factor-kappaB
$NO^{\bullet}$	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
PGE	Phloroglucinol equivalents
PG2	Prostaglandin 2
p-IkBα	pospho nuclear factor kB inhibitor α
PLE	Pressurized liquid extraction
PMS	Phenazine methosulfate
PON-1	Paraoxonase 1
RIPA buffer	Radioimmunoprecipitation assay buffer
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSM	Response surface methodology
SEM	Standart Error of the Mean
SFE	Supercritical fluid extraction
SNP	Sodium Nitroprusside
SOCS1	Suppressor of cytokine signalling
SOD	Superoxide dismutase
STAT5	Signal transducer and activator of transcription 5
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor-alpha
TBARS	Thiobarbituric acid reactive substances

TPhC	Total Phlorotannin Content
UAE	Ultrasonic-assisted extraction
UV radiation	Ultraviolet radiation
5-LOX	5-lipoxygenase

CHAPTER 1. INTRODUCTION

1.1 SEAWEEDS

Seaweeds, or macroalgae, are aquatic photosynthetic organism that present a variety of colours, shapes and sizes. They are classified in brown (*Phaeophyceae*), green (*Chlorophyceae*) or red (*Rhodophyceae*) according to the main pigments present in their composition, like fucoxanthin, chlorophylls and phycobilins, respectively[1].

Seaweeds have been used for centuries for direct consumption, mostly by East populations, in countries such as China, Japan and Korea, since they are considered a nutritius, but also a low calorie food. Furthermore, they have been used as biofertilizers and animal feed in agriculture and for extraction of phycocolloids to industrial exploitation[2–5].

1.1.1 Brown seaweeds and macroalgae *Fucus vesiculosus*

Fucus vesiculosus is a brown seaweed from Fucacea family, mostly known as bladder wrack. This species are naturally founded in the intertidal areas of cold- temperate waters coastlines of the North Sea, the western Baltic Sea, and the Atlantic and Pacific oceans[6]. The most common apliccation of this seaweed are, at therapeutic level, to treat goiter (i.e. a swelling of the thyroid gland caused by the lack of iodine), since it present high content of iodine that is necessary for the thyroid hormone production[7]. Moreover, due to their health potentialities and bioactive richness compounds, it has been used for the development of new functional foods to improve the nutritional composition. At the nutritional point of view, *F. vesiculosus* is recognized as a great source of carbohydrates (34-66 % DW_{algae}), fiber (4 – 59% DW_{algae}) and minerals (23 – 36% DW_{algae}), while its protein and lipid levels are in the range of 1 – 11% DW_{algae} and 1.2 – 4% DW_{algae}, respectively[8]. In addittion, it also present other nutritional elements such as vitamins and carotenoids, this seaweed have revealed high content of a specific carotenoid produced by brown seaweeds, fucoxanthin[8]. Among the secundary metabolite, *F. vesiculosus* is rich in phenolic compounds, in particularly phlorotannins, that are almost exclusively found in brown macroalgae, have been of great interest, since they have been related to numerous beneficial biological properties, including antioxidant[9–11], anti-inflammatory[12,13], antibacterial[14], anticancer[15] and antidiabetic[16].

1.1.2 Phlorotannins

Phlorotannins are usually stored in small vesicles called physodes in the cytoplasm of the cell and believed to play primary and secondary roles both at cellular and or systemic scales[17–19]. As primary functions, phlorotannins play a structural role, since they are present in the cell wall (although, they are more abundant in cytoplasm), and at the reproductive level, as they are also present on the surface of fertilized zygotes[20,21]. As secondary functions, these compounds play a protective role once they protect brown algae from stress conditions such as UV radiation and agains herbivorous species, such as the snail *Littorina obtusata*[22,23]. Chemically, phlorotannins are hydrophilic compounds formed from monomeric units of phloroglucinol (1,3,5-trihydroxybenzene) with molecular weights ranging from 126 Da to 650 kDa[17]. The biosynthetic pathway of these compounds is still not consensual; however, the biosynthesis of phloroglucinol occurs through the acetate-malonate pathway, also known as polyketide, in the Golgi complex[17]. Initially, in the acetate-malonate pathway, an acetyl-CoA molecule is converted to malonyl-CoA by the addition of a carbon dioxide molecule. This reaction leads to the modification of the acetyl methyl group into a reactive methylene group, which makes the polymerization process possible[24]. The polymerization gives rise to a polyketic chain that can undergo an intramolecular cyclization with the concomitant loss of a water molecule, resulting in a hexacyclic ring (triketide). Due to the instability of the triketide, it undergoes a tautomerization process, in order to make the aromatic ring more stable, giving rise to phloroglucinol (**Figure 1**)[24].

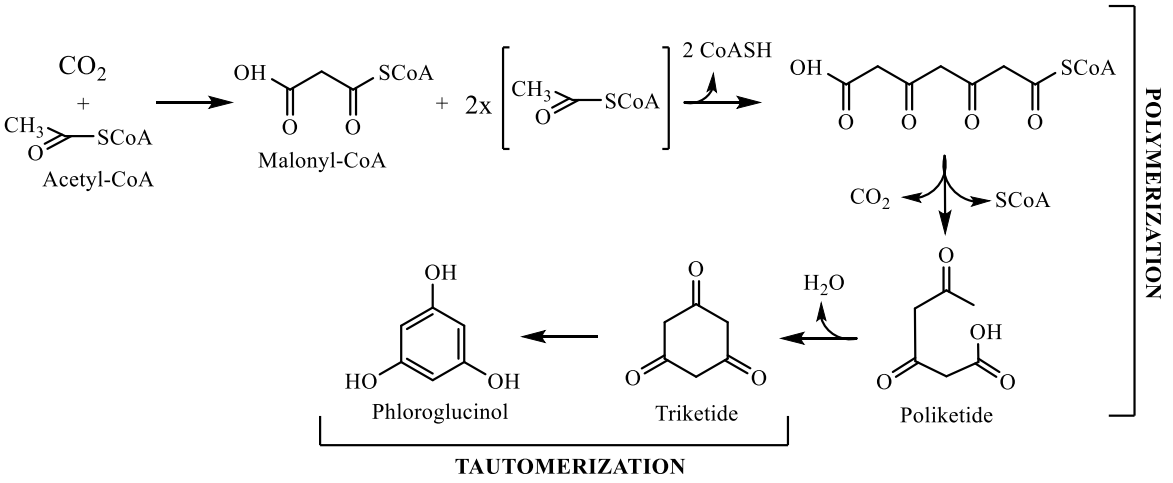


Figure 1. Phloroglucinol biosynthesis.

Phlorotannins may be dehydro-oligomers/ dehydro-polymers taking into account the C-C and/ or C-O-C oxidative coupling formed between the monomeric units of phloroglucinol[25].

These compounds are then classified in 4 sub-classes according to the type of linkage between phloroglucinol units and the number of hydroxyl groups (**Figure 2**): phlorethols and fuhalols (ether linkages), fucols (aryl-aryl linkages), fucophlorethols (aryl-aryl and ether linkages) and eckols and carmalols (dibenzodoxine linkage)[26].

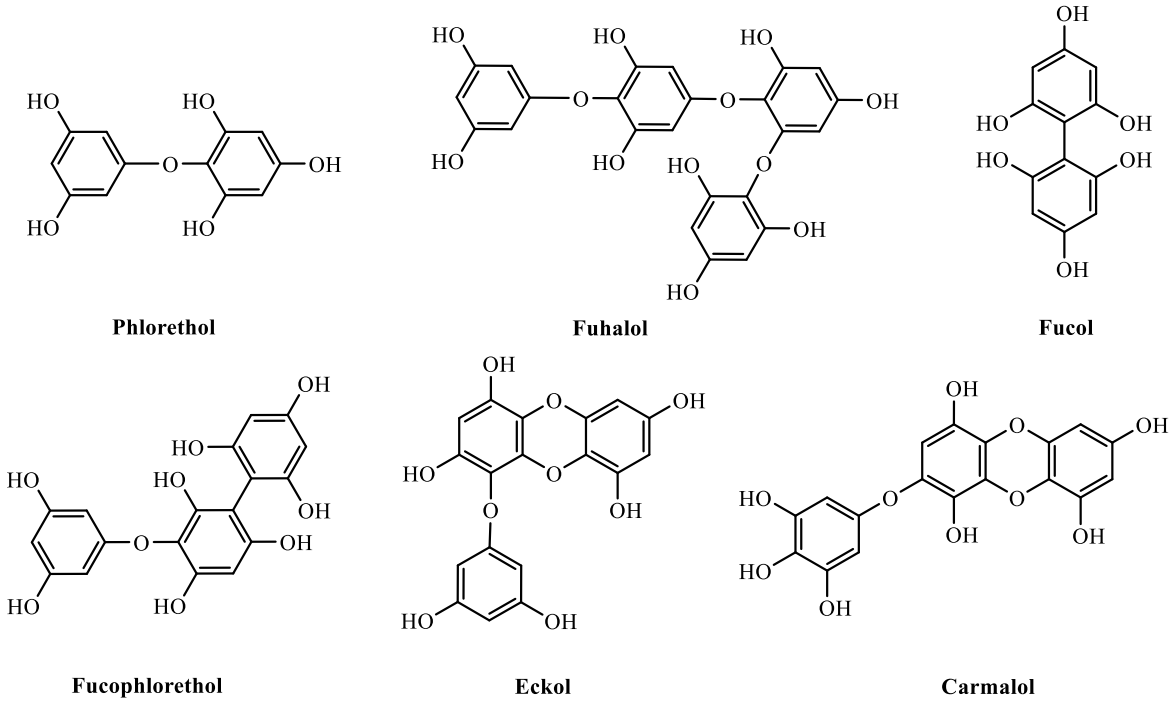


Figure 2. Classification of phlorotannins according to the type of linkage.

1.2 EXTRACTING METHODS OF PHLOROTANNINS

In general, the extraction of phlorotannins is traditionaly performed by the conventional solvent extraction method[16,27,28]. However, phlorotannins have peculiar characteristics that make their extraction a challenge, namely their chemical complexity, susceptibility to oxidation and interaction with other components of the matrix[18,19,26]. Solid-liquid extraction (SLE) of these phenolic compounds depends on a number of factors, such as solvent composition, solvent polarity, extraction time, temperature, solvent-solid ratio and particle size[16,29–31]. Regarding solvent composition, aqueous mixtures of acetone, ethanol or methanol are commonly used[32–34]. The time and temperature selected for the extraction process also influence the quality of the extracts, since they can lead to degradation or cause undesirable reactions, such as enzymatic oxidation, which compromises the stability of phlorotannins[35]. In addition to the more traditional solid-liquid extraction at room

temperature, there are other, more automated methods of extracting phlorotannins from seaweeds that have been widely studied, such as supercritical fluid extraction (SFE)[36,37], pressurized liquid extraction (PLE)[38], microwave-assisted extraction (MAE)[27,29], ultrasound-assisted extraction (UAE)[30,31], these aforementioned methods can also be coupled with a previous pre-treatment, such as enzyme assisted extraction (EAE)[39].

The SFE technique uses a fluid, normally SC-CO_2, which operates with temperature and pressure above its critical limit. Under these conditions the fluid acquires some new characteristics such as density, viscosity and diffusivity like a liquid, a gas and somewhere between a liquid and a gas, respectively. However, the viscosity of the fluid can be altered by changing the temperature and the pressure who consequently also influence the dissolving power[40]. This method is characterized by its selectivity and fastness in the extraction process; however, it contains some limitations, namely the low polarity of the supercritical CO_2 which limits the dissolution power of several compounds with a lower polarity, thus making difficult to extract them, even using co-solvents such as ethanol [37,40]. Thus, studies were carried out with brown seaweeds using this type of extraction for the recovery of phlorotannins. Leen and Chun[41] reported that the oil extracted from *Laminaria Japonica* using this methodology contained 214.61 ± 0.34 mg gallic acid equivalents (GAE)/100 g dry weight (DW) [41]. Studies carried by Saravana et al.[37] showed that, under optimized conditions of extraction with SC-CO_2, the recovered amounts of phlorotannins from *Saccharina japonica* accounted for 0.927 ± 0.026 mg phloroglucinol equivalents (PGE)/g DW_{algae}[37] (**Table 1**).

Much like SFE, the PLE technique uses high temperature and pressure values for the extraction process, accelerating the extraction and increasing the boiling point temperature of the solvent, respectively[39]. In addition, the rise of temperature increases the solubility and mass transfer rate, reducing the viscosity and the surface tension of solvents, spreading them over the biological matrix and improving the extraction rate[38,39]. The use of high values of temperature and pressure can be considered a disadvantage for the extraction of compounds with thermolabile characteristics, like phlorotannins[32]. Notably, the application of PLE with ethanol on the extraction of seaweeds *Saccharina longicruris* and *Ascophyllum nodosum* allowed to obtain extracts with values of total phenolics contents of 3.3 mg GAE/g $DW_{extract}$ and 50.2 mg GAE/g $DW_{extract}$, respectively[38] (**Table 1**).

Nowadays, MAE and UAE represent the techniques that allow the fastest and largest extraction of bioactive compounds, including phenolic compounds[27,42]. MAE technique has several advantages over the other methods: it allows the rapid heating of aqueous samples with non-ionizing electromagnetic radiation, a lower solvent use, a greater selectivity for the family of compounds of interest, a higher level of automation, and a superior efficiency and lower extraction times[29]. When optimizing the MAE extraction of phlorotannins from *Saccharina japonica* using ethanol as solvent He et al.[29] obtained a maximum yield value of 0.644 mg PGE/g DW_{algae}[29]. Moreover, the efficiency of MAE regarding SLE techniques was already highlighted by Magnusson et al.[27] when comparing the extraction of phloroglucinol from the species *Carpophyllum flexuosum*, *Carpophyllum plumosum* and *Ecklonia radiata* (8.6 ± 0.2, 7.5 ± 0.1 and 1.5 ± 0.2 g PGE/100g DW_{algae} vs 15.8 ± 0.3, 9.2 ± 0.6 and 2.0 ± 0.1 g PGE/100g DW_{algae} for SLE and MAE extraction respectively)[27] (**Table 1**).

Similarly to the MAE, the UAE is more efficient in extracting biomolecules in general than traditional methods, due to the acoustic cavitation. It also has a low cost of production, as well as low energy consumption levels and reduced quantity of solvent used. Furthermore, it requires considerably lower times for extraction[30]. Distinct authors have reported variable contents of phlorotannins/phenolic compounds in extracts from brown seaweed *Ascophyllum nodosum*, depending on the UAE conditions applied in the optimization process. In this regard, Kadam et al.[31,43,44] reported higher levels of a total phenolic content in this seaweed when the extraction optimization was carried out using a mathematical model, namelly response surface methodology (RSM) (84.54 ± 0.46 mg GAE/g_{db}[43] versus 143.12 mg GAE/g_{db}[31], respectively). In another study, under different optimum conditions the author described a total polyphenol content of 15.6 mg PGE/100 g_{db}[44], while Moreira et al.[45] reported recovery values of 3180 ± 131 mg PGE/100 g DW_{algae}[45] both using water as solvent extraction, although the difference between this values could be explain due the fact the harvest seaweed are from different sites, wich influences the production/amount of phlorotannins. Other brown seaweeds, such as *Ecklonia cava*, *F. vesiculosus* and *Hormosira banksia* have also been studied, for which values of phenolic contents were reported to be 4.76 ± 0.1 g GAE/100 g DW[46], total polyphenol content of 1571 ± 76 mg PGE/100 g DW_{algae}[42] and total phenolic contents of 23.12 ± 1.01 mg GAE/g DW_{algae}[30], respectively (**Table 1**).

Contrary to the above methods, the EAE is not an extraction method *per se*, but instead, it is a pre-treatment extraction process that is usually, coupled with the aforementioned methods, in order to improve the yields of extracted phlorotannins. Moreover, as this treatment has a relatively low-cost and it usually avoids the use of organic solvents on the extraction process, it can be considered as eco-friendly and non-toxic process[39,47]. Taking this into account, in general, the values of total phenolic content are always higher after the pre-treatment with EAE, in a variety of different seaweeds[48] (**Table 1**).The majority of studies applying this technique were done with *Sargassum muticum* and combining different methods already mentioned above. When EAE is coupled with PLE the extracts presented values of 5.018 mg PGE/g of $DW_{extract}$[39]; when coupled with UAE, extracts revealed higher total phenolic contents relatively to the control (200.5-300.3 $\mu g_{catechol\ acid\ equiv}$ /$g_{lyoph\ extract}$ for the different enzymes studied versus 235.0 ± 5.57 $\mu g_{catechol\ acid\ equiv}$ /$g_{lyoph\ extract}$)[49]; when coupled with SLE the extracts also showed higer total phenolic contents when compared to the control (3.8 – 6.4 g PGE/100 g DW_{algae} for the different enzymes tested versus 2.6 g PGE/100 g DW_{algae})[50]. The extracts obtained from seaweed *Ecklonia radiata* exhibited values of total phenolic contents higher with the EAE when coupled with or without MAE when compared to the conventional water extraction (3.7 to 4.2 g PGE/100 g DW versus 2.6 g PGE/100 g DW, respectively)[51]. In short the use of pretreatment with enzymes are considered an advanteges for the extraction of phenolic compounds, such as phlorotannins.

Table 1. Extraction methodologies applied to distinct brown macroalgae.

