NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

AFONSO PATRÍCIO MARQUES

BSc in Biochemistry

SEARCH FOR BIOACTIVE COMPOUNDS WITH ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY IN SEAWEED SPECIES FROM THE PORTUGUESE COAST

MASTER IN BIOCHEMISTRY

NOVA University Lisbon

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Search for bioactive compounds with antioxidant and antibacterial activity in seaweed species from the Portuguese coast

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Dedicated to my future.

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A destination is not reached with a single long step but many short ones. So last but most certainly not least, my great thanks to all the people who were a part of my journey not only in this thesis, but also up to this thesis. They are too numerous to mention all, but I hope none feel left out or that their part was unappreciated or ignored.

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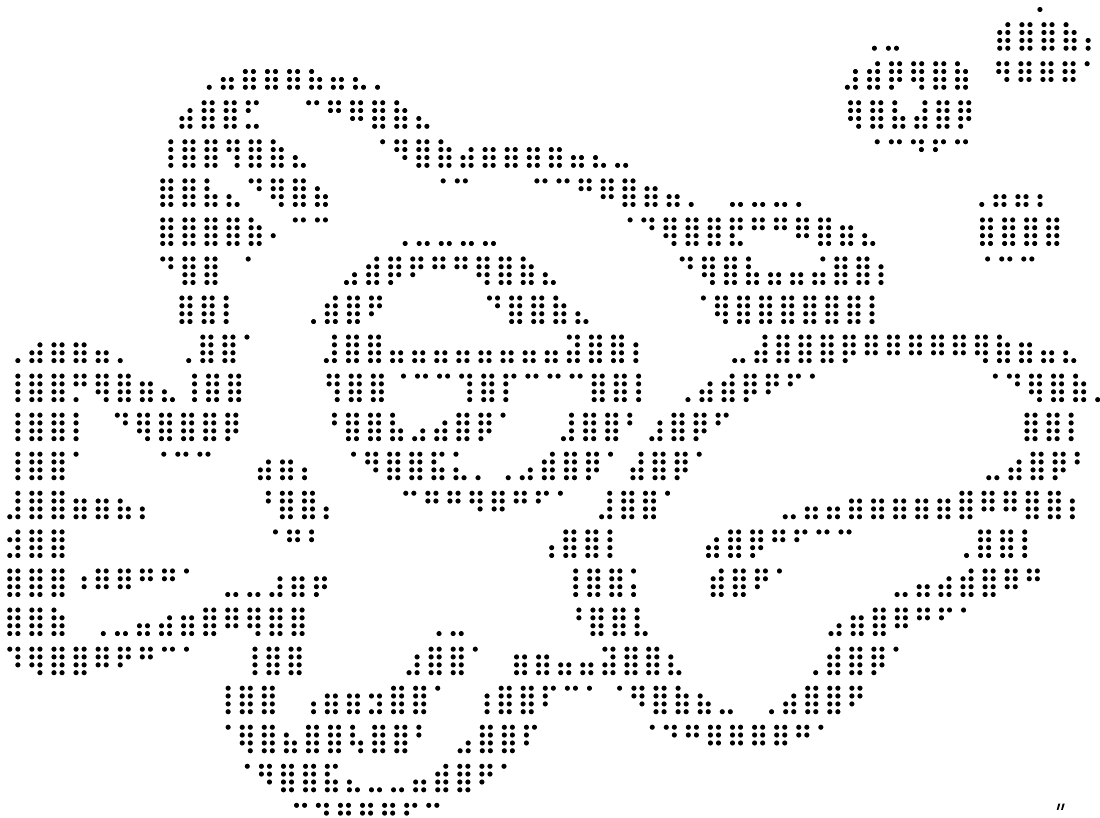

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(Francisco Seco)

ABSTRACT

The Portuguese coast has an enormous diversity of seaweed species, which offer great potential for the study of bioactive compounds. Of this bioactivity, compounds with antioxidant and antibacterial activity are the focus of our work due to their important role in human health. Antioxidant compounds protect against oxidative damage, which can induce cell death, while the antibacterial compounds present in algae can help fight antibiotic-resistant strains. Algae are known to contain molecules that have antibacterial properties. However, these are often left unidentified or only classified in terms of their class.