Extraction method	Algae	Conditions	Yield	Ref.
SFE	*Dilophus ligulatus*	SC-CO2: Optimum weight mass extract obtained with pressure 15MPa and 328 K.	Weight=44.3 mg	36
	Laminaria japonica	SC-CO2: Optimum: 40°C, 300 bar in the brown seaweed oil.	TPC of oil=214.61 ± 0.34 mg GAE/100 g DW	41
	Saccharina japonica	SC-CO2: Optimum for PT: 48.98°C, 300 bar, and 2.00% of water	TPhC=0.927 ± 0.026 mg PGE/g DW_{algae}	37
PLE	*Saccharina longicruris*, *Ascophyllum nodosum*	50°C with 100% ethanol at 1,500 psi for 5 min.	TPC=3.3 mg GAE/g $DW_{extract}$ (*S. longicruris*) TPC=50.2 mg GAE/g $DW_{extract}$ (*A. nodosum*)	38

	Fucus vesiculosus	40°C, 100 bar, extraction solvents were, respectively, MeOH and DCM, preceded by a water rinse phase.	↑ phlorotannin tetramer TIC in summer months	52
MAE	*Saccharina japonica*	Optimum: 55% ethanol, solid/liquid ratio 1:8, 25 min, irradiation power of 400 W, 60°C.	TPhC=0,644 mg PGE/g DW_{algae}	29
	Carpophyllum flexuosum, Carpophyllum plumosum and Ecklonia radiata	Optimum: water, biomass:solvent ratio 1:30, 160 °C, 3 min.	PGE=15.8 ± 0.3, 9.2 ± 0.6 and 2.0 ± 0.1 g PGE/100 g DW_{algae} (*Carpophyllum flexuosum,C. plumosum and E. radiata*, respectively)	27
UAE	*Ecklonia cava*	Optimum: 1:100 water as solvent, 12h, 30°C, 200W.	TPC= 4.76 ± 0.1 g GAE/ 100g DW_{algae}	46
	Ascophyllum nodosum	Optimum: 1:10 (s/w), 75.78 W cm^{-2}, 0.03 M HCl as solvent and 10 min.	TPC= 84.54 mg GAE/g_{db}	43
	Ascophyllum nodosum	Optimum: 1:10 (s/w), ultrasonic amplitude 114 μm, 0.03 M HCl as solvent and 25 min, with RSM.	TPC=143.12 mg GAE/g_{db}	31
	Ascophyllum nodosum	Optimum: 1:4 (s/w), intensity of 35.78Wcm^{-2}, distilled water as solvent, 15 min.	TPC= 15.6 mg PGE/100 g_{db}	44
	F. vesiculosus	Optimum: 35°C, 4 min, 80% amplitude, 30(w/w).	TPC= 1571±76 mg PGE/100 g DW_{algae}	42
	Hormosira banksii	Optimum: 30°C, 60 min and power 60%, or 150 W.	TPC=23.12 mg GAE/ g DW_{algae}	30
	Ascophyllum nodosum	Optimum: 50°C, 4 min, 80% amplitude, 30(w/w).	TPC=3180 ± 131 mg PGE/100 g DW_{algae}	45
EAE	*Ecklonia cava, Ishige okamurae, Sargassum fullvelum, Sargassum horneri, Sargassum coreanum, Sargassum thunbergii, Scytosipon lomentaria.*	Coupled with SLE: Enzymes: five carbohydrases and five proteases, 12 h SLE: 1 ml of seaweed enzymatic extracts+1 ml of 95% EtOH+5 ml of distilled water.	↑ TPC w/ carbohydrases in *Ecklonia cava, Ishige okamurae, Sargassum fullvelum Sargassum coreanum, Sargassum thunbergii;* ↑ TPC w/ proteases in *Sargassum horneri,* Scytosipon *lomentaria* (but some variability between values of enzyme hydrolysis)	48
	Ecklonia radiata	Coupled with MAE Enzymes: three carbohydrases and three proteases MAE: 50 °C; 3 h.	TPC=4.4 g PGE/100 g DW	51
	Sargassum muticum	Coupled with PLE. Enzymes: proteases and carbohydrases, alkaline hydrolysis PLE optimum: 160°C and 95% ethanol; pressure, 1500 psi; 120 °C.	TPC=94.0 mg GAE/g of extract; TPhC=5.018 mg PGE/g of extract	39

	Sargassum muticum	Coupled with UAE Enzyme: two proteases and two carbohydrases UAE: 60 min, 50 °C.	TPC= 235.0 ± 5.57 $\mu g_{catechol\ acid\ equiv}/g_{lyoph\ extract}$ (UAE) TPC= 261.9 ± 29.42, 300.3 ± 11.67, 289.7 ± 18.46, 200.5 ± 7.40 $\mu g_{catechol\ acid\ equiv}/g_{lyoph\ extract}$ (for the enzymes)	49
	Sargassum muticum	Coupled with SLE Enzymes: two proteases and six carbohydrases SLE: 0.5 mL of samples + 0.5 mL 95% ethanol + 2.5 mL H_2O.	TPC= 2.6 ± 0.0% DW (control) TPC= 3.8 ± 0.0, 4.2 ± 0.0%, 3.8 ± 0.0%, 4.5 ± 0.0%, 4.8 ± 0.0%, 4.7 ± 0.0%, 6.4 ± 0.5%, 5.0 ± 0.1% DW (for the enzymes)	50

SC-CO2- supercritical carbon dioxide; TPC- total phenolic contents; GAE- gallic acid equivalents; MeOH-methanol; DCM-dichloromethane; TIC- Total ion count; PGE- phloroglucinol equivalent; TPhC- total phlorotannin content; RSM- response surface methodology; DW-dry weight; $g_{lyoph\ extract}$-gram of lyophilized extract

Since phlorotanins are particularly susceptible to oxidation, several other factors other than the extraction method, such as the storage and preparation of algae for analysis, should be considered in order to maximize their recover from macroalgae. In order to avoid oxidation, extraction can be carried out under a nitrogen atmosphere or with solvents containing antioxidants, like $K_2S_2O_5$ or ascorbic acid[25]. Koivikko et al.[32] found that the addition of 0.3% of ascorbic acid to the extraction solvent led to a more satisfactory chromatographic profile of *F. vesiculosus* phlorotannins, with increases in both the number of detected peaks and their intensity[32].

Concerning the conditions of sampling and storage of the extracts, special care is also necessary, as these processes can influence the quantity and quality of the phlorotannins and also their bioactivities[53]. In fact, Cruces et al.[53] analysed the effect of different preservation methods on the extraction of phlorotannins from *Lessonia spicata*. Among freezing, freeze-drying, silica drying, oven drying and air drying, the authors reported that lyophilized samples allowed the recovery of higher levels of phlorotannins, possibly because this preservation methodology minimizes the degradation of thermolabile molecules[53].

Since several variables influence extraction of phlorotannins, the optimal operating extraction parameters may be estimated through the use of a statistical optimization method. The response surface methodology (RSM) makes use of the quantitative data of an appropriate experimental design to determine and simultaneously solve the multivariate equation. In order to minimize the number of experiments, this methodology relies on a

mathematical model where all the interactions that occur between the test variables are taken into account[54]. This type of approach enables a considerable reduction of cost and execution time in the experimental projects with more than two variables[55]. One of the RSM models most used for experimental planning is the Box-Behnken design (BBD). The main advantage of this experimental design is that the experiments are not carried out under extreme conditions, i.e., the combinations between the different factors are never in their higher or lower levels, since this type of combination usually gives unsatisfactory results[56].

1.3 BIOLOGICAL PROPERTIES OF BIOACTIVE COMPOUNDS

As previously mentioned, brown macroalgae are particularly rich in bioactive compounds, from wich phlorotannins, fucoxanthin and fucoidans can be highlighted. In particular, phlorotannins have been demonstrated to exert numerous bioactive activities, including the ability to counteract oxidative stress, cancer, allergies, diabetes, inflammation, viral and microbial infections, among others[57,58]. Given these characteristics, such compounds have been used in the development of new drugs and nutraceuticals, as well as in cosmetics[57]. The antioxidant and anti-inflammatory activities are the most studied biological properties of phlorotannin-rich extracts and of isolated phlorotannins and hence, these will be discussed in more detail in the next sections.

1.3.1 Antioxidant activity

1.3.1.1 Overview

Oxidative stress is a biological condition characterized by an impairment in redox homeostasis, caused by an imbalance between the production of reactive oxygen and nitrogen species (RONS) and their removal through enzymatic or non-enzymatic systems[59]. The production of reactive oxygen species (ROS) occurs mostly in the mitochondria and includes radical and non-radical species[60]. Radical species comprise the hydroxyl group ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), among others, whereas non-radical species comprise hydrogen peroxide (H_2O_2), oxygen ($^1O_2$) and hypochlorous acid (HOCl)[59].

On the other hand, the most produced reactive nitrogen species (RNS) are the radical nitric oxide ($NO^{\bullet}$), the non-radical peroxynitrous acid (ONOOH) and peroxynitrite ($ONOO^{-}$), which are quite dangerous for the central nervous system (CNS)[59,61,62].

Oxidative stress can arise from physiological or non-physiologic causes, whichever the case, the formed oxidants must be removed in order to allow a return to homeostasis in the cell, and thus inhibit oxidation of other molecules[61,63]. This removal is performed by antioxidant systems, wich may be enzymatic or non-enzymatic[59,64]. The first encloses, enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT)[59]. SOD is considered a detoxification enzyme and one of the most powerful promotors of antioxidant activity in cells, acting as the first line of defense against ROS. This enzyme are responsible for the dismutation of two molecules of $O_2^{\bullet-}$ in O_2 and H_2O_2 , in order to limit the availability of $O_2^{\bullet-}$ converting rapidly the superoxide radical to H_2O_2, keeping its levels reduced [65,66]. As regards to the peroxide it can be converted into oxygen and water by CAT or by the GPx enzyme wich is responsible for reducing peroxides through glutathione (GSH)[65,66].

Regarding the non-enzymatic antioxidant system, numerous compounds take part, including ascorbic acid, α-tocopherol, carotenoids and phenolic compounds[59]. These compounds have the ability to scavenging free radicals from the bloodstream or through cell membranes[67,68].

1.3.1.2 Antioxidant activity and brown seaweeds

Since continuous inactivation of the oxidants is required, ingestion of antioxidants through the diet might be one solution in order to maintain the equilibrium and an appropriate antioxidant state[63]. In this context, brown macroalgae might hold a relevant rule due to their richness in antioxidants, including phlorotannins[69–74].

In general, the antioxidant capacity of such compounds has been mostly clamied based on the scavenging ability of radicals towards 1,1-diphenyl 1,2-picrylhydrazyl ($DPPH^{\bullet}$), hydroxyl, superoxide, alkyl and peroxyl, and can be evaluated in *in vitro* assays[57]. Other methods capable of determining the antioxidant activity are 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), β-carotene bleaching inhibition, oxygen radical absorption capacity, reducing power and chelating activity of metals[75]. These methods also have been applied to compare the antioxidant capacity between the phlorotanninsand others bioactive compounds and the conventional antioxidants. Different

studies have been described for different types of seaweeds from Fucacea family, including *F. vesiculosus*. Hefferman and coworkers[76] established the antioxidant potential of four brown seaweeds as *Fucus serratus, Fucus vesiculosus, Himanthalia elongata* and *Cystoseira nodicaulis*, through ferric reducing antioxidant power (FRAP) and DPPH methods[76]. According to the different antioxidant assays the authors established the following potential: *F. vesiculosus>Himanthalia elongata>Fucus serratus>Cystoseira nodicaulis* This results indicates that *F. vesiculosus* is a seaweed with great potentiality for scavenging radicals, a fact that the authors also associated to the high TPC (231.95 ± 8.97μg PGE/mg sample)[76]. Studies conducted by O'Sullivan et al.[33] determined the antioxidant activity through FRAP, β-carotene bleaching and the DPPH scavenging assay in *Ascophyllum nodosum*, *Laminaria hyperborea*, *Pelvetia canaliculata*, *Fucus vesiculosus* and *Fucus serratus*[33]. Among the different seaweeds, *F. serratus* was the most promissing, followed by *F. vesiculosus* (113.4 ± 18.5 and 109.8 ± 17.7 AAE/g DW, respectively). *F. vesiculosus* and *A. nodosum* were also the most promissing algae with regard to the scavenging ability towards DPPH radicals and to the preventing of β-carotene bleaching[33]. Moreover, in *in vitro* asssays, the H_2O_2-stimulated Caco-2 cells all the extracts presented great protection against ROS formation through the increase of SOD and CAT activity and raise of GSH production. The authors demonstrated a decrease in the DNA damage, particularly with extracts from *F. vesiculosus* and *F. serratus* origin[33]. In another study, O'Sullivan et al.[77] tested the same seaweeds in tert-butyl hydroperoxide (*tert*-BOOH)-stimulated Caco-2 cells and demonstrated a similar behavior for SOD but not CAT or GSH and in DNA damage was also the *F. vesiculosus* and *F. serratus* with better results[77]. Overall, both studies revealed great results for *F. vesiculosus* in terms of antioxidant activity, confirming the potentiality of this seaweed for food and pharmacological applications[33,77]. Moreover, in *in vivo* experiments, Zaragozá et al.[78], reported that sprague–Dawley rats treated for four weeks with 200 mg/kg body weight/day revealed an increase of antioxidant activity in non-enzymatic antioxidant protection in plasma (29% in reducing power, 33% in paraoxonase 1 (PON-1) and 25% in superoxide scavenging activity) and in enzymatic antioxidant protection in erythrocytes (the extracts were able to increase SOD activity but not CAT activity remaining this similar to the control group)[78]. This work confirms the versatility of *F. vesiculosus* to protect organisms against oxidative stress. Notably, among the two polyphenolic extracts, Zaragozá et al.[78] highlighted that the one enriched with fucoxanthin revealed better results, pointing to a possible

enrichment of functional food or for pharmacological aplicability, since this seaweed present a enormous variety of bioactive compounds[78]. In addition to *F. vesiculosus*, different types of seaweeds have been investigated also to determine their antioxidant properties. Maneesh and coworkers[79] have investigated the antioxidant properties of *Sargassum wightii*[79]. They realised an *n*-hexane extraction with further fractioning in ethyl acetate-methanol and chloroform and tested the antioxidant properties with the iron ion chelating ability, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. The fraction with the best results was ethyl acetate-methanol with values of IC_{50} of 0.52 mg/mL, 0.82 mg/mL and 0.32 mg/mL for iron ion chelating ability, $ABTS^{\bullet+}$ and DPPH, respectevely, moreover this fraction was the one with higher phenolic content[79]. Another study with the brown seaweeds *Turbinaria conoides* (TC) and *Turbinaria ornata* (TO) where the extraction process were realised with methanol and further purificated with *n*-hexane, dichloromethane and ethyl acetate were submitted to antioxidant assays[80]. The fraction with greater results for the $DPPH^{\bullet}$, hydroxyl radical and peroxide radical assay was obtained for the EtOAc fraction in both seaweeds (IC_{50} of 0.77 and 0.21 mg/mL; IC_{50} of 0.47 and 0.44 mg/mL and IC_{50} 1.5 and 1.9 mg/mL for TC and TO, respectively).Yet, methanol fraction was more promissing than ethyl acetate regarding scavenging ability towards $ABTS^{\bullet+}$ assay and iron chelating ability for TC (IC_{50} of 2.31 mg/mL versus 6.29 μg/mL and 0.53 mg/mL versus 1.07 mg/mL, respectively). However, for TO it was the ethyl acetate fraction with better results than methanol (IC_{50} of 3.16 versus 5.08 μg/mL and 0.46 versus 1.03 mg/mL, respectively). Moreover they analysed the lipid peroxidation through thiobarbituric acid reactive substances (TBARS) assay. The TBARS inhibitory activity were greater for ethyl acetate fraction than methanol for TO, however for TC it was methanol fraction with better values than ethyl acetate[80]. Thus, although the fraction with the highest phenolic content was EtOAc also the methanol fraction presented great antioxidant activity so the properties presented for the different extracts were not only due to the phenolic compounds. The author Vijayabaskar and coworkers[81], also studied the antioxidant properties in methanol extracts of *Turbinaria ornata*[81]. Their research demonstrated values of scavenging activity of 84.27 ± 2.17%, 61.86 ± 1.27% and 72.58 ± 3.45% for the $DPPH^{\bullet}$ assay, superoxide anion assay and $ABTS^{\bullet+}$ assay, respectevely. The results revealed best antioxidant activity than the standard gallic acid but not for the hidroxyl radical assay and $NO^{\bullet}$ assay[81]. Studies carried out with methanol extracts of *Ecklonia cava* were further submited to fraction with *n*-hexane, chloroform and ethyl acetate and also demonstrated

great antioxidant properties[82]. According to Senevirathne[82], the most promissing fraction was ethyl acetate for DPPH•, hydrogen peroxide and nitric oxide radical inhibition (IC_{50} 0.013 mg/mL, 0.009 mg/mL and 0.33 mg/mL, respectively). Instead, methanol extract and chloroform fraction were the best regarding the ability to scavenge the hidroxyl radical (IC_{50} 0.023 ± 0.003 mg/mL). Moreover, for the superoxide anion assay and iron chelating assay it was also the methanol extract (IC_{50} 0.051± 0.003 and 0.436±0.03 mg/mL, respectevely)[82]. Once more, as methanol showed the highest levels of phenolic compounds, the high antioxidant ability of ethyl acetate fraction was atributed to the combined presence of phenolic and non-phenolic components[82]. Moreover, studies conducted with the brown seaweed *Sargassum siliquastrum* in order to determine their antioxidant capacity with different extraction solvents (*n*-hexane, chloroform, ethanol, and water) were also assessed[83]. In more detail, for the DPPH• radical scavenging assay all the different extracts showed higher ability to scavenge this radical, although for TBARS assays and metal ion chelating ability it was the chloroform and etahnol extract. In addition the reducing power assay, for with the ethanol extract revealed similar effect as ascorbic acid at 10 mg/mL wich means that ethanol extracts have similar antioxidant effects as the commonly used acid ascorbic indicating high potential for natural purposes [83]. In the case of *Himanthalia elongata* among the different methanol extracts (20 - 80%) the one with the highest FRAP and antioxidant capacity against DPPH radical, metal ions, lipid peroxidation and hydrogen peroxide radicals was the 60% methanol extract (11.7 ± 0.21 mg TE/g, EC_{50} 57.2 ± 0.48, EC_{50} 1,982.0 ± 8.73, EC_{50} 546.3 ± 7.61 and EC_{50} 361.7 ± 6.30, respectevely), this fraction was also the one with the higher value of phenolics content[84]. Numerous other studies have focused on the antioxidant activity of phlorotannins isolated from brown algae. In the seaweed *Ecklonia stolonifera*, the DPPH• assay for the compounds dioxinodehydroeckol, dieckol and phlorofucofuroeckol A showed superior radical scavenging activity (EC_{50} = 8.8 ± 0.4, 6.2 ± 0.4 and 4.7 ± 0.3 μM, respectively) than L-ascorbic acid (EC_{50} = 10.3 ± 0.5 μM)[10]. However, Shibata et al.[9] demonstrated that in the *Japanese Laminariaceae*, the phlorotannins eckol, phlorofucofuroeckol A, dieckol and 8,8'-bieckol were more effective eliminators of DPPH• (EC_{50}=26, 12, 13 and 15 μM, respectively) when compared to catechin, ascorbic acid and α-tocopherol (EC_{50} = 32, 30 and 52 μM, respectively). These authors also found that these compounds had a better ability to eliminate $O_2^{\bullet -}$ (EC_{50} = 10.7,

8.4, 7.6 and 6.5 μM, respectively) than resveratrol, ascorbic acid and α-tocopherol (EC_{50} = 21, 62 and 12 μM, respectively)[9].

Furthermore, an overview of numerous studies on the antioxidant activity of phlorotannins isolated from brown algae at the cellular and/or systemic level is summarized in **Table 2**.

Table 2. Phlorotannins identified in brown algae and their antioxidant activity assessed in *in vivo* and *in vitro* models.

Compound	Alga	Antioxidant activity
Phloroglucinol	*Ecklonia cava, Eisenia bicyclis Ecklonia stolonifera, Sargassum muticum*	↓ Aβ-induced oxidative damage in PC12 cells[69]; ↓ ROS in human leukocytes[70]; ↓ Aβ-induced ROS formation in HT-22 cells[85]; ↓ MPO and ↑ CAT in chronic cystitis bladder in Sprague-Dawley rats[86]; ↑ Nrf2, CAT and GPx in 6-OHDA-treated SH-SY5Y cells[87]; ↓ ROS and lipid peroxidation and ↑ SOD and CAT in UVB radiated HaCaT cells[88]; ↑ GSH and ↓ DNA oxidative damage in H_2O_2-treated HT1080 cells[89]; ↓ DNA oxidative damage (Comet assay) in H_2O_2-treated L5178 cells[71]; ↓ ROS and lipid oxidation (TBARS) in AAPH-treated zebrafish[11];
Eckol	*E. cava, Ecklonia kurome, E. bicyclis, E. stolonifera*	↑ GSH, HO-1 and Nrf2 in H_2O_2-treated HepG2 cells[90]; ↑ SOD in H_2O_2-induced HeLa cells[91]; ↓ Aβ-induced oxidative damage in neurons[69]; ↓ intracellular ROS in tacrine-treated HepG2[72]; ↓ ROS and lipid peroxidation in HaCaT cells irradiated with UVB radiation[92]; ↓ DNA oxidative damage (Comet assay) in H_2O_2-treated L5178 cells[71]; ↓ ROS and lipid oxidation (TBARS) in AAPH-treated zebrafish[11];
Dieckol	*E. cava, E. bicyclis, E. stolonifera*	↓ Aβ-induced oxidative damage in PC12 cells[69]; protected HepG2 against t-BHP-induced oxidative stress[73]; ↓ROS and ↑ SOD, HO-1, CAT, NAD(P)H dehydrogenase and Nrf2 in H_2O_2-treated HepG2 cells[93]; ↑ HO-1 in mice livers[93]; ↓ ROS and lipid oxidation in high glucose-treated zebrafish[94]; ↓ lipid oxidation and ↑ SOD, CAT and GPx in mice liver tissues[95]; ↓ ROS in UVB radiated zebrafish[96]; ↓ DNA oxidative damage in H_2O_2-treated L5178 cells[71]; ↓ ROS and lipid oxidation (TBARS) in AAPH-treated zebrafish[11];
Eckstolonol	*E. cava*	↓ ROS, lipid oxidation and DNA damage and ↑ SOD and CAT in UVB radiated HaCaT cells[97]; ↓ ROS and lipid oxidation (TBARS) in AAPH-treated zebrafish[11];
Phlorofucofuroeckol A	*E. bicyclis, E. stolonifera, E. cava*	↓ Aβ-induced oxidative damage in PC12 cells[69]; protected HepG2 against t-BHP-induced oxidative stress[73]; ↓ ROS in LPS-stimulated Raw 264.7[10]; ↓ ROS high glucose-

		treated zebrafish[94]; ↓ ROS and ↑ GSH in H_2O_2 treated Raw 264.7 and ↓ MPO in TNF-α treated HL-60[98];
Triphloroethol A	*E. cava*	↓ UVB-induced oxidative stress in HaCaT[92]; ↓ ROS and lipid oxidation (TBARS) in AAPH-treated zebrafish[11];
Diphlorethohydroxycarmalol	*I. okamurae*	↓ ROS in H_2O_2 treated Raw 264.7 and ↓ MPO in TNF-α treated HL-60[74];
2-phloroeckol	*E. stolonifera*	↓ intracellular ROS in tacrine-treated HepG2[72];
7-phloroeckol	*E. bicyclis, E. cava*	↓ Aβ-induced oxidative damage in PC12 cells[69]; ↓ ROS and ↑ GSH in H_2O_2 treated Raw 264.7 and ↓ MPO in TNF-α treated HL-60[98];
6,6′-bieckol	*E. bicyclis, E. cava, Ishige okamurae*	protected HepG2 against t-BHP-induced oxidative stress[73]; ↓ ROS high glucose-treated zebrafish[94]; ↓ ROS and ↑ GSH in H_2O_2 treated Raw 264.7 and ↓ MPO in TNF-α-treated HL-60[74,98];
8,8′-bieckol	*E. bicyclis, E. cava*	protected HepG2 against t-BHP-induced oxidative stress[73]; ↓ ROS in LPS-stimulated Raw 264.7[99];
2,7″-phloroglucinol-6,6′-bieckol	*E. cava*	↓ DNA damage in H_2O_2-treated Vero cells[100];

Aβ – Amyloid-β, 6-OHDA – 6-hydroxydopamine, AAPH - 2,2'-Azobis(2-amidinopropane) dihydrochloride, t-BHP - tert-Butyl hydroperoxide, LPS – lipopolyssacharide, TNF-α – Tumor necrosis factor-α, ROS – Reactive oxygen species, GSH – glutathione, MPO – Myeloperoxidase, CAT – Catalase, HO-1 – Heme oxygenase, SOD – Superoxide dismutase, GPx – Glutathione peroxidase, Nrf2 – Nuclear factor (erythroid-derived 2)-like 2;
Cell lines: PC12 – pheochromocytoma, HepG2 – human hepatocyte, HeLa – human cervical cancer, L5178 – murine leukemia, HT1080 – fibrosarcoma, HaCaT – human keratinocyte, SH-SY5Y – Human neuroblastoma, HT-22 – mouse hippocampal, Raw 264.7 – Murine macrophage, HL-60 – Human promyelocytic leukemia

The different phlorotannins studied have been shown to be capable of acting as antioxidants at various levels. Not only they scavenge free radicals in the intracellular environment chelate metals[11,69,72], they also inhibit pro-oxidant enzymes and redox-sensitive mediators[86], and increase auto-antioxidant defences through positive regulation of phase II detoxifying enzymes[90,93,95]. The most studied parameter to measure the antioxidant activity is the formation of ROS. This parameter was evaluated by the scavenging activities of DPPH, alkyl, hydroxyl, and superoxide radicals assays and it was verified a decrease of ROS in the presence of all the phlorotannins[10,11,70,72,93,94,98]. In addition, a decrease of DNA oxidative damage in cells treated with compounds like phloroglucinol[71,101], eckol[71], dieckol[71] and 2,7″-phloroglucinol-6,6′-bieckol[100], was also verified, through the Comet assay. The lipid peroxidation was also analysed by some researchers and a decrease was noticed when cells were treated with compounds, such as dieckol, phlorofucofuroeckol A and 6,6′-bieckol[94], and with phloroglucinol, eckol, dieckol, eckstolonol, triphloroethol A[11]. Some of the studies revealed an increase of the antioxidant peptide GSH when cells were treated with phloroglucinol[89], eckol[90], phlorofucofuroeckol A[98], 7-phloroeckol[98] and 6,6′-

bieckol[74,98].The activity of some detoxifying enzymes was also assayed by some authors, who noted an increment in the expression of SOD and HO-1, in cells treated with eckol[90,91] and dieckol[93]; and CAT and NADP(H) dehydrogenase in response to dieckol[93]. Other studies reported a decrease of MPO activity, an enzyme present in neutrophils that produces HOCl from H_2O_2 and Cl^-, in TNF-α treated cells with phlorofucofuroeckol A[98], diphlororethohydroxycarmalol[74], 7-phloroeckol[98] and 6,6′-bieckol[74,98]. Ryu et al.[87] analysed the content of the GPx and CAT, wich were reported to be increased in 6-OHDA-treated cells by phloroglucinol, most probably through the transcription factor Nrf2[87]. Kang et al.[95] reported a raise on the activity of the enzyme GPx, SOD and CAT in mice liver treated with dieckol, which was accompanied by the decrease of lipid peroxidation[95]. In addition, some of these phlorotannins, like phloroglucinol[88], eckol[92], dieckol[96], eckstolonol[97], triphloroethol A[92] were demonstrated to be able to protect UVB irradiated cells or animal models, through the decrease of ROS formation, lipid peroxidation and DNA damage. So, we can assume that these compounds have a protective role against UVB radiation, not only in the seaweeds itself but also in cells and organism subjects at this type of radiation[22,88,92,96,97].

Taken together, the integrated analysis of the literature highlights that independently of the type of oxidative stress-induced, all studied phlorotannins have the ability to decrease ROS formation and/or to increase the antioxidant defences. Still, to the present, there are only few studies focusing on the involved regulatory mechanisms and although the transcription factor Nrf2 seems to be involved in the modulation of the expression of antioxidant proteins, more investigation is necessary to better understand this regulation. In addition, phloroglucinol, eckol and eckstolonol have shown the ability to reduce lipid peroxidation and DNA damage in UVB irradiated cells, which may eventually justify their future application in protection against UVB radiation not only in plants but in others organisms. The versatility of these phlorotannins make them an attractive option for future industrial applications in food, cosmetics and pharmaceuticals, particularly with the increasing consumer awareness and demand for functional foods and nutraceuticals. Thus, this socioeconomic pressure and health benefits make phlorotannins and brown seaweed a good choice for future food applications.

1.3.2 Anti-inflammatory activity

1.3.2.1 Overview

The inflammatory process is part of the complex biological response of the bodies-tissues to noxious stimuli, such as pathogens. Under physiological conditions, it is a protective response that involves cells of the immune system, blood vessels and various inflammatory mediators[102]. These mediators amplify the inflammatory response and, determine the type of inflammation, and its clinical impact. The main function of this process is to eliminate the cause of the cellular lesion as well as removing necrotic cells and damaged tissues, and initiate tissue repair[103]. Inflammation is a generic response and therefore it is considered an innate immunity mechanism, compared to adaptive immunity, which is specific for each pathogen[102].

Inflammation can be classified as acute or chronic, depending on the nature of the stimulus and efficacy of the initial stimulus elimination reaction or tissue damage. Acute inflammation is the body's initial response to harmful stimuli and is achieved by increased plasma velocity and increased movement of leukocytes (especially granulocytes) from the blood to the injured tissues[104]. A series of biochemical events spreads and matures the inflammatory response, involving the local vascular system, the immune system and several cells within the injured tissue[104]. Chronic inflammation may be the result of ineffective acute inflammation process or might be related with chronic diseases, such as atherosclerosis, rheumatoid arthritis, osteoarthritis and cancer[103]. It is characterized by the presence of lymphocytes and macrophages, proliferation of blood vessels and destruction of tissues, leading to a progressive and permanent change in the tissues structure[102].

Inflammation depends on the action of mediators such as histamine, released by mast cells, and $NO^{\bullet}$, produced by endothelial cells, macrophages and some neurons[104–106]. Histamine has vasodilatory capabilities and is responsible for increased vascular permeability leading to a localized and immediate increase in blood supply[104]. Similarly, $NO^{\bullet}$ also promotes vasodilation through a series of intra- and extracellular events that result in a relaxation response by vascular smooth muscle cells[106]. After actuation of these systemic mediators, local production of inflammatory mediators with vasodilatory capacity responsible for increased capillary permeability and chemotaxis (the chemical process by which polymorphonuclear cells, neutrophils and macrophages are attracted to the lesion site)[103]. The above referred polymorphonuclear cells have the ability to phagocytose the

inflammatory agents and to produce more proinflammatory chemical mediators, such as cytokines (such as tumour necrosis factor (TNF), interleukins (ILs) and chemokines), prostaglandins, leukotrienes and bradykinin (BK)[102,107–109]. One of the most characterized mediators is tumour necrosis factor-α (TNF-α). Its main function is the activation of nuclear factor-kB (NF-kB), which is responsible for the transcription of several genes that code for other pro-inflammatory mediators[110]. Among these mediators are TNF-α, ILs, chemokines, adhesion molecules and inflammatory enzymes, such as cyclooxygenase-2 and inducible nitric oxide synthase (COX-2 and iNOS, respectively), that further amplify the pro-inflammatory state[104,110]. In addition to TNF-α, other cytokines, such as ILs, which are proteins produced by leukocytes, are involved in the activation or suppression of the immune system and the induction of division of other cells[111]. In the inflammatory process, the cytokines TNF-α, IL-1, IL-6, IL-12, IFN-γ and chemokines, which function as potent mediators and/or regulators of inflammation, are present mainly for the ability to recruit and activate specific subpopulations of leukocytes[105].

Arachidonic acid-derived metabolites are locally active cellular mediators comprised among others by prostaglandins and leukotrienes[107,108]. Prostaglandins are synthesized via the cyclooxygenase pathway and involve systematic and vascular inflammatory reactions[107]. In this biosynthetic pathway, arachidonic acid undergoes the action of the cyclooxygenases COX-1 and COX-2[107]. As result, these substances act as hormones and are capable not only of increasing capillary permeability, but also of stimulating chemotaxis, attracting cells, such as macrophages, which are specialized in phagocytosis of the resulting cellular debris from the inflammatory process[107]. Leukotrienes, on the other hand, are synthesized via the lipoxygenase pathway and have vascular effects[108].

In addition to these mediators, blood elements like, platelets play an important role in the coagulation cascade, activated to control possible bleeding from the lesion[112]. The presence of adhesion factors at the surface of the internal endothelial cells lining the blood vessels controls the adhesion and diapedesis of circulating monocytes and other inflammatory cells[104].

1.3.2.2 Anti-inflammatory activity and brown seaweeds

In addition to antioxidant activity, phlorotannins are also closely related and associated with numerous inflammatory events. These compounds have demonstrated a potent effect on

cellular and *in vivo* models associated with inflammation-related diseases including osteoarthritis, atherosclerosis and cancer[58]. Phlorotannins revealed not only the ability to inhibit the expression of proinflammatory cytokines and scavenging radicals activity, typical of inflammation, but also to regulate the expression and/or activity of important enzymes, as well as the activation of transcription factors. In this regard, numerous studies hava been conducted with seaweeds extracts to assess their anti-inflammatory potentialities. Studies performed with a water phlorotannin- rich fermented extract from *Ecklonia cava*, with 70% ethanolic extract of *S. horneri* and with 30% of ethanol extract of *Sargassum fusiforme* and further fractioned with ethyl acetate in LPS-stimulated RAW 264.7 macrophages revealed the ability to inhibit the $NO^{\bullet}$ production[113–115], the expression of PGE2, iNOS and COX-2 and also the pro-inflammatory cytokines IL-1β and IL-6 for the first two extracts[114,116]. Furthermore, the pre-treatment of LPS-stimulated RAW 274.7 macrophages with 70% ethanolic extract was able to downregulate the pro-inflammatory cytokines TNF-α, and transcriptional factors NF-kB and phosphorylation of ERK1/2 and JNK[114]. Karadeniz et al.[117] also realised an ethanol extraction of *Sargassum horneri* followed by liquid partitioning with water, *n*-buthanol, 85% methanol and *n*-hexane[117]. In this case, all the fractions revealed the ability to inhibit the protein expression of MMP-2 and MMP-9[117]. In another from Kim and coworkers[118], performed with the same seaweed, the authors demonstrated great anti-inflammatory activity for the 80% methanol extract. Moreover, after purification, the subsequent purified *n*-hexane, chloroform and ethyl acetate fractionswere shown to decrease the $NO^{\bullet}$ production for the crude extraction and purified fractions in LPS-stimulated RAW 264.7[118]. Kellogg et al.[119] conducted anti-inflammatory ability in murine macrophage RAW 264.7 with 80% methanol crude extract of *Fucus distichus* followed by partitioning with hexane, ethyl acetate and 1-butanol. All the different fractions were able to reduce the expression of TNFα, IL-10, MCP-1, and COX-2 and reduced iNOS, although it was the hexane and ethyl acetate fractions wich demonstrated most significant reduction in inflammatory mediators expression[119]. In short, the different studies realised in macrophages RAW 264.7 showed the ability to reduce the $NO^{\bullet}$ production as also the expression of pro-infalammatory citokynes mediators responsible for the inflammatory state, although it should be noted the use of ethanol and methanol extracts followed with purification wich also revealed pontential to reduce inflammation. In addition, Dutot et al.[120] realised a pholorotannin-rich extract at 0.2% of *Ascophyllum nodosum* and observed a decrease in

TNF-α and IL-6 release in LPS-stimulated Human leukemic monocytes revealing the potentiality of this type of compounds to contest inflammation mediators[120]. Maneesh et al.[79] studied the anti-inflammatory activity of *Sargassum wightii* seaweed with chemical cyclooxygenase inhibition assay and 5-lipoxygenase (5-LOX) inhibition assay. The extraction process was realised with *n*-hexane then the residue was subsequntly extracted with ethyl acetate-methanol mixture and finally extracted with chloroform. Among the different fractions, ethyl acetate-methanol fraction was more promissing towards COX-1, 2 and 5-LOX (IC_{50} 0.03-0.05 mg/ml and IC_{50} 0.03 mg/ml, respectively) than the chloroform fraction[79]. Likewise, according to Lee et al.[121], after 96% ethanol extraction of *Laminaria japonica* and further partitioning with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol the *n*-hexane fraction showed the ability to reduce the production of $NO^{\bullet}$ and PGE2 secretion in LPS-stimulated macrophages. Moreover, this fraction was able to inhibit the expression of protein enzymes like iNOS and COX-2, pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β), transcriptional factors NF-kB and phosphorylation of IkB-α and signalling proteins of MAPK and Akt pathways. However, the main compound present in this fraction was identified has fucoxanthin, so thus indicate that other compounds besides phlorotaninns or phenolics in general also have the ability to reduce the inflammatory state, like carotenoids such as fucoxanthin, starting with an ethanolic extract[121]. Moreover, other studies more complexes with *in vivo* and *ex vivo* models have already starting to be exploited. In this sense, Bahar et al.[122] conducted three different type of extraction, cold water, hot water and 80% of ethanol in *Fucus vesiculosus* in order to determine the anti-inflammatory properties of the seaweed. Among the three crude extracts, in TNF-α challenged Caco-2 cells, the cold water extract was the one with a maximum inhibition of IL-8 production. The same was observed for the LPS-stimulated *ex-vivo* porcine colonic tissue were the same extract inhibited the expression of inflammatory mediators (PTGS2, C5, LYZ), cytokines (IL17A, IL8), chemokines (CCL2, CXCL2, CXCL10, CXCL11), cell adhesion molecules (ICAM1, VCAM1), toll like receptors (TLR4, TLR7) and components of NF-κB (NFKB1, RELB), MAPK (MAP3K8) and AP-1 (CJUN) pathways[122]. Moreover, the same author showed better anti-inflammatory activity for the 80% ethanol extracts from *Ascophyllum nodosum* with a inhibition of the expression of cytokines (IL8, TNFA, IL1B, IL18 and CSF1) chemokines (CXCL10 and CCL5), components of NF-κB pathway (NFKB2 and IKBKB), and inflammatory mediators (PTGS2 and MIF) in the TNF-α challenged Caco-2 cells[34]. The

study conducted in LPS-stimulated *ex-vivo* porcine colonic tissue also revealed a downregulation in pro-inflammatory mediator such as LYZ, IL8, PTGS2, TLR6, CXCL10, IL6, CXCL11, ICAM, NFKB1 and CXCL2[34]. Studies carried out with other seaweeds like *Cystoseira compressa* also revealed a decrease on the production of inflammatory mediators[123]. Mhadhebi et al.[123] performed a methanol extraction of *C. compressa*, with subsequent fraction with chloroform, ethyl acetate and methanol. The fractions with the best results was the chloroform and ethyl acetate fraction , with a inhibitory effect on rat paw edema 3h after carrageenan injection of 63.7 to 76.2% and from 65.4 to 78.3%, respectively, in more detail, the fraction with higher total phenolic content was ethyl acetate[123]. Thus, among the different studies ethanol extracts and phenolic-rich fractions showed great potential to treat diseases with inflammation associated, potenciating their pharmacological properties as a natural product.