In this work, seven species of algae were selected (based on their abundance and availability on the Portuguese coast) and collected: *Chondrus crispus*, *Codium adhaerens*, *Codium tomentosum*, *Cystoseira tamariscifolia*, *Fucus spiralis*, *Porphyra umbilicalis* and *Ulva intestinalis* for screening bioactive compounds, namely those with antioxidant power and antibacterial activity. The samples were collected at "Magoito" beach and "Avencas - Parede" beach (Portugal) during low tide.

One of the most important steps in researching bioactive compounds is choosing the most suitable and efficient extraction method(s) for the target compound classes. Therefore, in the first phase, various extraction methods were tested and optimized (e.g. aqueous, methanolic, microwave-assisted-extraction (MAE) and Soxhlet). The extracts obtained were then analyzed in a preliminary phase using qualitative phytochemical tests. From this initial screening, a profile was obtained regarding the presence of a series of classes of compounds (e.g. alkaloids, carbohydrates, coumarins, flavonoids, glycosides, phenols, phytosterols, among others). The antioxidant potential of the extracts was then characterized using the FRAP, DPPH and ABTS assays. The results showed variability depending on the type of assay. For instance, in the DPPH assay, a greater antioxidant power was observed in *Ulva sp.* in extracts obtained through MAE and in *C. tamariscifolia* in extracts with hexane obtained by Soxhlet. However, in the FRAP assay, *C. tamariscifolia* showed greater reducing power in methanolic and methanol extracts obtained by Soxhlet extraction, greater than the other species analyzed. Antibacterial activity assays (inhibition zone assay) showed antibacterial activity in ethyl

acetate extracts (Soxhlet) in all algae species analyzed. However, in *C. tamariscifolia* methanolic extracts also showed antibacterial activity. Results of the soluble protein content performed by Bradford and Lowry assays showed different results, also depending on the extraction procedure. Overall, higher protein content was determined by Lowry method in *C. tamariscifolia* and *P. umbilicalis* extracts aqueous and MAE, respectively. Regarding the total phenolic content, results show that *C. tamariscifolia* has the highest content in most of the extraction procedures. Moreover, *C. tomentosum* showed the highest content in lipids. Based on the previous results three seaweed species (*C. tamariscifolia U. intestinalis, P. umbilicalis*), were selected for further GC-MS (FAMES and other compounds) and proteomic analyses. The GC-MS analyses identified 18 FAMES in *U. intestinalis*, 29 in *C. tamariscifolia* and 23 in *P. umbilicalis*. Additionally, Soxhlet extracts allowed to putatively identify several other compounds, some of them related to antioxidant and antibacterial activity but also with anti-inflammatory or anticancer activity, among others. The proteomic results show that most of the proteins identified in the three selected species are mainly related with translation, biosynthetic and metabolic processes. Proteins related to heat shock stress were found in all seaweed species and proteins related to antioxidant activity were also identified in all seaweed species.

Therefore, the present work contributes with relevant information regarding the presence of bioactive compounds in algae from the Portuguese coast. The results obtained allow us to select the most promising species to carry out future work in terms of elucidation and purification of compounds of interest to the pharmaceutical or food industry, for example.

Keywords: antioxidant activity, antibacterial activity, macroalgae, bioactivity, extraction methods

Resumo

A costa portuguesa apresenta uma enorme diversidade de espécies de algas marinhas, que oferecem um grande potencial para o estudo de compostos bioativos. Desta bioatividade, compostos com atividade antioxidante e antibacteriana são o foco do presente trabalho devido ao seu importante papel na saúde humana. Os compostos antioxidantes protegem contra os danos oxidativos, que podem induzir a morte celular, enquanto os compostos com atividade antibacteriana presentes nas algas podem ajudar a combater estirpes resistentes aos antibióticos. Sabe-se que as algas contêm moléculas com propriedades antibacterianas. No entanto, muitas vezes não são identificadas ou são classificadas apenas em termos de sua classe.