Moreover, the effects of the different types of isolated phlorotannins against different proinflammatory markers evaluated through various cellular and *in vivo* models of inflammation and inflammation-related diseases is in **Table 3**.

Table 3. Phlorotannins identified in brown algae and their anti-inflammatory activity in cellular and animal models of inflammation.

Compound	Alga	Anti-inflammatory activity
Phloroglucinol	*Eisenia bicyclis, Sargassum muticum*	↓ HMGB1 release, CAMs expression and adhesion of leucocytes to HUVEC cells[124]; ↓ PGE2 and COX-2 in human whole blood[70]; ↓ TNF-α and IL-6 in chronic cystitis bladder in Sprague-Dawley rats[86]; ↓ IL-1β, IL-6, TNF-α and PGE2 in Raw 264.7[89]; ↓ MMP-2 and -9 and NF-kB in HT1080 cells[101]; ↓ $NO^{\bullet}$ in LPS-stimulated Raw 264.7 cells[125]; ↓ IL-1β, IL-6, TNF-α, COX-2 and iNOS in LPS-induced HepG2 cells[126];
Eckol	*E. bicyclis, Eisenia arborea*	↓ HMGB1 release, CAMs expression and adhesion of leucocytes to HUVEC cells[124]; ↓ AA, TPA and OXA-induced edema in ICR mice ear[127]; ↓ $NO^{\bullet}$ in LPS-stimulated Raw 264.7 cells[125];
Dieckol	*E. bicyclis. E. cava*	↓ HMGB1 release, CAMs expression and adhesion of leucocytes to HUVEC cells[124]; ↓ NF-kB in LX-2 cells[128]; ↓ $NO^{\bullet}$, iNOS and COX-2 in high glucose-treated zebrafish[94]; ↓ $NO^{\bullet}$, iNOS, COX-2 and NF-kB in high glucose-treated HUVECs[129]; ↓ histamine release in IgE-sensitized KU812F cells[130]; ↓ $NO^{\bullet}$, iNOS, p-IKKα/β, p-IkBα and NF-kB in

		LPS-stimulated Raw 264.7 cells[13,125]; ↓ JNK, MMP-9 and AP-1 in TPA-induced SK-Hep1 cells[131]; ↓ IL-1β, TNF-α, NO•, PGE2, iNOS, COX-2, NF-kB and p38-MAPK in LPS-stimulated BV2 cells[132];
Fucofuroeckol-A	*E. bicyclis*	↓ IL-6, TNF-α, MCP-1, NO•, PGE2, iNOS, COX-2, NF-kB and MAPK in LPS-stimulated Raw 264.7[133];
6,6′-bieckol	*E. cava, E. bicyclis*	↓ NO•, PGE2, iNOS, COX-2, TNF-α, IL-6 and NF-kB in LPS-stimulated Raw 264.7[134]; ↓ histamine release in IgE-sensitized KU812F cells[130]; ↓ ICAM-1, VCAM-1, TNF-α, IL-1β, COX-2, p-ERK1/2, p-JNK and NF-kB in LPS-induced HDPC cells[135];
7-phloroeckol	*E. bicyclis*	↓ NO• in LPS-stimulated Raw 264.7 cells[125];
8,8′-bieckol	*E. cava, E. arborea*	↓ NO•, PGE2, iNOS, IL-6 and NF-kB in LPS-stimulated Raw 264.7[99]; ↓ AA, TPA and OXA-induced edema in ICR mice ear[127];
Dioxinodehydroeckol	*E. bicyclis*	↓ NO• in LPS-stimulated Raw 264.7 cells[125];
Diphlorethohydroxycarmalol	*Ishige okamuarae*	↓ IL-6, NF-kB and STAT5 and ↑ SOCS1 in LPS-stimulated Raw 264.7[136];
Phlorofucofuroeckol A	*E. stolonifera, E. arborea, E. bicyclis*	↓ NO•, PGE2, iNOS, and COX-2, in LPS-stimulated Raw 264.7[10,125]; ↓ ICAM-1, VCAM-1, TNF-α, IL-1β, p-ERK1/2, p-JNK and NF-kB in LPS-induced HDPC cells[135]; ↓ AA, TPA and OXA-induced edema in ICR mice ear[127]; ↓ Akt and p38-MAPK in LPS-treated Raw 264.7 cells[12];
Phlorofucofuroeckol B	*E. arborea*	↓ AA, TPA and OXA-induced edema in ICR mice ear[127]

LPS – lipopolysaccharide, AA – arachidonic acid, TPA – 12-O-tetradecanoylphorbol-13-acetate, OXA – oxazolone, IgE – immunoglobulin E, HMGB1 – high mobility group protein 1, CAMs – Cell adhesion molecules, ICAM-1 – intracellular adhesion molecule 1, VCAM-1 – vascular cell adhesion molecule, PGE2 – prostaglandin E2, TNF-α – tumor necrosis factor α, IL-6 – interleukin 6, IL-1β – interleukin 1β, NO• – nitric oxide, COX-2 – cyclooxygenase-2, iNOS – inducible nitric oxide synthase, MMP – matrix metalloproteinase, MMP-2 - matrix metalloproteinase 2, MMP-9 - matrix metalloproteinase 9, MCP-1 – monocyte chemoattractant protein-1, p-IKKα/β – phospho-IkB kinase, p-IkBα – pospho nuclear factor kB inhibitor α, NF-kB – nuclear factor kB, JNK – c-Jun N-terminal kinases, p-JNK – phospho-c-Jun N-terminal kinase, p-ERK1/2 – phospho-extracellular signal–regulated kinase 1 and 2, p38-MAPK - p38 mitogen-activated protein kinases, AP-1 – activator protein 1, STAT5 - signal transducer and activator of transcription 5, SOCS1 - suppressor of cytokine signaling 1, Akt - protein kinase B;
Cell lines: Raw 264.7 – murine macrophage, HUVEC - human umbilical vein endothelial cells, HT1080 – human fibrossarcoma, HepG2 – human liver hepatocellular carcinoma, LX-2 - human hepatic stellate, KU812F – human basiphilic cells, SK-Hep1 – human hepatic adenocarcinoma, BV2 – microglial cells, HDPC – human dental pulp cells

The algae of the genus Eisenia and Ecklonia have been the most studied in this context, perhaps due to their wide distribution and ecological importance. The majority of the phlorotannins obtained from this kind of seaweeds have revealed the ability to inhibit the expression of cellular adhesion molecules, like, ICAM-1 and VCAM-1, and cytokines expression, namely, TNF-α, IL-1β and IL-6 in several cells of the immune system[89,124,126,132,135]. In addition, some authors have also reported that the phlorotannins dieckol, fucoforoeckol-A, 6,6′-bieckol and phlorofucofuroeckol A have been shown to be able of inhibiting the release of NO• and PG2, as well as the enzymes responsible for their

synthesis, i.e., iNOS and COX-2, respectively[10,132–134]; however, some authors only refer the inhibition of the release of NO•[125] and PG2[99], and others mediators, like, histamine[130], without any information about the enzymatic pathway involved. The anti-inflammatory capacity of these compounds also seems to involve the modulation of the transcription factors NF-kB and AP-1, which regulate of the gene expression of some proinflammatory mediators[128,131]. Yayeh et al.[13] reported that dieckol decreases the p-IKK α/β and p-IkB-α content, as well as the nuclear subunit p-NF-kBp65 in LPS stimulated murine macrophages[13]. Some researchers showed that in addition to the NF-kB inhibition, some compounds, like dieckol, fucofuroeckol-A, 6,6′-bieckol and phlorofucofuroeckol A, have been demonstrated to inhibit MAPK pathway[12,131–133,135], through the inhibition of ERK1/2 and JNK phosphorylation and with no alterations reported in p38 MAPK signalling[131,135]. In contrast, Jung et al.[132] have described that dieckol inhibited p38 kinase activation, whereas p-ERK-1/2 and p-JNK was not affected in LPS-stimulated microglial cells[132]. Phlorofucofuroeckol A also has the ability to inhibited activation of p38-MAPK and Akt, a downstream player of PI3K, in LPS-treated murine macrophages[12]. Akt phosphorylates and inhibit IKKα, activating the NF-kB pathway, and so the transcription of target proinflammatory genes occurs. In addition to phlorofucofuroeckol A, dieckol also showed the ability to decrease the levels of p-PI-3K and p-Akt[131].

Although other compounds, such diphlorethohydroxycarmalol, which is exclusively isolated from *Ishige okamuarae*. This compound showed potent anti-inflammatory effects on LPS-stimulated Raw 264.7 macrophages through the inhibition of the signal transducer and activator of transcription 5 (STAT5), and by increasing the suppressor of cytokine signalling 1 (SOCS1)[136]. The SOCS1 is a negative feedback regulator of JAK-STAT signalling. In this study the inhibition of JAK2-STAT5 was accompanied by the inhibition of NF-kB, with the consequent reducing of the pro-inflammatory cytokine IL-6, but not TNF-α[136].

These compounds are capable of inhibiting the expression of numerous cytokines and other pro-inflammatory mediators, like NO• and PG2, and their respective enzymes. However, many studies have also shown the inhibition of numerous signalling pathways, like NF-kB, MAPK and Akt-PI3K pathway. In addition, there are some reports where phlorotannins, demonstrate the ability of inhibiting transcriptional factors, such as NF-kB and STAT5, preventing the formation of pro-inflammatory mediators. In short, phlorotannins show great

potential as anti-inflammatory compounds and therefore their inclusion in the development of new drugs is a very probable outcome in the near future.

1.4 SCOPE OF THIS WORK

Phytochemicals from brown macroalgae origin, including phlorotannins, are under the spotlight of much research due to their numerous potential therapeutic properties, which include antioxidant effects, through scavenging of ROS or enhancement of intracellular antioxidant defences, and anti-inflammatory effects, through inhibition of several pro-inflammatory mediators. In parallel, new extracting methodologies applied to these compounds are also been investigated, having in mind the improve of its efficiency and greenness. Notably, most of such studies were carried out on algae from the genus *Ecklonia* and *Eisinia* while numerous other species of brown seaweed remain to be studied regarding their potential to serve as a source of bioactive compounds, including of phlorotannins.

Based on these challenges, the main aims of this work were:

i) Optimize the extraction process of phlorotannins from *F. vesiculosus* using MAE technique and a green solvent, namely ethanol;

ii) Characterize the main bioactive components obtained from *F. vesiculosus* under the optimized MAE conditions;

iii) Evaluate the biological potentialities, namely antioxidant and anti-inflammatory activity, of crude extract obtained under optimized MAE conditions and/or its purified fractions.

CHAPTER 2. EXPERIMENTAL SECTION

2.1 Chemicals

Ground *F. vesiculosus* from July 2017 was purchased from Algaplus Lda. Acetone, ethanol, methanol, *n*-hexane, ethyl acetate, acetonitrile HPLC grade, hydrochloric acid, glacial acetic acid, sodium hydroxide and N-(1-naphtyl)ethylenediamine dihydrochloride were acquired from Fisher Chemical (Pittsburgh, PA, USA). The 2,4-dimethoxybenzaldehyde (DMBA), phloroglucinol, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS-NH_4), Nitrotetrazolium Blue chloride (NBT), Phenazine methosulfate (PMS), Dulbecco's Modified Eagle Medium (DNEM), lipopolysaccharide (LPS) from *E. coli* — serotype 026:B6, resazurin, phosphate buffered saline (PBS) reagents (sodium salt, sodium chloride, potassium chloride, disodium hydrogenophosphate and potassium dihydrogenophosphate), radioimmunoprecipitation assay (RIPA) buffer (Tris–HCl, Nonidet P-40, sodium chloride, sodium deoxycholate, sodium dodecyl sulfate and ethylenediamine tetraacetic acid), ascorbic acid and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium persulfate, potassium di-hydrogen phosphate, phosphoric acid and Gallic acid were acquired from Panreac (Barcelona, Spain). Beta-nicotinamide adenine dinucleotide (β-NADH), Sodium nitroprusside (SNP) acid and sulfanilamide were purchased from Acros Organics (Hampton, NH, USA) and dimethyl sulfoxide (DMSO) were acquired from Honeywell Riedel-de Haën (Charlotte, NC, USA). All reagents were of analytical grade or of the highest available purity.

2.2 Methods

2.2.1 Single-factor experiments using microwave-assisted extraction (MAE)

The method development for the extraction of seaweed phlorotannins by MAE was based on the work of He et al.[29]. The extraction process was performed by systematically varying one condition at a time, namely the concentration of ethanol (0, 20, 40, 60, 80, 100% (v/v)), the solvent-solid ratio (40, 60, 80, 100, 120, 140 and 160 (mL/g)), extraction temperature (25, 50, 75, 100, 125 and 150 °C) and irradiation time (1, 3, 5, 10, 15, 20 and 25 min). When one variable was not studied, it was kept constant. The constant values for irradiation time, solvent-solid ratio and microwave power were 20 min, 40 mL/g and 400 W, respectively, and samples were heated to the target temperature within a 2-minute ramp. After extraction, the extract was recovered by filtration through cotton to remove the solid residues and then

through a G4 glass filter and collected in a falcon tube to be analysed the TPhC as described in section 2.2.5.1. Experiments were performed with a focused microwave system with an Ethos MicroSYNTH Microwave Labstation (Milestone Inc.) using an 80 mL reactor at atmospheric pressure and samples were stirred under constant agitation throughout the extraction process.

2.2.2 Experimental design for optimization of phlorotannins microwave-assisted extraction

An RSM based on a three-level-three-factor Box-Behnken experimental design (BBD) was employed in this study to optimize the phlorotannin extraction process, namely considering the effects of solvent concentration (%, v/v, X_1), temperature (°C, X_2) and extraction time (min, X_3). The factor levels of these three variables were coded as -1 (low), 0 (central point or middle) and +1 (high), respectively, according to the single-factor tests outlined above (**Table 4**).

Table 4. Independent variables and their coded levels used in the BBD.

Symbols	Independent Variables	Levels		
		-1	0	+1
X_1	Solvent concentration (%, v/v)	40	60	100
X_2	Temperature (°C)	75	100	125
X_3	Time (min)	1	3	5

A total of 15 different experiments, including three replicates at central point (**Table 5**), were conducted in a randomized order. Using the response surface methodology, the experimental design and analysis of variance (ANOVA) were carried out in the statistical software JMP, version 10.0.0, to generate the following second order polynomial equation that represents the total phlorotannin content as a function of the coded independent variables:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_1 X_1 + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{k} \beta_{ij} X_i X_j$$

were Y is the predicted response; β_0 is the constant coefficient; β_i, β_{ii}, β_{ij} are the linear, quadratic and interactive coefficients of the model, respectively; and X_i and X_j are the coded independent variables.

Table 5. Box-Behnken experiment design matrix.

Extract No.	Independent Variables		
	X_1	X_2	X_3
1	40	125	3
2	60	100	3
3	80	125	3
4	60	75	5
5	80	100	5
6	60	125	1
7	80	75	3
8	60	100	3
9	40	100	1
10	40	100	5
11	60	125	5
12	60	75	1
13	40	75	3
14	60	100	3
15	80	100	1

Model adequacy was evaluated using the coefficient of determination (R^2) and the lack-of-fit test represented at 5% level of significance, accordingly. Three-dimensional response surface plots and two-dimensional contour plots were used for visualization of the effects of independent variables and their mutual interactions in the responses. To validate the accuracy of the models, triplicate experiments were carried out at the optimal conditions predicted for TPhC, and the obtained experimental data were compared to the values predicted by the corresponding regression model.