Neste trabalho foram selecionadas sete espécies de algas (com base na sua abundância e disponibilidade na costa portuguesa) e recolhidas: *Chondrus crispus, Codium adhaerens, Codium tomentosum, Cystoseira tamariscifolia, Fucus spiralis, Porphyra umbilicalis e Ulva intestinalis* para averiguar a presença de compostos bioativos, nomeadamente aqueles com atividade antioxidante e antibacteriana. As amostras foram recolhidas na praia do "Magoito" e na praia das "Avencas - Parede" (Portugal) durante a maré baixa.

Uma das etapas mais importantes na pesquisa de compostos bioativos é escolher o(s) método(s) de extração mais adequado(s) e eficiente(s) para as classes de compostos alvo. Portanto, na primeira fase, vários métodos de extração foram testados e otimizados (por exemplo, aquoso, metanólico, assistida por micro-ondas (MAE) e Soxhlet). Os extratos obtidos foram então analisados numa fase preliminar através de testes fitoquímicos qualitativos. A partir desta triagem inicial, foi obtido um perfil quanto à presença de uma série de classes de compostos (e.g. alcaloides, carboidratos, cumarinas, flavonoides, glicosídeos, fenóis, fito esteróis, entre outros). O potencial antioxidante dos extratos foi então caracterizado por ensaios FRAP, DPPH e ABTS. Os resultados mostraram variabilidade dependendo do tipo de ensaio. Por exemplo, no ensaio com DPPH, foi observado maior poder antioxidante em *U. intestinalis* em extratos obtidos por MAE e em *C. tamariscifolia* em extratos com hexano obtidos por Soxhlet. Porém, no ensaio FRAP, *C. tamariscifolia* apresentou maior poder redutor nos extratos metanólicos e metanólicos obtidos por extração Soxhlet, superior às restantes espécies analisadas.

Ensaios de atividade antibacteriana (ensaio de zona de inibição) mostraram atividade antibacteriana em extratos de acetato de etilo (Soxhlet) em todas as espécies de algas analisadas. Porém, em *C. tamariscifolia* os extratos metanólicos também apresentaram atividade antibacteriana. Os resultados do teor de proteína solúvel realizados pelos ensaios de Bradford e Lowry apresentaram resultados diferentes, dependendo também do procedimento de extração. No geral, o maior teor de proteína foi determinado pelo método Lowry nos extratos aquoso e MAE de *C. tamariscifolia* e *P. umbilicalis*, respetivamente. Em relação ao teor de fenólicos totais, os resultados mostram que *C. tamariscifolia* apresenta o maior teor na maioria dos procedimentos de extração. Além disso, a mesma espécie também apresenta o maior teor de lipídios. Com base nos resultados anteriores, três espécies de algas marinhas (*C. tamariscifolia U. intestinalis, P. umbilicalis*) foram selecionadas para posterior análise por GC-MS (FAMES e outros compostos) e análise proteómica. As análises de GC-MS identificaram 32 FAMES em *U. intestinalis*, 23 em *C. tamariscifolia* e 18 em *P. umbilicalis*. Adicionalmente, os extratos de Soxhlet permitiram identificar vários compostos, alguns deles relacionados com atividade antioxidante e antibacteriana, mas também com atividade anti-inflamatória ou anticancerígena, entre outros. Os resultados de análise proteómica mostram que a maioria das proteínas identificadas nas três espécies selecionadas estão principalmente relacionadas com processos de tradução, biossíntese e metabolismo. Proteínas relacionadas com o stress por choque térmico foram encontradas em todas as espécies de algas marinhas e proteínas associadas com atividade antioxidante foram também encontradas em toda as algas analisadas com.

Assim, o presente trabalho contribui com informações relevantes sobre a presença de compostos bioativos em algas da costa portuguesa. Os resultados obtidos permitem selecionar as espécies mais promissoras para a realização de trabalhos futuros em termos de elucidação e purificação de compostos de interesse para a indústria farmacêutica ou alimentar, por exemplo.

Palavas chave: atividade antioxidante, atividade antibacteriana, macroalgas, bioatividade, métodos de extração

CONTENTS

1 INTRODUCTION 29

1.1 Macroalgae 29

1.2 Macroalgae species in the Portuguese coast 30

1.2.1 *Chondrus crispus* 30

1.2.2 *Codium adhaerens* 31

1.2.3 *Codium tomentosum* 32

1.2.4 *Cystoseira tamariscifolia* 32

1.2.5 *Fucus spiralis* 33

1.2.6 *Porphyra umbilicalis* 34

1.2.7 *Ulva intestinalis* 34

1.3 Bioactivity 35

1.3.1 Antibacterial activity 35

1.3.2 Anti-inflammatory activity 36

1.3.3 Antioxidant activity 37

1.3.4 Antiviral activity 37

1.3.5 Bioactive compounds in seaweeds 38

1.4 Importance of extraction methods 43

1.4.1 Solid liquid extractions 44

1.4.2 Ultrasound - Assisted Extraction 44

1.4.3 Microwave Assisted Extraction 45

1.4.4 Soxhlet extraction 46

1.5 Gas Chromatography – Mass spectrometry 46
1.6 Objectives 47
2 MATERIALS AND METHODS 49
2.1 Seaweed species collection 49
2.2 Samples treatment 49
2.2.1 Sample preparation 50
2.2.2 Extractions 50
2.3 Antibacterial and biochemical assays 54
2.3.1 Antibacterial activity assays 54
2.3.2 Total soluble protein 56
2.3.3 Phytochemical assays 57
2.3.4 Antioxidant assays 61
2.3.5 GC-MS 64
2.3.6 Short length fatty acids 64
2.3.7 Lipidic content (gravimetric method) 65
2.3.8 Proteomics 65
2.4 Statistical analysis 67
3 RESULTS 69
3.1 Extraction yield 69
3.2 Antibacterial assays 70
3.3 Protein quantification 71
3.4 Phytochemical assays 73
3.5 ABTS assay 74
3.6 DPPH 75
3.7 FRAP assay 76
3.8 Total Phenolic Content (TPC) assay 76
3.9 Total tannin content (TTC) assay 77
3.10 GC-MS 78
3.11 FAMEs 81

3.12 Short chain fatty acids 85
3.13 Lipid content 85
3.14 Proteomics 85
3.14.1 *Porphyra umbilicalis* 85
3.14.2 *Ulva intestinalis* 87
3.14.3 *Cystoseira tamariscifolia* 90
4 DISCUSSION 93
5 CONCLUSIONS AND FUTURE PROSPECTS 101

LIST OF FIGURES

Figure 1 - Representative image of *C. crispus* collected from the Portuguese shore (Magoito, Portugal). 31
Figure 2 - Representative image of *C. adhaerens* collected from the Portuguese shore (Parede, Portugal). 31
Figure 3 - Representative image of *C. tomentosum* collected from the Portuguese shore (Parede, Portugal) 32
Figure 4 - Representative images of *C. tamariscifolia* collected from the Portuguese shore (Parede, Portugal) 33
Figure 5 – Representative image of *F. spiralis* collected from the Portuguese shore (Magoito, Portugal) 33
Figure 6 - Representative image of *P. umbilicalis* collected from Portuguese shore (Magoito, Portugal). 34
Figure 7 - Representative image of *U. intestinalis* collected from the Portuguese shore (Magoito, Portugal) 35
Figure 8 - Scheme representing the different extraction methods and seaweed species which went through specific extractions. 51
Figure 9 - *C. crispus* extraction using distilled water. 52
Figure 10 - Extractions using methanol before filtration. 52
Figure 11 - Microwave reactor used in extractions. 53
Figure 12 - Soxhlet apparatus for the extraction of selected seaweeds. 54
Figure 13 - Zone of inhibition test diagram (created using Biorender.com). 56
Figure 14 - Average extraction yields of the extraction methods assayed (H_2O: aqueous extracts, MeOH: methanolic extracts, MAE: MAE extracts; Sox Hex: hexane extracts through Soxhlet, Sox Etac: ethyl acetate extracts through Soxhlet, Sox MeOH: methanol extracts through Soxhlet, Sox total: sum of all Soxhlet extracts.) 70
Figure 15 - Example of extract presenting a small hallo surrounding the sample imbued in paper filter. 71