2.2.3 Extraction and purification of phlorotannins under optimal MAE and conventional solvent extraction

The MAE extract was prepared following the optimum conditions determined through the response surface method. Briefly, 0.6 g of dried algal powder were dispersed in 60 mL of

57% ethanol solution in the microwave flasks, and incubated in MAE for 5 min at 75 °C under constant agitation. Afterwards, the mixture of the different flasks were combined, obtaining a total of 3.6 g of dried algal powder dispersed in 360 mL. The conventional solvent extraction of *F. vesiculosus* was performed according to the optimum conditions established by Catarino et al.[16]. Briefly, 30 g of dried algal powder (DW) were dispersed in 2100 mL of 70% acetone solution with 1% of glacial acetic acid, and incubated for 3 h at room temperature under constant agitation. The combined mixture obtained with MAE and conventional solvent extraction was filtered through cotton to remove the solid residues and then through a G4 glass filter. Afterwards the extract was concentrated in a rotary evaporator to about 100 mL and further defatted using *n*-hexane (1:1, v/v) several times until a colourless nonpolar fraction was obtained. The resulting, aqueous phase was submitted to liquid-liquid extraction with ethyl acetate (1:1, v/v) also several times. Finally, the solvents from the three fractions, including aqueous residue, were removed by rotary evaporation. Dried hexane (Hex), ethyl acetate (EtOAC) and aqueous (Aq) fractions were subsequently stored at -20 °C until further analysis.

2.2.4 Characterization of the extracts

2.2.4.1 Determination of Total Phlorotannin Content (TPhC)

Quantification of total phlorotannins was carried out according to the 2,4-dimethoxybenzaldehyde (DMBA) colorimetric method previously described[17]. Briefly, equal volumes of the stock solutions of DMBA (2%, m/v) and HCl (6%, v/v), both prepared in glacial acetic acid, were mixed prior to use (work solution). Afterwards, 250 µL of this solution was added to 50 µL of each extract in a 96-wells plate and the reaction was incubated in the dark, at room temperature. After 60 min, the absorbance was read at 515 nm in an automated plate reader (Biotek Instrument Inc, USA) and the phlorotannin content was determined by using a regression equation of the phloroglucinol linear calibration curve (0.06 – 0.1 mg/mL). The results were expressed as mg phloroglucinol equivalents/g dry seaweed (mg PGE/g DW).

2.2.4.2 Characterization by UHPLC-DAD-ESI/MS analysis

Chromatographic analysis of phlorotannins and pigments of the purified fractions (Hex and EtOAc dissolved in methanol and Aq dissolved in water) from *F. vesiculosus* were filtered throught a nylon filter of 0.22 µm (Whatman™, Buckinghamshire, UK) and further was

carried out in Ultimate 3000 (Dionex Co., San Jose, CA, USA) apparatus consisting of an autosampler/injector, a binary pump, a column compartment and an ultimate 3000 Diode Array Detector (Dionex Co.,San Jose, CA, USA), coupled to a Thermo LTQ XL (Thermo Scientific, San Jose, CA, USA) ion trap mass spectrometer equipped with an ESI source. The LC separation was conducted with a Hypersil Gold (ThermoScientific, San Jose, CA, USA) C18 column (100 mm length; 2.1 mm i.d.; 1.9 μm particle diameter, end-capped) maintained at 30 °C.

The analysis of phlorotannins was performed according to the described by Catarino et al.[16] using a binary solvent system composed of (A) acetonitrile and (B) 0.1% of formic acid (v/v) for phlorotannins. The solvent gradient started with 5–40% of solvent (A) over 14.72 min, from 40–100% over 1.91 min, remaining at 100% for 2.19 more min before returning to the initial conditions for the phlorotannins analysis and solvent gradient started with 15–28% of solvent B over 3.9 min, increasing to 100% in 2.2 min and maintaining this value up to 25 min, followed by the return to the initial conditions for the pigments and carotenoids analysis. The flow rate was 0.2 mL min^{-1} and UV–Vis spectral data for all peaks were accumulated in the range of 200–700 nm while the chromatographic profiles were recorded at 280 nm. Control and data acquisition of MS were carried out with the Thermo Xcalibur Qual Browser data system (ThermoScientific, San Jose, CA, USA). Nitrogen above 99% purity was used, and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative mode with the ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 275 °C. The full scan covered the mass range from m/z 100 to 2000.

The analysis of pigments was performed according to the described by Silva et al.[137] using a gradient elution of 0.1% of formic acid (v/v) (solvent A) and acetonitrile:methanol (70/30) (solvent B). The solvent gradient consisted of a series of linear gradients, starting with 15–28% of solvent B over 3.9 min, increasing to 100% in 2.2 min and maintaining this value up to 25 min, followed by the return to the initial conditions, with a total running time of 20 min. The chromatographic profiles were recorded at 655 and 450 nm. Control and data acquisition of MS system was as described above for phlorotannins analysis.

2.2.5 Antioxidant properties

2.2.5.1 $ABTS^{\bullet+}$ discoloration assay

The total antioxidant activity of the crude extract and subsequent purified fractions were measured using an adaptation of the $ABTS^{\bullet+}$ discoloration assay based on the procedure described by Catarino et al.[28]. Briefly, a stock solution of $ABTS^{\bullet+}$ was prepared by reacting the ABTS-NH_4 aqueous solution (7 mM) with 2.45 mM potassium persulfate in the dark at room temperature for 12–16 h to allow the completion of radical generation. This solution was then diluted with distilled water until its absorbance reached 0.70 ± 0.05 at 734 nm. Afterwards, 50 µL of each sample (EtOAC and Hex dissolved in DMSO, Aq dissolved in water and crude dissolved in 57% ethanol) were mixed with 250 µL of the diluted $ABTS^{\bullet+}$ solution in a 96-well microplate. The mixture was then allowed to react for 20 min in the dark, at room temperature and the absorbance was then measured at 734 nm in an automated plate reader (Biotek Instrument Inc, USA). The percentage of inhibition of $ABTS^{\bullet+}$ was calculated for the crude extract and purified fractions using the equation described by Yen and Der Duh[138] as follows:

$$\%\ ABTS^{\bullet+}\text{scavenging} = \frac{A_c - A_e}{A_c} \times 100$$

where Ac is the absorbance of the control (without extract addition) and Ae is the absorbance of the extract. Ascorbic acid was used as the reference compound. The concentration of the extract/standard able to inhibit 50% of $ABTS^{\bullet+}$ (IC_{50}) was then calculated by plotting the percentage of inhibition against the plant extract concentrations. Moreover, attending that ethanol, DMSO and water interfere on the quantification method, ascorbic acid tested in the same solvent conditions was used as reference and the results were expressed as mg of ascorbic acid equivalents per mL (mgAAE/mL).

2.2.5.2 Superoxide scavenging assay

In a 96 well plate, 75 µL of nitroblue tetrazolium (NBT), 100 µL of β-NADH, 75 µL of each sample (EtOAC and Hex dissolved in DMSO, Aq dissolved in water and crude dissolved in 57% ethanol) and 75 µL of phenazine methosulfate (PMS) were mixed and incubated for 5 min, at room temperature. The absorbance was then measured at 560 nm in an automated plate reader (Biotek Instrument Inc, USA). The IC_{50} value for superoxide scavenging activity was determined by plotting the percentage of inhibition of superoxide radicals generation in

the presence of the crude extract and subsequent purified fractions and calculated using the equation described by Yen and Der Duh[138] previously mentioned. Moreover, attending that ethanol, DMSO and water interfere on the quantification method, gallic acid tested in the same solvent conditions was used as reference and the results were expressed as mg of galic acid equivalents per mL (mgGAE/mL).

2.2.5.3 Chemical NO• scavenging assay

The NO• scavenging method was adapted from Catarino et al.[28]. Briefly, in a 96 well plate, 100 µL of each sample (EtOAC and Hex dissolved in DMSO, Aq dissolved in water and crude dissolved in 57% ethanol) were mixed with 100 µL of sodium nitroprusside (3.33 mM in 100 mM sodium phosphate buffer pH 7.4) and incubated for 15 min under a fluorescent lamp (Tryun 26 W). Afterwards, 100 µL of Griess reagent (0.5% sulphanilamide and 0.05% naphthyletylenediamine dihydrochloride in 2.5% H_3PO_4) were added to the mixture, which was allowed to react for another 10 min in the dark. The absorbance was then measured at 562 nm in an automated plate reader (Biotek Instrument Inc, USA). The IC_{50} value for NO• scavenging activity was determined by plotting the percentage of inhibition of nitrite generation in the presence of the crude extract and subsequent purified fractions and calculated using the equation described by Yen and Der Duh[138], previously mentioned. Moreover, attending that ethanol, DMSO and water interfere on the quantification method, ascorbic acid tested in the same solvent conditions was used as reference and the results were expressed as mg of ascorbic acid equivalents per mL (mgAAE/mL).

2.2.6 Anti-inflammatory properties

2.2.6.1 Cell culture

The murine macrophage cell line RAW 264.7, from American Type Culture Collection (ATCC number: TIB-71), was cultured in DMEM supplemented with 10% (v/v) non-inactivated fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Along the experiments, cells were monitored by microscopic observation in order to detect any morphological change.

2.2.6.2 Determination of cell viability by resazurin assay

Assessment of metabolically active cells was performed using 7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt (resazurin) colorimetric assay as previously reported[139]. Briefly,

RAW 264.7 cells (60×10^5 cells/well) were plated and allowed to stabilize for 12 h in 200 μL medium in 96 well plates. Following this period, cells were either maintained in culture medium (control) or incubated with 5 different concentrations (0.006 – 0.100 mg/mL) of the fractions to be tested. After 20 h, a resazurin stock solution (500 μM in phosphate buffered saline) was added to a final concentration of 50 μM and cells further incubated at 37 °C for 3 h, in a humidified atmosphere of 95% air and 5% CO_2. The bioreduction of the dye was quantified by absorbance measurement at 570 nm, with a reference wavelength of 620 nm in an automated plate reader (Biotek Instrument Inc, USA).

2.2.6.3 Measurement of nitrite production

The production of nitric oxide ($NO^{\bullet}$) was measured by the accumulation of nitrite in the culture supernatants, using a colorimetric reaction with the Griess reagent[140]. Briefly, 100 μL of culture supernatants were diluted with equal volumes of Griess reagent [0.1% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride and 1% (w/v) sulphanilamide containing 5% (w/v) H_3PO_4] and maintained during 30 min, in the dark. The absorbance at 550 nm was measured in an automated plate reader (Biotek Instrument Inc, USA). Culture medium and LPS-stimulated culture medium was used as controls and nitrite concentration was determined from the percentage of the control for the different concentrations (0.006 – 0.100 mg/mL).

2.2.6.4 Cell lysates and Western blot analysis

A Raw 264.7 cells (60×10^5 cells/well) were plated and, after stabilizing for 12 h, cells were either maintained in culture medium (control), or pre-incubated with 0.100 mg/mL of EtOAc fraction, or 0.050 mg/mL of Hex fraction - concentrations that inhibited $NO^{\bullet}$ generation and were noncytotoxic, for 1 h and then 50 ng/mL LPS was added. Cells were lysed with RIPA buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 2 mM ethylenediamine tetraacetic acid) freshly supplemented with 1 mM dithiothreitol, protease and phosphatase inhibitor cocktails and incubated for 30 min in ice. The nuclei and the insoluble cell debris were removed by centrifugation at 4 °C, at 12.000×g for 10 min. The postnuclear extracts were collected and used as total cell lysates. Protein concentration was determined by the RC-DC™ Protein Assay (Bio-Rad, CA, USA). Briefly, to 5 μL of cell lysates or protein standard (bovine serum albumin; concentrations ranging from 0.1 to 10.0 mg/mL), 50 μL of Reagent A' (mixture of

reagent S with reagent A in a 20 μL:1 mL ratio), was added, which was followed by mixture, and 400 μL of Reagent B was added, followed by mixture. Afterwards, 200 μL of each solution was added in a 96-wells plate and incubated in the dark, at room temperature. After 15 minutes, the absorbance was measured at 750 nm in an automated plate reader (Multiskan GO, Thermo Scientific, MA, USA). For the Western blot analysis, equivalent amounts of protein (40 μg) of each sample, earlier precipitated with cold (- 20 °C) acetone, were diluted in 15 μL of loading buffer (125 mM Tris, pH 6.8; 4% SDS (w/v); 15% glycerol (v/v); 20% β-mercaptoethanol (v/v); 0.1% bromophenol blue) and incubated at 100 ºC for 5 minutes. Subsequently, loaded samples were electrophoresed in 12.5% polyacrylamide gels as described by Laemmli[141]. After adequate separation, gels were blotted onto a nitrocellulose membrane (Amersham™ Protan™, GE Healthcare Lifesciences) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 2 hours at 200 mA. After membrane preparation, non-specific binding was blocked for 1h in 5% (w/v) non-fat dry milk in TBS-T (100mM Tris, 1.5 mM NaCl, 0.5% Tween 20). Immediately, membranes were incubated with primary antibody, diluted 1:1000 in 5% (w/v) non-fat dry milk in TBS-T, for 1 hour at room temperature, followed by overnight incubation at 4ºC (mouse anti-iNOS, (MAB9502), R&D Systems; goat anti-COX2, sc-1746, Santa Cruz; rabbit anti-p38-MAPK,(# 9212S) Cell Signalling Technology; rabbit anti-NF-kB-p65, (ab16502), Abcam; rabbit anti-p-IKB-α, 2859S, Cell Signaling Technology; all antibodies are polyclonal, unless otherwise stated). Thereafter, membranes were washed 3 x 10 minutes in TBS-T, which was followed by incubation with secondary HRP-conjugated anti-rabbit (GE Healthcare Life Sciences; diluted 1:1000 in 5% (w/v) non-fat dry milk in TBS-T) for 1 hour at room temperature. Finally, membranes were again washed 3 x 10 minutes in TBS-T and immunoreactive bands were detected by enhanced chemiluminescence (WesternBright™ ECL, Advansta) according to the manufacturer's procedure. Images were recorded using a ChemiDoc™ Imaging System (Bio-Rad) and analysed with Image Lab (version 5.0, Bio-Rad). The optical densities obtained were expressed in arbitrary units. Equal protein loading was confirmed by Ponceau S staining or with β-actin antibody (rabbit anti- β-actin, ab8227, Abcam).

2.2.7 Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) of three similar and independent experiments performed in duplicate. JMP and Minitab software were used to

construct the BBD and to analyse the results. Data from single-factor experiments and BBD were analysed using ANOVA ($p<0.05$) followed by Tukey's *post hoc* test.

CHAPTER 3. RESULTS

3.1 SINGLE-FACTOR EXPERIMENTS ON MAE

This part of the study aimed to determine the optimal conditions for TPhC extraction by MAE with a greener solvent, namely ethanol, from the algae *Fucus vesiculosus*, using BBD. Taking into account the different variables that mainly could affect the phlorotannin extraction[27], preliminary single-factor experiments were realised to specify the selected factors in the BBD experiment.

Different concentrations of ethanol were tested in the range of 0 to 100% (v/v). According to **Figure 3** (A), the recovery of TPhC from *F. vesiculosus* increased almost proportionally between 20 to 60% ethanol (1.23 ± 0.031 to 1.59 ± 0.034 mg PGE/g DW_{algae}), being the maximum TPhC yield obtained for this last concentration. In turn, the use of ethanol above 60% resulted in a decrease of TPhC by approximately 1.40 mg PGE/g DW. Based on this, the concentration of ethanol to study the next variable was 60%. Moreover, considering these results, for the BBD experiment, an ethanol concentration range between 40-80% was selected.

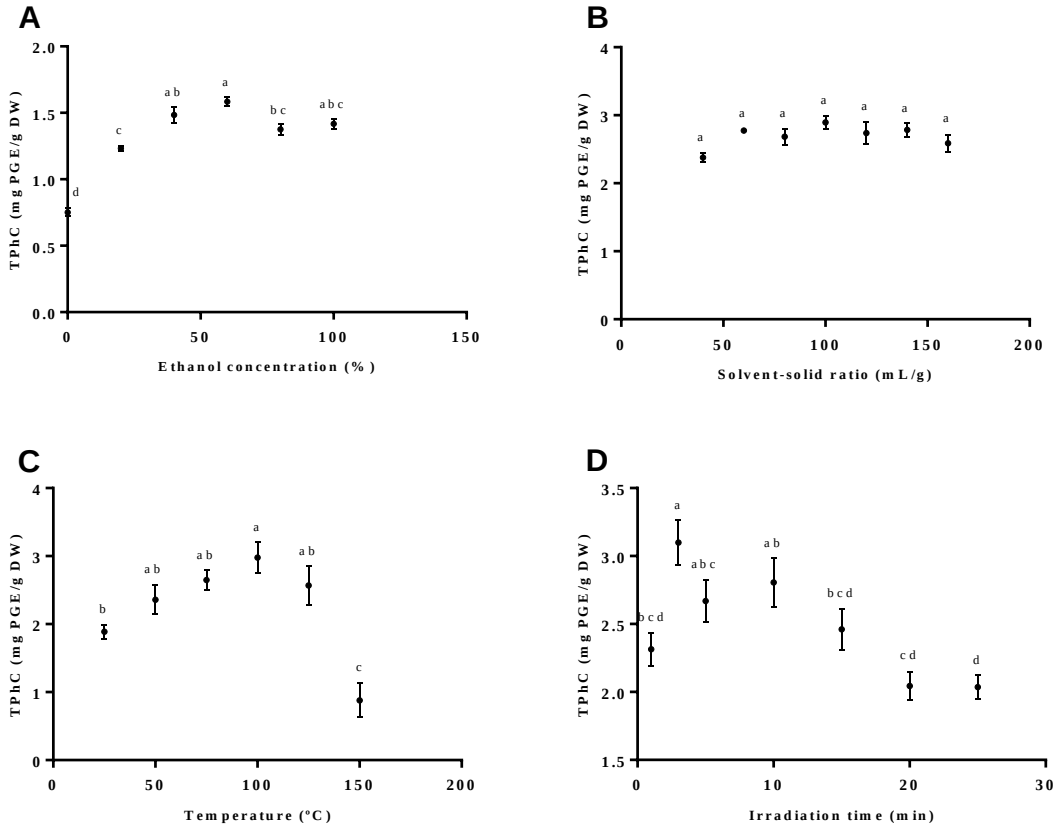


Figure 3. Effect of (A) ethanol concentration, (B) solvent-solid ratio, (C) temperature and (D) irradiation time on the recovery of phlorotannins from *F. vesiculosus*, in the single-factor experiments.

The effect of different solvent-solid ratios on the recovery of phlorotannins from *F. vesiculosus* was tested in the range of 40 to 160 mL/g. As represented in **Figure 3** (B), the variation of this parameter did not influence significantly the amount of TPhC, which accounted for approximately 2.7 mg PGE/g DW_{algae} from 60 to 160 mL/g. Yet, attending that a maximum point of TPhC was obtained at 100 mL/g (2.90 ± 0.091 mg PGE/g DW_{algae}), this solvent-solid ratio was selected for the following factors study and for the BBD experiment as well.