Figure 16 - String network showing protein interactions found for *P. umbilicalis*. 87
Figure 17 - String network showing protein interactions found for *U. intestinalis*. 89
Figure 18 - String network showing protein interactions found for *C. tamariscifolia*. 92

LIST OF TABLES

Table 1 - Extraction determined for the different extraction methods tested on seaweed species expressed as percentage relative to the mass of algae used (mean ± standard deviation)...... 69
Table 2 - Qualitative results of antibacterial assays in samples that showed at least one positive result.. 71
Table 3 - Results of protein quantification in the seven species of macroalgae analyzed, expressed as mg soluble protein /g DW (mean ± standard deviation). 73
Table 4 - Results of phytochemical qualitative assay results in seven analyzed species of macroalgae selected for analysis. 74
Table 5 - Results of ABTS assay in the selected seaweed species analyzed and expressed as percentage (%) of inhibition (mean ± SD).. 74
Table 6 - Results of DPPH assay in the selected species of seaweed analyzed and expressed as percentage (%) of inhibition (mean ± SD).. 75
Table 7 - Results of FRAP assay determined in the selected seaweed species analyzed and expressed as mg TE/g DW (mean ± SD). 76
Table 8 - Results of TPC assay in the seaweed species s analyzed and expressed as mg GAE/g DW (mean ± SD).. 77
Table 9 - Results of TTC assay in the selected seaweed species analyzed and expressed as µg CE/g DW (mean ± SD)... 77
Table 10 - Results of GC-MS in the three selected species of macroalgae analyzed................. 79
Table 11 - Results of FAMEs in the three selected species of macroalgae analyzed................. 82
Table 12 - Lipid content determined in the seven species of macroalgae analyzed and expressed percentage of dry weight (% of DW) (mean ± SD) 85
Table 13 - Biological processes and molecular functions of proteins identified in *P. umbilicalis*. 86
Table 14 - Biological processes and molecular functions of proteins identified in *U. intestinalis*. 88

Table 15 - Biological processes and molecular functions of proteins identified in *C. tamariscifolia*. 91

LIST OF EQUATIONS

Equation 1 - ABTS Inhibition determination 61

ABBREVIATIONS AND ACRONYMS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
$ABTS^+$	Oxidized ABTS
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
C24 side chain	Fatty acid side chain 24 carbons long
C-Ring	Carbon ring
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DPPH ●	DPPH radical
DW	Dry weight
FA	Formic acid
FAMEs	Fatty acid methyl esters
FASP	Filter-aided sample preparation
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
GC	Gas chromatography
GST	Glutathione S-transferase
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IV	Intravenous

LA	Liquid agar
LB	Liquid broth
MAE	Microwave assisted extraction
MIC	Minimum inhibition concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass Spectrometry
OD	Optical density
Prob	Probability
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SLE	Solid-liquid extraction
sp.	Species
TCEP	tris(2-carboxyethyl) phosphine
TE	Trolox equivalent
TEAB	Triethylammonium bicarbonate buffer
TPTZ	2,4,6-Tripyridyl-S-triazine
TSA	tryptic soy agar
TSB	tryptic soy broth
UAE	Ultrasound assisted extraction

1

INTRODUCTION

1.1 Macroalgae

Macroalgae, also known as seaweeds, are autotrophic and photosynthetic aquatic organisms, varying in size from a few centimeters to up to one hundred meters in length (Gomez-Zavaglia *et al.*, 2019). Unlike their terrestrial counterparts, they lack conductive tissues and instead extract nutrients through all their exposed surfaces, which distinguishes them from terrestrial plants (Schepers *et al.*, 2020).

Macroalgae are divided into three main taxonomic groups: brown algae (*Phaeophyta*), green algae (*Chlorophyta*) and red algae (*Rhodophyta*) (Cikoš *et al.*, 2018). This classification is based on their chemical composition and the pigments they contain. For example, red algae have phycobilin, green algae contain chlorophyll, and brown algae have fucoxanthin (Mohamed *et al.*, 2012).

In the past, compounds from seaweed were commonly used as gelling, thickening, and emulsifying agents in diverse food products. Nevertheless, today, seaweeds are recognized as a reservoir of health-enhancing compounds. The specific beneficial components they contain vary based on environmental factors like water temperature, salinity, nutrient levels, and light availability (Kadam *et al.*, 2013).

Seaweeds offer a wealth of bioactive compounds and secondary metabolites that are promising for applications in the food, cosmetic, and pharmaceutical sectors, leading to a notable increase in the study of the bioactivities and bioprospection of these organisms to understand their health benefits and related mechanisms (Nova *et al.*, 2020). These include proteins, polyphenols, pigments, minerals, and noteworthy carbohydrates like carrageenan and alginate. Additionally, certain species boast significant vitamin content, such as vitamin C and vitamin B12 (Wendin *et al.*, 2020).

1.2 Macroalgae species in the Portuguese coast

In mainland Portugal, there is a clear latitudinal gradient, moving from north to south. This gradient is characterized by an increase in sea temperatures and photosynthetically active radiation, and a decrease in wave exposure and nutrient levels. These variations function as drivers for alterations in the presence of seaweed species (Ramos *et al.*, 2012).

Along the coast, there is an overlap of seaweeds, with species coexisting from both the northern European coast and southwestern Atlantic European coast. Additionally, a substantial number of cold-water and warm-water species have their southernmost or northernmost habitat limits within this region (Berecibar, 2011).

Portuguese seaweeds represent a natural resource that remains untapped in terms of potential economic advantages. Collecting seaweed that wash up on beaches for fertilization is an ancient practice, with the first documented references dating back to the 14th century. While this tradition is still sporadically observed in the northern regions of the country, it continues to be of limited prominence (Gaspar *et al.*, 2019).

More recently, Berecibar (2011) compiled a comprehensive record of macroalgae species found on the Portuguese coast, encompassing 548 taxa, comprising 527 species, 19 varieties, and two forms within three distinct biogeographical regions: the Mediterranean, the Atlantic (mainland Portugal), and the Macaronesian region (the archipelagos). However, in recent decades, there have been notable changes in the algae population along mainland Portugal. This trend aligns with a global pattern, where cold water species are retreating towards higher latitudes, while warm water species are expanding into new territories simultaneously.

Some examples of seaweed frequently found on the Portuguese coast and with potential to be explored in terms of bioactive compounds or for incorporation into food products are: *Chondrus crispus* Stackhouse, 1797; *Codium adhaerens* C. Agardh, 1822; *Codium tomentosum* Stackhouse, 1797; *Cystoseira tamariscifolia* Hudson Papenfuss, 1950; *Fucus spiralis* Linnaeus, 1753; *Porphyra umbilicalis* Kützing, 1843; and *Ulva intestinalis* Linnaeus, 1753

1.2.1 *Chondrus crispus*

Chondrus crispus Stackhouse, 1797, often referred to as Irish moss, is a small red sea algae species which can be found in pools in the mid-intertidal in some locations; widely distributed in the northwestern and northeastern Atlantic (Fish & Fish, 2011) and within the Portuguese mainland coast in the northernmost regions (Gaspar *et al.*, 2019).