It is expected that temperature affects the extraction process of thermolabile compounds such as phlorotannins[35]. Taking this into account, different temperatures were selected between 25 to 150 ºC. As represented in **Figure 3** (C), a linear increase in the recovery of total phlorotannin content was obtained between 25 to 100 ºC (1.89 ± 0.104 to 2.98 ± 0.235 mg PGE/g DW_{algae}). However, temperatures of extraction above 100 ºC, namely 125 and 150 ºC, caused a decrease in TPhC (2.57 ± 0.28 and 0.878 ± 0.251 mg PGE/g DW_{algae}, respectively). Hence, for the analysis of the next variable, the temperature was set on 100 ºC, which presented the maximum TPhC yield. Additionally, for the BBD experiment, the interval chosen was 75-125 ºC.

Moreover, the influence of the irradiation time was considered for the interval of 1 to 25 min. As depicted in **Figure 3** (D), while the raising of irradiation time up to 3 min was paralelled by an increase in the amount of TPhC, the opposite tendency was registered for longer extraction periods (3.10 ± 0.168 to 2.04 ± 0.087 mg PGE/g DW_{algae}). Based on these results, the irradiation time interval selected for the BBD experiment was 1-5 min.

3.2 ANALYSIS OF THE RESPONSE SURFACE METHODOLOGY

3.2.1 Fitting the model

The experimental values obtained for the TPhC recovery, represented in **Table 6**, were fitted to a quadratic polynomial model (equation (1)). This equation allowed the determination of the optimum conditions for the extraction process, in order to obtain the maximum

phlorotannin recovery, and also determine the different correlations, which are related to the independent variables interactions, and respective responses.

Table 6. Experimental TPhC values obtained from Box-Behnken design matrix.

Extract No.	Independent Variables			Experimental TPhC (mg PGE/g DW_{algae})
	X_1	X_2	X_3	
1	40	125	3	1.17
2	60	100	3	2.58
3	80	125	3	1.61
4	60	75	5	3.09
5	80	100	5	1.99
6	60	125	1	2.37
7	80	75	3	2.16
8	60	100	3	2.58
9	40	100	1	2.60
10	40	100	5	1.95
11	60	125	5	0.85
12	60	75	1	2.52
13	40	75	3	2.42
14	60	100	3	2.58
15	80	100	1	2.35

The experimental data allowed the determination of the coefficients of the model, which were evaluated for statistical significance using statistical analysis of variance (ANOVA) and are listed in **Table 7**. Accordingly, the independent variables with higher impact in the TPhC were the temperature ($p<0.001$) and time ($p<0.01$), while the ethanol concentration revealed no effect. Moreover, significant interactive effects between ethanol concentration and temperature ($p<0.05$) and between temperature and time ($p<0.001$) were observed. The variables ethanol concentration and temperature also showed a significant quadratic effect on the phlorotannin recovery ($p<0.01$, for both).

$$TPhC = 2.58 - 0.004X_1 - 0.52X_2 - 0.24X_3 - 0.36{X_1}^2 - 0.38{X_2}^2 - 0.0001{X_3}^2 + 0.17X_1X_2 + 0.073X_1X_3 - 0.36X_2X_3 \quad (1)$$

The statistical analysis revealed a high F-value (43.77) and simultaneously, a low *p*-value ($p<0.001$), meaning that the model is significant. Furthermore, the coefficient of multiple

determination (R^2) for the response TPhC was 0.99 and the adjusted determination coefficient (R^2_{Adj}) was 0.96, since these values are similar, suggesting that there is a good correlation between the observed and predicted values for TPhC. Taking this into account, the fitted model may be assumed as trustworthy and capable to predict the TPhC response.

Table 7. Regression coefficients and results of ANOVA analysis of the model.

Parameter	Regression Coefficient
β_0	2.58 ***
X_1	-0.004
X_2	-0.52 ***
X_3	-0.24 **
$X_1 X_2$	0.17 *
$X_1 X_3$	0.073
$X_2 X_3$	-0.52 ***
$X_1 X_1$	-0.36 **
$X_2 X_2$	-0.38 **
$X_3 X_3$	-0.0001
R^2	0.99
R^2_{Adj}	0.96
Model F-value	43.77
Model *p*-value	<0.001

β_0 – constant coefficient; X_1 – ethanol concentration (%); X_2 – temperature (°C); X_3 – time (min). *, **, *** represent statistical significance with $p<0.05$; 0.01 and 0.001; respectively.

3.2.2 Effect of the independent variables on the TPhC

The effects of the independent variables and their mutual interactions on the TPhC can be visualized on the three-dimensional response surface plots and two-dimensional contour plots shown in **Figure 4**, respectively. Each plot demonstrates the effects of two independent variables on the target response while the third variable is maintained at its zero level.

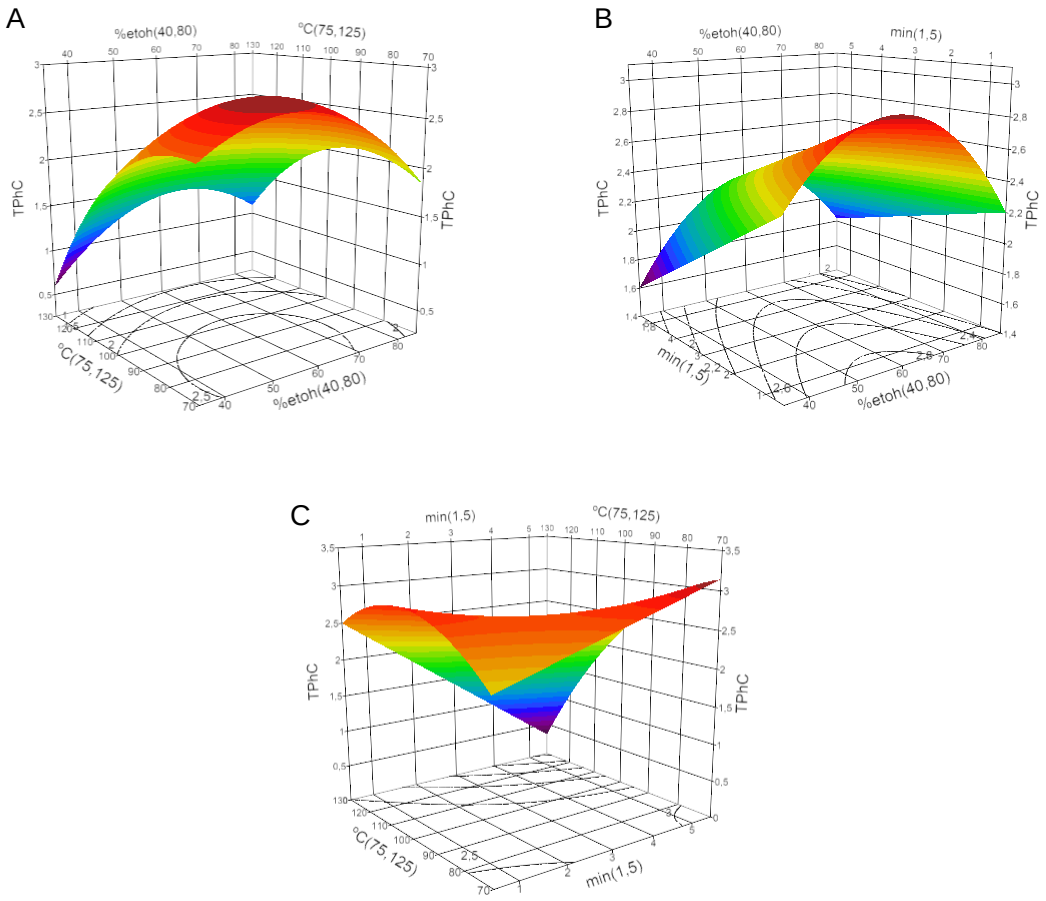


Figure 4. Response surface and contour plots for the total phlorotannin content (TPhC in mg PGE/g DW_{algae}) from *F. vesiculosus* extracts with respect to (A) ethanol concentration (%, X_1) and temperature (°C, X_2); (B) ethanol concentration (%, X_1) and time (min, X_3); (C) temperature (°C, X_2) and time (min, X_3). The third variable of each graph was kept at its zero level.

According to the results of the regression coefficient shown in **Table 7**, the interaction between ethanol concentration *versus* temperature, and temperature *versus* time revealed a significant effect on the TPhC ($p<0.05$ and $p<0.001$, respectively). As observed in **Figure 4** (A), there was an increase of the TPhC for variations of temperatures between 125 to 75 °C, and of ethanol between 50 and 60%. In addition, both independent variables, ethanol concentration and temperature, had a significant quadratic effect ($p<0.01$, for both). **Figure 4** (B) also demonstrated an increase of TPhC for short time extractions (1 to 5 min) and a quadratic effect for ethanol concentrations between 50 and 70%. Moreover, a higher level of TPhC was obtained at lower temperatures (75 °C) and for longer time (5 min) (**Figure 4** (C)). As well, the quadratic effect of temperature was also registered in this figure.

3.2.3 Optimization and validation of the models

The optimum MAE conditions for the extraction of phlorotannins from *F. vesiculosus* were determined according the quadratic polynomial model (equation (1)). The predicted conditions were ethanol concentration at 57% (v/v), temperature at 75 °C and time at 5 min, with a theoretical maximum value of phlorotannin content of 3.01 ± 0.25 mg PGE/g DW_{algae}. These optimum conditions mentioned above were tested to validate the adequacy of the model prediction, in triplicate, and the experimental value of 3.16 ± 0.06 mg PGE/g DW_{algae} were obtained for TPhC. The results presented demonstrate a good correlation between the experimental and the predicted values, confirming the appropriateness of this model, which is trustworthy and precise.

3.3 COMPARISON BETWEEN MAE AND CONVENTIONAL SOLVENT EXTRACTION

In order to conclude about the feasibility of using MAE and ethanol as a alternative extraction method for the extraction of phlorotannins from *F. vesiculosus*, the yields of mass and of TPhC obtained under optimal conditions of MAE and of conventional solvent extraction (as previously established by Catarino et al.[16] i.e., a solvent-solid ratio of 70 mL/g with a 70% of acetone with 1% of glacial acetic acid at room temperature for 3 h), were compared for the crude extracts. Moreover, the richness of TPhC in the crude extracts and in the corresponding purified fractions, *n*-hexane (Hex), ethyl acetate (EtOAc) and the aqueous residue (Aq) were evaluated (**Table 8**).

Table 8. Mass yield and total phlorotannin content of the crude extract and the purified fractions obtained by MAE and conventional solvent extraction of *F. vesiculosus*.

Extraction method	Sample	Yield (%)*	TPhC (mg PGE/g $DW_{extract}$)
MAE	Crude extract	31.29 ± 4.99	9.75 ± 1.76
	Hex	2.39	1.68 ± 0.83
	EtOAc	2.17	1.01 ± 0.18
	Aq	29.00	4.88 ± 0.57
Conventional	Crude extract	26.36 ± 4.29	10.68 ± 1.48
	Hex	4.68 ± 0.22	4.38 ± 0.79

	EtOAc	0.93 ± 0.03	17.02 ± 2.13
	Aq	22.68 ± 0.76	3.94 ± 0.18

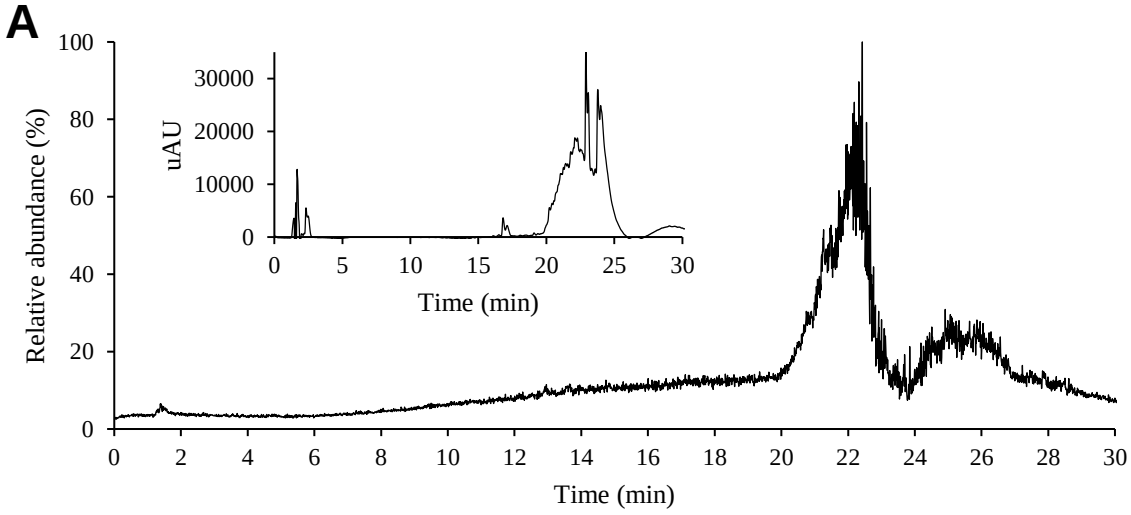
*Results are expressed as w/w of algal powder.
Hex – *n*-Hexane fraction; EtOAc – ethyl acetate fraction and Aq – aqueous fraction

Under optimal conditions, the mass yield of the crude extracts obtained by the two distinct methods were close to 30%, albeit tending to be superior in MAE technique. Atending to this similar mass yield, and to the equal amount of recovered phlorotannins in the two methods, namely 3.16 ± 0.17 mg PGE/g DW_{algae} and 3.16 ± 0.74 mg PGE/g DW_{algae} for MAE and conventional solvent extraction, respectively (data not shown), overall the TPhC of the crude extracts were close (about 10.68 ± 1.48 mg PGE/g $DW_{extract}$), albeit tending to be higher in the conventional method.

Surprisingly, contrarily to what was observed for the conventional crude extract (for which the purification process resulted in a concomitant loss of mass but an enrichment in phlorotannins in the EtOAc fraction), both mass yields and TPhC in MAE EtOAc fraction were simillar to those of the Hex fraction.

3.4 UHPLC-DAD-ESI/MS CHARACTERIZATION OF THE PURIFIED FRACTIONS

The different purified fractions were submitted to UHPLC-DAD-ESI/MS analysis for further characterization. The chromatographic profile of the Hex, EtOAc and Aq fraction from MAE are represented in **Figure 5**.



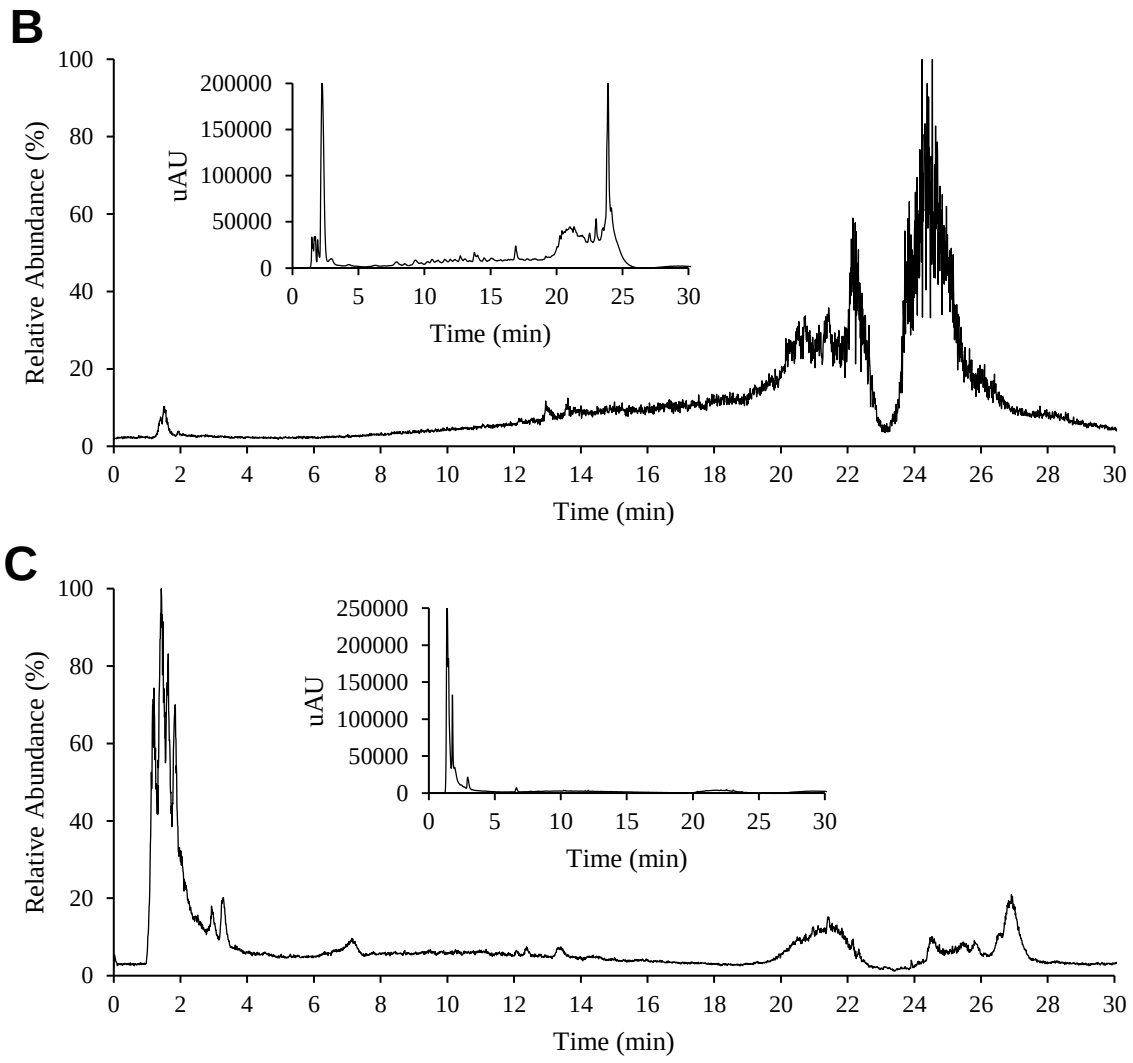


Figure 5. Total ion and UV at 280 nm (inset) chromatographic profiles and of (A) Hex fraction (B) EtOAc fraction and (C) Aq fraction from *F. vesiculosus*

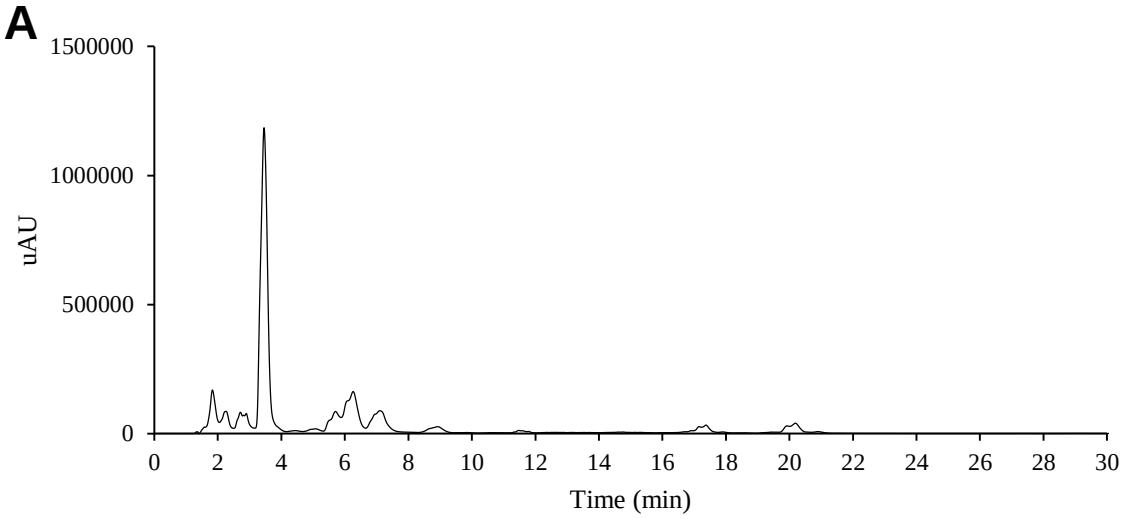
No clear separation of signal was obtained in the UV chromatogram and TIC, most probable due to the low amount of multiple phlorotannins in the fractions, even upon the purification procedure (1 - 5 mg PGE/$DW_{extract}$) and to the low ionization ability of tannins in mass spectrometry. Nonetheless, the presence of phlorotannins in the EtOAc and Aq fractions was suggested by the UV spectrum of distinct chromatographic zones that presented a UVmax close to 270 nm (data not shown)[142]. Taken this, the identification of phlorotannins in the MAE purified fractions was performed in Selected Ion Monitoring (SIM) mode, at *m/z* of known phlorotannins previously described for *F. vesiculosus* by Catarino et al.[16]. In agreement with the UV spectrum data typical of phlorotannins in Hex and Aq fractions, some $[M-H]^-$ corresponding to such compounds were also detected, while they were absent from the Hex fraction (**Table 9**).

Table 9. Possible identification of phlorotannins present in the different purified fractions Hex, EtOAc and Aq fraction.

Retention time (min)	$[M-H]^-$ *m/z*	Probable compound	Hex	EtOAc	Aq
2.90	247	Dibenzodioxine-1,3,6,8-tetraol	-	-	+
11.08	497	Tetrafucol	-	+	+
	529	Hydroxytetrafuhalol	-	+	+
11.6	317	Phlorotannin derivative	-	+	+
12.2	610	Unidentified	-	+	+
13.0	723	Unidentified	-	+	-
13.5	837	Unidentified	-	+	+
	869	Trifucotriphlorethol	-	+	+
	899	Phlorotannin derivative	-	+	-
14.24	950	Unidentified	-	+	-
15.0	587	Unidentified	-	+	-
16.6	603	Fucofurotriphlorethol	-	-	-

+ - detected; - - not detected

The analysis of UV spectra of the last part of the chromatogram (22-24 min) also revealed data compatible with the presence of pigments (**λ_{max}** in visible**)**, namely in the Hex and EtOAc fractions. Taking this into account, these two fractions were also analysed by a method of analysis apropriate for pigments separation. As can be observed in **Figure 6** and **Table 10**, this analysis allowed to conclude that the Hex fraction dominated by fucoxanthin.



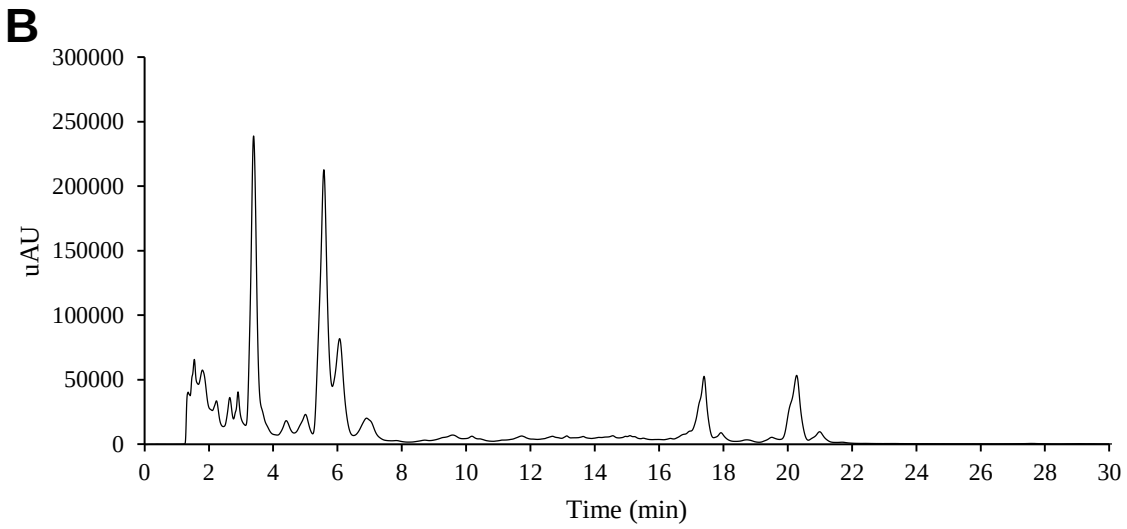


Figure 6. UV spectrum at 430 nm of (A) Hex fraction and (B) EtOAc fraction

In addition to fucoxanthin, the EtOAc had others relevant pigments, namely pheorbidin *a*, 15-hydroxy-lactone chlorophyll *a*, pheophytin *b* and pheophytin *a*.

Table 10. Possible identification of some pigments present in Hex and EtOAc fractions.

Retention time (min)	**λ_{max}**	**$[M-H]^+$ *m/z***	**Pobable compound**	**Hex**	**EtOAc**
2.9	449, 583, 632	609.4	Chlorophyll *c*	+	-
3.4	448, 465	659.3	Fucoxanthin	+	+
5.7	408, 607, 665	593.4	Pheorbidin *a*	+	+
6.9	418, 610, 653	925.4	15-hydroxy-lactone chlorophyll *a*	-	+
17.4	434, 610, 652	885.7	Pheophytin *b*	+	+
20.3	408, 608, 665	871.7	Pheophytin *a*	+	+

+ - detected; - - not detected

3.5 ANTIOXIDANT ACTIVITIES

The MAE crude extract and its purified fractions were evaluated for their potential antioxidant capacity throught different chemical methods, namely $ABTS^{\bullet+}$, superoxide and $NO^{\bullet}$ scavenging assays.

According to the results shown in **Figure 7** (A), all samples exhibited considerable potential to scavenge the $ABTS^{\bullet+}$, with 50% of inhibition being achieved for concentrations bellow 0.2 mg/mL. When expressing data in mg of AAE/mL (to eliminate solvent reaction interferences), it is clear that the sequency of potency was EtOAc>Hex>Aq>Crude. Moreover, please note that the potency of EtOAc fraction was about twice that of Hex and close to ten times that of the crude extract.

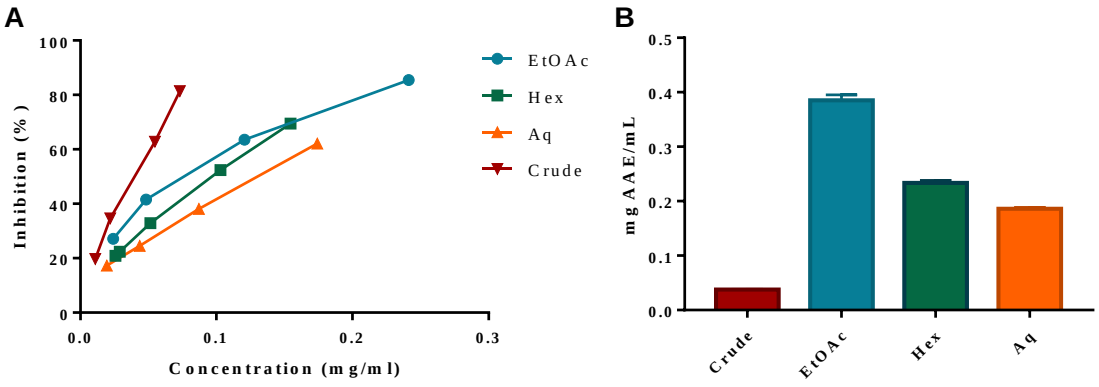


Figure 7. Ability of MAE crude extract and purified fractions to scavenge $ABTS^{\bullet+}$ radical, expressed as (A) percentage of inhibition (mg/mL) and (B) mg AAE/mL. The samples were dissolved in DMSO (EtOAc and Hex), water (Aq) or in 57% ethanol (crude), as described in 2.2.5.1.

All the purified fractions also showed considerably more ability to scavenge the superoxide radical than the crude extract (**Figure 8** (A)), albeit this was clearly inferior to that of the commercial antioxidant (IC_{50} values of 0.420 ± 0.008 mg/mL versus 0.004 mg/mL for EtOAc and ascorbic acid, respectively under the same experimental conditions).

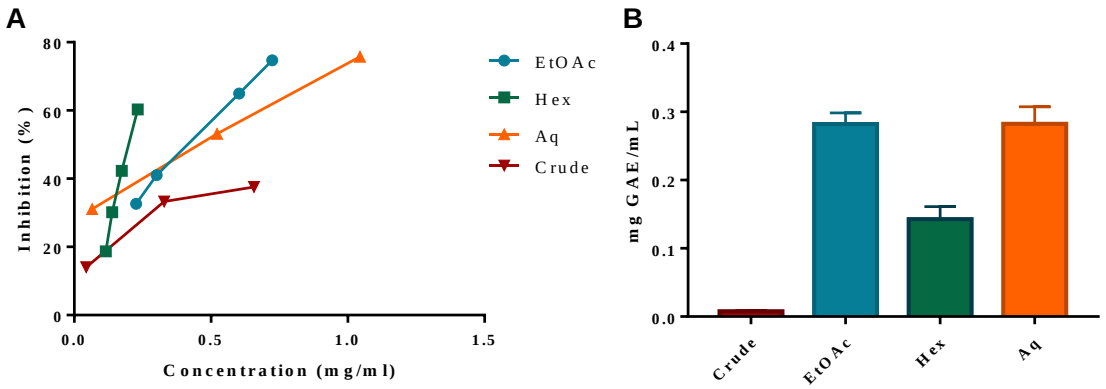


Figure 8. Ability of MAE crude extract and purified fractions to scavenge superoxide radical, expressed as (A) percentage of inhibition (mg/mL) and (B) mg GAE/mL. The samples were dissolved in DMSO (EtOAc and Hex), water (Aq) or in 57% ethanol (crude), as described in 2.2.5.2.

In turn, all samples exhibited high potential to scavenge $NO^{\bullet}$ (**Figure 9**). This was particular relevant for EtOAc and Hex fractions, for which values were about twenthy five and ten times that of the crude extract, respectively (IC_{50} values of 0.115 ± 0.005 and 0.102 ± 0.005 mg/mL, respectively).

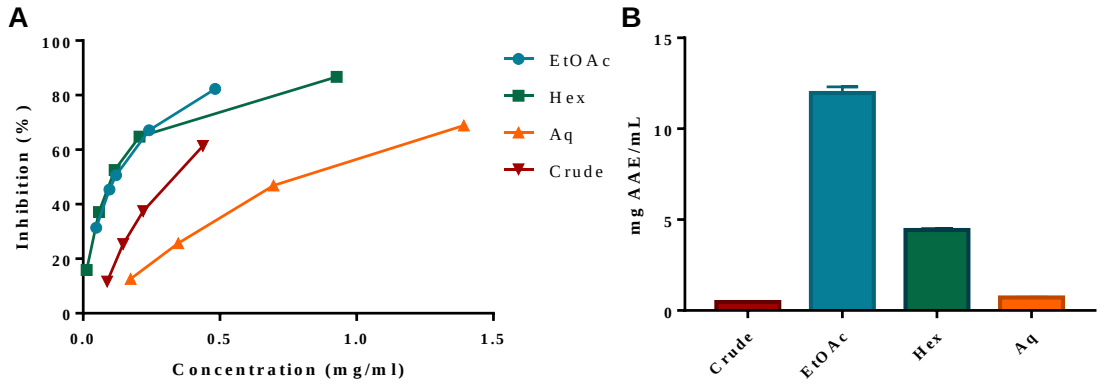


Figure 9. Ability of MAE crude extract and purified fractions to scavenge $NO^{\bullet}$ radical, expressed as (A) percentage of inhibition (mg/mL) and (B) mg AAE/mL. The samples were

dissolved in DMSO (EtOAc and Hex), water (Aq) or in 57% ethanol (crude), as described in 2.2.5.3.

Taken together, these results allowed to highlight the superior antioxidant ability of the purified fractions with respect to the crude extract. Also, having in mind the high potential of EtOAc and Hex fractions towards NO•, and the fact that this radical is closely associated with inflammatory processes, these two samples were selected for further studies on LPS-stimulated RAW 264.7 macrophages.

3.6 ANTI-INFLAMMATORY ACTIVITIES

The anti-inflammatory capacity of the two most promissing fractions regarding antioxidant potential, and in particular to the ability to scavenge the NO• (EtOAc and Hex fractions), was evaluated on LPS-stimulated RAW 264.7 macrophages. The effects produced by the EtOAc and Hex fractions are represented in **Figure 10**.

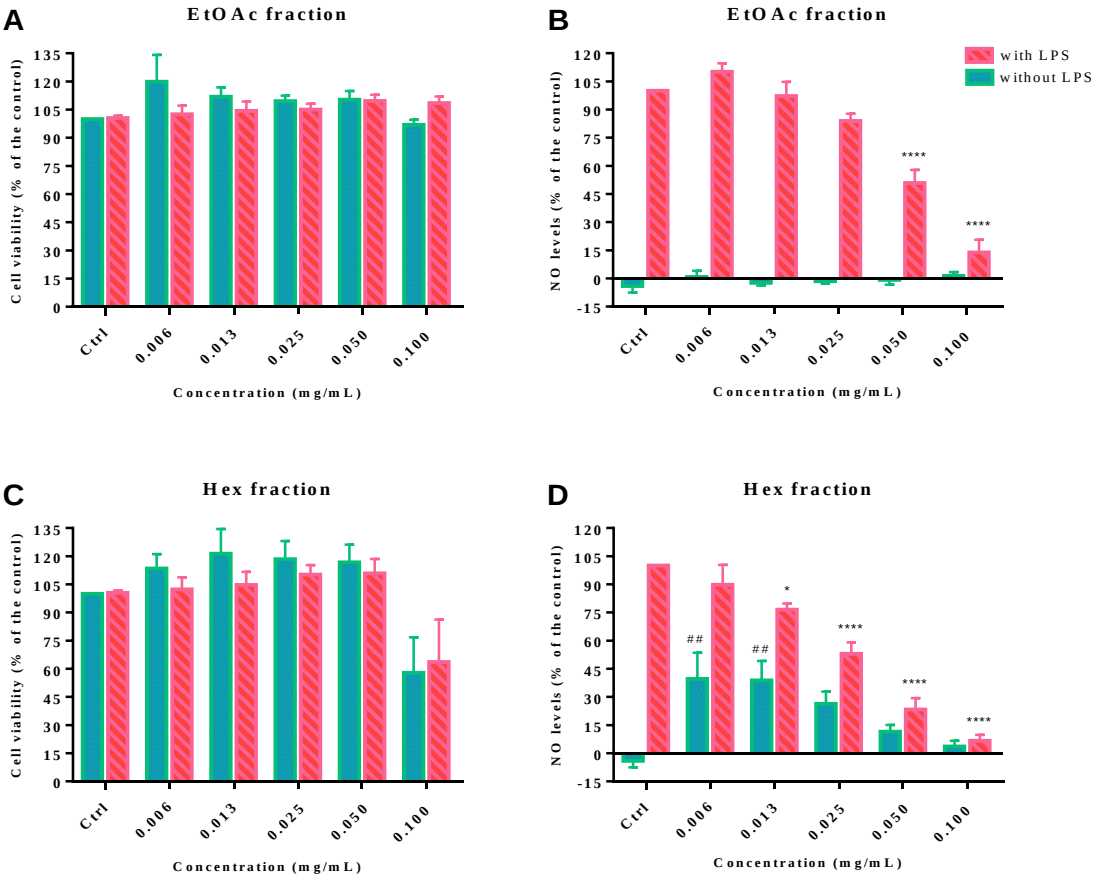


Figure 10. Effects of the pre-treatment with EtOAc and Hex fractions (0.006, 0.013, 0.025, 0.050 and 0.100 mg/mL), on the cell viability and NO• levels on RAW 264.7 cells after 24h incubation with or without LPS. Statistical analysis was performed by one-way ANOVA, followed by Tukey's *post-hoc* test. *, **, **** represent statistical significance with $p<0.05$; 0.01 and 0.0001; respectively, compared to the control with LPS; ##, represent statistical significance with $p<0.01$ compared to the control without LPS. Data represent mean ± SEM of 3 independent assays.

As can be observed in **Figure 10** (B) and (D), under normal conditions, macrophages produce low levels of nitrites, but when exposed to LPS, levels of NO• increased significantly when compared with the control.