Previous studies have shown that extracts of this species collected in the red sea coast have antioxidant, anti-inflammatory and antitumoral activity (Alkhalaf, 2021).

Figure 1 - Representative image of *C. crispus* collected from the Portuguese shore (Magoito, Portugal).

1.2.2 *Codium adhaerens*

Codium adhaerens C. Agardh, 1822 is a green seaweed species growing as an encrusting spongy, felt-like mat on rocky surfaces, that can be found in the Atlantic European coast in the lower shore area (Fish & Fish, 2011) and within the Portuguese mainland coast it can be found in the most southern regions (Gaspar *et al.*, 2019).

Previous studies showed antioxidant activity in extracts of samples collected in Pag, Croatia (Radman *et al.*, 2021) and in the southeast coast of India (Sudha *et al.*, 2014) along with anticoagulant activity in the Moroccan coast (El Asri *et al.*, 2021).

Figure 2 - Representative image of *C. adhaerens* collected from the Portuguese shore (Parede, Portugal).

1.2.3 *Codium tomentosum*

Codium tomentosum Stackhouse, 1797 is a green seaweed species with solid fronds, with a spongy and felt-like texture, native to the northeast Atlantic coast and inhabits rock pools and lower seashores throughout the year (Rey *et al.*, 2020).

Some studies showed that extracts of this species, collected in Peniche (Portugal) contain the compound loliolide, a neuroprotective therapeutic agent, (Silva *et al.*, 2021) and many other compounds with anti-inflammatory (Rabecca *et al.* 2022) and antioxidant (Rey *et al.* 2020) activity.

Figure 3 - Representative image of *C. tomentosum* collected from the Portuguese shore (Parede, Portugal)

1.2.4 *Cystoseira tamariscifolia*

Cystoseira tamariscifolia Hudson Papenfuss, 1950 is a brown seaweed which presents an olive-green color that darkens when dry but shows an iridescent blue-green color when underwater with many small, spine-like structures giving rough texture and bushy appearance. It can be found on the Atlantic coast and all along the mainland Portuguese coast (Gaspar *et al.*, 2019) along with other species of Cystoseira which are easily distinguishable by their color under water (Fish & Fish, 2011).

Samples collected from Čiovo, Croatia showed great antioxidant and antibacterial activity against *Listeria monocytogenes*, *Staphylococcus aureus* (*S. aureus)*, and *Salmonella enteritidis* (Cagali *et al.*, 2022), samples collected in the southern Portuguese coast showed anti-radical activity (Custódio *et al.*, 2016) and samples collected in Cornwall, United Kingdom showed cytotoxic activity against various cancer cell lines (Mansur *et al.*, 2019).

Figure 4 - Representative images of *C. tamariscifolia* collected from the Portuguese shore (Parede, Portugal)

1.2.5 *Fucus spiralis*

Fucus spiralis Linnaeus, 1753 is a brown seaweed with twisting fronds and a smooth margin tipped by rounded structures. It is commonly found on sheltered rock formations on high shore. It is present throughout the Atlantic coast (Fish & Fish, 2011) including the Portuguese coast (Gaspar *et al.*, 2019).

Samples collected in Brazil have shown that seaweed extracts contained terpenes and phenolic compounds with antifungal activity against *Colletotrichum lagenarium* and *Aspergillus flavus* (Peres *et al.*, 2012), while samples collected in the Atlantic coast of Morocco presented antibacterial and antifungal activity and other fractions extracted with petroleum ether showed a high anticancer activity associated to cytotoxicity mechanisms which is thought to be linked to the high presence of fatty acids (Grozdanic *et al.*, 2020).