The treatment of LPS-stimulated cells with EtOAc fraction revealed a decrease in NO• production for increased concentrations of extracts applied (0.006 to 0.100 mg/mL) and demonstrated no significantly influence in the NO• production (**Figure 10** (B)). The higher concentration applied, 0.100 mg/mL, showed an inhibition of NO• production of 85%, approximately. Furthermore, the cellular viability was not affected by the pre-treatment with EtOAc fraction indicating no toxicity for the tested concentrations (**Figure 10** (A)). As EtOAc fraction, also Hex fraction, demonstrated a decrease in the NO• production, in LPS treated cells, for higher concentrations applied (0.006 to 0.100 mg/mL), however, the different concentrations applied of this fraction also increased the production of NO• (**Figure 10** (D)). In this case the higher concentration applied, 0.100 mg/mL, showed an inhibition of NO• production of 93%, approximately (**Figure 10** (D)). Besides that, the exposure of the macrophages RAW 264.7 to the higher concentration (0.100 mg/mL) revealed a decrease in the cellular viability in cells treated with and without LPS, while the remaining concentrations did not. Thus, the concentration of Hex fraction which reduced the NO• production in, approximately, 77% with no cellular toxicity was 0.050 mg/mL.

Based on the overall results of NO• production and cellular viability of macrophages RAW 264.7, EtOAc and Hex fractions at 0.100 mg/mL and 0.050 mg/mL, respectively, were further studied regarding their ability to modulate inflammation. In particular, the expression of the enzymes iNOS and COX-2, as well as of p38MAPK and members from the NF-kB pathway were evaluated by Western blot and results are presented in **Figure 11**.

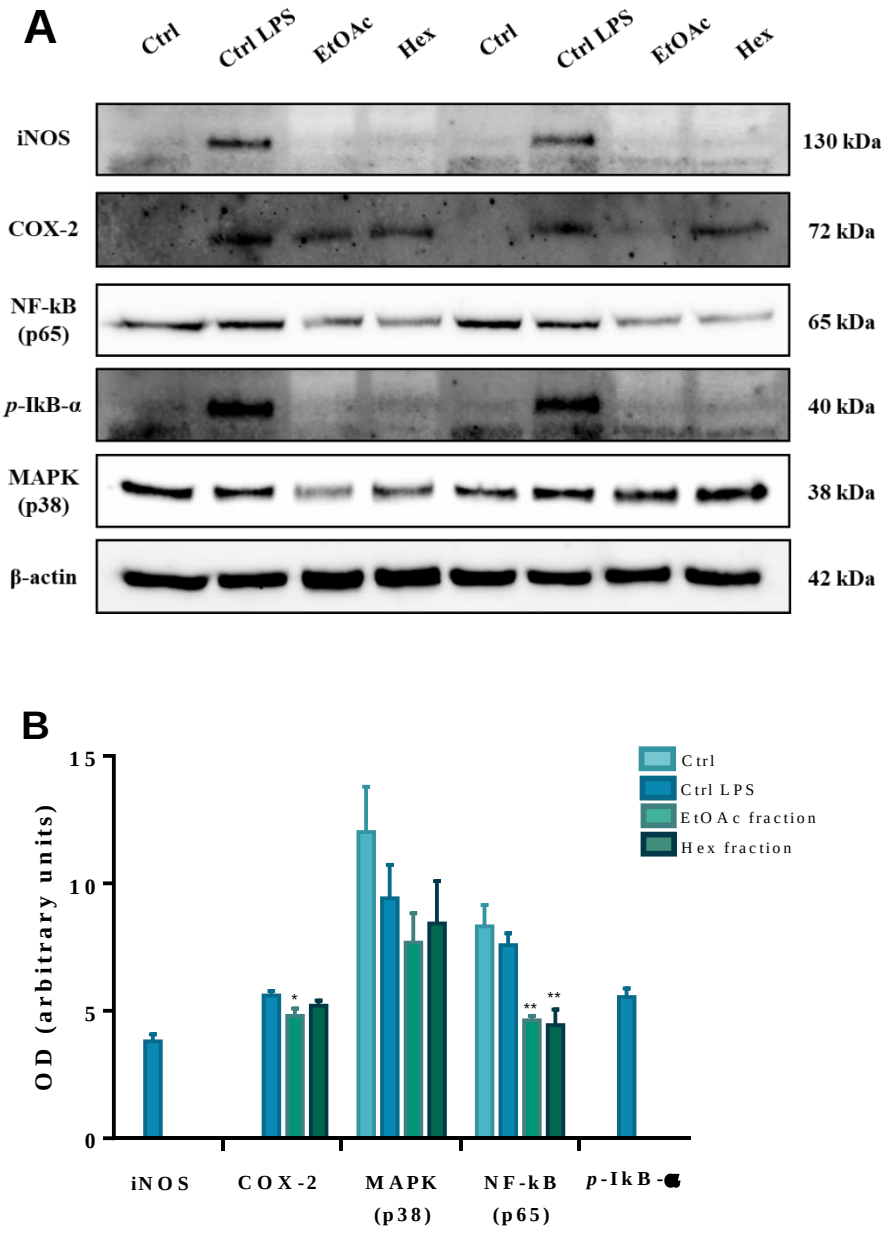


Figure 11. Effect of the EtOAc and Hex fractions (at 0.100 mg/mL and 0.050 mg/mL, respectively) on the expression of iNOS and COX-2 enzymes and on MAPK and NF-kB pathways in LPS-stimulated RAW 264.7 cells. (A) Representative western blots obtained in the analysis of iNOS, COX-2, p38MAPK, NF-kB p65 subunit and *p*-IkB-α. β-actin was analyzed to confirm equal protein loading ; (B) Values of optical density are presented in arbitrary units for each protein analyzed. Statistical analysis was performed by one-way ANOVA, followed by Tukey's *post-hoc* test. *, ** represent statistical significance with

$p<0.05$ and 0.01; respectively, compared to the control with LPS. Data represent mean ± SEM of 3 independent assays.

As expected, under normal conditions, macrophages did not express iNOS nor COX-2. However, upon stimulation with LPS, their expression was clearly noticed, indicating that macrophages entered in an inflammatory stage. Notably, the treatment of the LPS-stimulated macrophages with EtOAc and Hex fractions reversed, at least partially, the inflammation state. This effect was more notorious for iNOS, since after the pretreatment with this fraction there was no expression verified. Although the effect of the COX-2 was not so evident, it was possible to verify a decrease in the protein expression levels of approximately 14% and 7% for EtOAC and Hex fractions, respectively.

As regards to the MAPK pathway, the different fractions revealed a slight decrease in the expression of the signalling protein p38MAPK, although was not statistical significant. Nonetheless, EtOAc and Hex fractions caused a significant decrease in the content of the transcriptional factor NF-kB subunit p65 in total cell lysates ($p<0.01$), and completely inhibited the phosphorylation of IkB-α, suggestive of the down-regulation of NF-kB signaling.

CHAPTER 4. DISCUSSION

Phlorotannins are a class of phenolic compounds present exclusively in brown seaweeds, which are claimed to exert potent antioxidant and anti-inflammatory properties. So far, the majority of works point out that, simillarly to tannins in general, phlorotannins are preferentially extracted with hydroacetonic mixtures[16]. Hence, one of the main aims of this work was to evaluate the feasibility of using greener solvents to recover these high-valuable compounds from *F. vesiculosus*. As a tentative to maxime this recover, microwave-assisted extraction was evaluated, since this techique has some advantages regarding regular stirring, namely it allows the rapid heating of aqueous samples with non-ionizing electromagnetic radiation, a lower solvent use, a greater selectivity, a higher level of automation, and a superior efficiency and lower extraction times[29].

According to preliminary single factor experiments, solvent-solid ratio was shown to not significantly affect the recovery of phlorotannins, while considerable impacts were observed when varying the ethanol concentration in the range of 0 - 100%, the temperature 25 – 150 °C and the time 1 - 25 min, with maximal values being obtained for 60%, 100 °C and 3 min, respectively. Concerning the effect of ethanol concentration, the maximum TPhC was obtained for ethanol concentration of 60% (v/v) slightly higher than the revealed by the optimisation of He et al.[29] (55% v/v) for MAE of *Saccharina japonica*. Although, Magnusson et al.[27] showed that water is a better solvent than ethanol for the extraction of polyphenols in MAE, however, it can be notice that, for *F. vesiculosus*, hydroethanolic mixtures are better than use only water which can be related to the polarity of the solvent, indicating that the type of solvent influences the recovery of TPhC from the seaweeds during the extraction process (**Figure 3** (A)). Afterwards, a maximum TPhC was herein obtained for the extracting temperature of 100 °C, which is higher than that obtained by He et al.[29] (60 °C) for *Saccharina japonica* and lower than that described by Magnusson et al.[27] (160 °C) for *Carpophyllum flexuosum,Carpophyllum plumosum* and *Ecklonia radiata*, using the same technique. Such differences can be explained due to the fact that these compounds are thermolabile and because the different seaweeds present a different phlorotannin profile between each other. Lastly, the effect of time in the phlorotannin MAE showed a maximum TPhC for 3 min, which is similar to that described by Magnusson et al.[27].

According to the BBD model, the predicted optimal conditions of extraction were set as ethanol concentration of 57% (v/v), temperature of 75 °C and a time of 5 min. The ethanol concentration was similar to that obtained in the preliminary single factor experiments (60%

v/v) and to the described by He et al.[29] (55% v/v) for the macroalgae *Saccharina japonica*. In turn, the optimal temperature was lower than that established in the preliminary experiments (100 °C), while the extraction time was superior to the same preliminary studies. The herein gathered results from the BBD experiment also allowed to conclude that the main interactions between the different variables were temperature versus time, followed by ethanol concentration versus temperature. This could be due to the fact that the variables ethanol concentration and temperature revealed a quadratic effect. In the case of ethanol concentration, it is clear that the presence of water and ethanol provides a polar medium more suitable for the phlorotannin extraction than just ethanol[143]. Regarding temperature, since phlorotannins are thermolabile compounds, and according to the phlorotannin profile present on *F. vesiculosus*, the optimum temperature for the extraction process was 75 °C. To the best of our knowledge, there is no previous data regarding the extraction of *F. vesiculosus* with MAE thus hampering the comparison of data. Naturally, the optimum conditions obtained for the MAE were quite distinct than those settled by Catarino et al.[16] for the conventional solvent extraction using hydroacetonic mixtures (acetone of 70% (v/v), solvent-solid ratio of 70 mL/g at temperature 25 ºC and time of 3 hours). Nonetheless, it must be noted that the herein stabilished conditions for MAE allowed the recovery of equal amounts of phlorotannins than conventional solvent extraction (3.16 ± 0.17 mg PGE/g DW_{algae} and 3.16 ± 0.74 mg PGE/g DW_{algae}, respectively), hence indicating that the use of MAE associated to green solvents may serve as a good alternative to the extraction of phlorotannins from *F. vesiculosus*. When purified by sequential extraction with *n*-hexane, ethyl acetate and water, the optimun MAE crude extract was partitioned in three purified fractions, namely, Hex, EtOAc and Aq. In opposition to what has been commonly described for these compounds (and phenolic extracts in general[16]), the EtOAc fraction was not enriched in phlorotannins with respect to the crude extract. This result could be due to an experimental error, or to the loss or degradation of phlorotannins along the purification procedures. Albeit that, both the UV spectra and the analyses in the SIM mode were consistent with the presence of several phlorotannins previously reported for the same algae, particularly in EtOAc and Aq fractions. Besides phlorotannins, the HPLC-DAD-ESI/MS also allowed the detection of pigments in Hex and EtOAc fractions. In fact, the first was mostly dominated by fucoxanthin while pigments chlorophyll *c*, pheorbidin *a*, 15-hydroxy-lactone chlorophyll *a*, pheophytin *b* and pheophytin *a* were also main relevant components

in both fractions. In fact, the presence of pigments in the crude extract and its derived fractions could be expected, due to their high solubility in ethanol[144]. This has also been shown by other authors in the case of algae. E.g. Lee et al.[121], after partitioning an 96% ethanol extract from *Laminaria japonica* with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol, the author conclude that the first was mainly rich in the carotenoid fucoxanthin.

After characterization, the crude extract and the purified fractions revealed antioxidant potential, namely towards $ABTS^{\bullet+}$, superoxide radical sccavenging and $NO^{\bullet}$ scavenging assays. To the author knowledge, studies reported in the literature with *F. vesiculosus* only have performed as chemical antioxidant methods the FRAP, $DPPH^{\bullet}$ and β-carotene bleaching assays[33,76]. Overall, result highlighted a stronger antioxidant effect for EtOAc was the best fraction in all tests, especially for $NO^{\bullet}$ scavenging assay, which was shown to be more effective than the commercial antioxidant ascorbic acid. The Hex fraction also showed high antixidant potential in two of these tests namely superoxide radical scavenging and $NO^{\bullet}$ sacavenging assays. Thus, the results suggest that phlorotannins are not the only compounds responsible for *F. vesiculosus* antioxidant effects, since they were not been detected in Hex fraction. Considering the compounds identified in the two fractions, and that fucoxanthin is known to be a potent antioxidant, it is feasible to hyphothesie that this carotenoid may play a key role in the antioxidant properties of *F. vesiculosus* extracts[145].

The potential anti-inflammatory effects of EtOAc and Hex fractions were evaluated in a commonly used inflammatory cell model, i.e. LPS-stimulated macrophages RAW 264.7. For non-toxic concentrations of both fractions, the production of $NO^{\bullet}$ decreased in 93% and 77%, respectively (for concentrations of 0.100 and 0.050 mg/mL, respectively). Please note that both purified *F. vesiculosos* Hex and EtOAc fractions revealed good potential to inhibit inflammatory mediators and, particularly the latter, showed further potential to effectively regulate the pro-inflammatory iNOS and diminish the COX-2 expression, which seem to be mediated by the inhibition of the transcriptional factor NF-kB. Indeed, the gathered results highlights the inhibition of the transcriptional factor NF-kB is mediated throught inhibition of the IkB-α phosphorylation presented for both fractions. Indeed, the activation of NF-kB requires the phosphorylation of IkBs at critical serine residues. Once phosphorylated IkB is recognized by ubiquitin and targeted for proteolysis, allowing NF-kB heterodimers to translocate to the nucleus where activates the expression of taget genes that codify to pro-inflammatory mediators as COX-2 and iNOS[12,133,146]. Data suggest that EtOAc and Hex

fractions, particularly EtOAc one, exert their anti-inflammatory activity through the inhibition of IkB phosphorylation. In addition, the samples also seemed to downstream regulated the expression of signalling proteins like p38MAPK, although this effect was not statistic significant. Some reports have also reported similar effects for extracts of other seaweed. For example, Lee et al.[121] showed that *n*-hexane fraction of *Laminaria japonica* inhibits the expression of pro-inflammatory proteins like iNOS and COX-2, pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β), transcriptional factors NF-kB and phosphorylation of IkB-α and signalling proteins of MAPK and Akt pathways[121]. Moreover, studies carried out with *n*-hexane and ethyl acetate fractions of *Fucus distichus* were able to reduce the expression of TNFα, IL-10, MCP-1, and COX-2 and reduced iNOS[119].

In conclusion, findings from the present dissertation suggest that EtOAc and Hex fractions of *F. vesiculosus* have potent antioxidant and anti-inflammatory activities. In the case of EtOAc fraction, these biological activities could be due to the presence of phenolic compounds, like phlorotannins, and probably to diverse pigments. Regarding, Hex fraction, since the presence of phlorotannins was almost undetected, the antioxidant and anti-inflammatory activities from this fraction are probably mostly associated to the carotenoid fucoxanthin.

CHAPTER 5. CONCLUSIONS AND FUTURE PROSPECTS

In this work, microwave-assisted extraction was used to maximize the extraction of phlorotannins from *Fucus vesiculosus* using hydroethanolic mixtures as solvent. The optimum conditions were obtained using single-factor experiments followed by surface response methodology (Box-Behnken design) and settled as ethanol concentration at 57% (v/v), temperature at 75 °C and time at 5 min. When compared to the yield of extraction obtained under optimized conditions for conventional solvent extraction with hydroacetonic solvent, the MAE extraction allowed the recover of similar amounts of phlorotannins (3.16 ± 0.74 mg PGE/g DW_{algae} versus 3.16 ± 0.17 mg PGE/g DW_{algae}, respectively) . Yet, opposing to previous works, the application of sequential purification with *n*-hexane and ethyl acetate did not result in enrichment of phlorotannins in the EtOAc fraction. Nevertheless, phlorotannins species previously detected in *F.vesiculosus* were herein detected by SIM mode analysis, mostly in the EtOAc fraction. Curiosly, HPLC-DAD-ESI/MS analysis also allowed to conclude that this fraction contained several pigments, including chlorophyll *c* and fucoxanthin that are exclusively to brown algae. Of note, the latter carotenoid was the dominant compound detected in the Hex fraction.

The crude extract and the purified fractions were evaluated for their antioxidant properties in different in chemico methods through $ABTS^{\bullet+}$, superoxide radical sccavenging and $NO^{\bullet}$ scavenging asays. In general, Hex and EtOAc fractions revealed better results, with the last fraction presenting higher antioxidant potential than the comercial antioxidant ascorbic acid for the $NO^{\bullet}$ scavenging assay.

Finally, the anti-inflammatory activity was evaluated for the EtOAc and Hex fractions in LPS-stimulated macrophages. Both samples reduced the $NO^{\bullet}$ production without affecting the cellular metabolism (0.006 to 0.100 and 0.006 to 0.050 mg/mL, respectively). Furthermore, at 0.100 mg/mL (EtOAc) and 0.0050 mg/mL (Hex) downregulated inflammation-mediating enzymes, namely iNOS (both Hex and EtOAc) and COX-2 (EtOAc). Moreover, the results suggests that this effect can be related to the regulation of NF-kB signaling, since the expression of p65 subunit was significantly decreased in the cellular lysates of the pre-treated macrophages, besides the complete abolish of the *p*-IkB-α phosphorylation. Furthermore, the MAPK pathway was also analysed and showed a trend to decreased levels of p38MAPK, although not significant.

Nevertheless, the work performed in this master thesis stands as a starting point for novel potential applications of *F. vesiculosus*. First, more work is needed regarding the chemical

characterization. Furthermore, it is necessary to complement the results obtained regarding the antioxidant potential of the extracts, either *in cellular* and *in vivo* models, to further understand their effect on a molecular level. Moreover, as regards to anti-inflammatory activity, the search for other molecular targets modulated by EtOAc and Hex fractions should be accomplished in order to complement the herein gathered information to fees future *in vivo* studies.

In a wider perspective, investigation of the applicability of seaweeds like *Fucus vesiculosus*, could lead to a development of nutraceutics and pharmacological applications to treat a wide spectrum of disorders and/or diseases.

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