Figure 5 – Representative image of *F. spiralis* collected from the Portuguese shore (Magoito, Portugal)

1.2.6 *Porphyra umbilicalis*

Porphyra umbilicalis Kützing, 1843, is an edible seaweed with olive to brown-purple fronds unevenly lobed and divided from a central holdfast. It can be found throughout the world with many different names in different countries such as Nori in Japan.

Aqueous extracts from samples collected in the Portuguese coast (center region) showed antioxidant (Freitas *et al.*, 2022) and antigenotoxic (Santos *et al.*, 2019) activity and samples collected in the Chinese coast showed antifungal activity (De Corato *et al.*, 2017).

Figure 6 - Representative image of *P. umbilicalis* collected from Portuguese shore (Magoito, Portugal).

1.2.7 *Ulva intestinalis*

Ulva intestinalis Linnaeus, 1753, commonly known as sea lettuce, is a green seaweed with elongated fronds taking on a tubular and sometimes inflated shape that can be also found in regions with lower salinity of northwestern Europe (Fish & Fish, 2011).

Samples collected in the west coast of Ireland showed antioxidant activity associated to phenolic compounds (Tierny *et al.*, 2013) while other studies have shown anticoagulant, antioxidant and antitumor activity (Davoodi *et al.*, 2021).

Figure 7 - Representative image of *U. intestinalis* collected from the Portuguese shore (Magoito, Portugal)

1.3 Bioactivity

1.3.1 Antibacterial activity

The global proliferation of antibiotic-resistant bacteria, particularly of the nosocomial ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), has garnered widespread attention from both governments and the scientific community. This surge in antibiotic resistance stands as a critical healthcare challenge of the 21st century, leading to increased rates of illness and death (Rossi & Ciofalo, 2020).

This predicament has arisen due to the overuse and improper administration of antibiotics, as well as the prevalence of nosocomial infections. Notably, many of these drug-resistant bacteria fall under the category of Gram-negative pathogens. To combat this pressing issue, various strategies are currently under investigation and development to mitigate and slow the emergence of bacterial resistance including the bioprospection of new molecules with antibacterial activity, from novel sources. Many studies have documented the antimicrobial activity of extracts from various plants from molecules such as alkaloids (Kalea asıo lu *et al.*, 2013), coumarins (Küpeli *et al.*, 2020), proteins (Sun *et al.*, 2018), terpenes (Sun *et al.*, 2018), resins (Das *et al.*, 2023) and many others. However, fewer publications delve into the activity of specific molecules and their underlying mechanisms of action. This makes algae an appealing subject for exploration in this context (Alibi *et al.*, 2021).

Staphylococcus aureus is a Gram-positive bacterium that is typically arranged in clusters and is facultative anaerobic. It is commonly found on the skin and mucous membranes of humans, with approximately half of all adults being carriers and 15% having persistent

colonization in their nasal passages. Certain groups, such as healthcare workers, diabetics, IV drug users, hospitalized patients, and those with weakened immune systems, have higher rates of colonization (Taylor & Unakal, 2023).

Escherichia coli (*E. coli*) is a versatile Gram-negative bacterium that exhibits a wide range of genetic diversity and adaptability. It can be found in both commensal and pathogenic forms within the same species. Commensal *E. coli* strains are non-pathogenic and are part of the normal gut microbiota in humans and animals. In contrast, pathogenic *E. coli* strains can be divided into diarrheagenic and extraintestinal variants, each with different pathotypes and hybrid strains (Braz *et al.*, 2020).

These pathogenic *E. coli* strains can either be facultative or obligate pathogens. Facultative pathogens are typically found in the intestinal tract but can cause opportunistic infections when they leave their natural habitat, resulting in various extraintestinal infections. Obligate pathogenic variants, on the other hand, are specialized to cause infections under specific conditions, ranging from mild diarrhea to life-threatening cases (Braz *et al.*, 2020).

Pathogen evolution in cases of antimicrobial resistance and persistent human infections occurs in the milieu of the host environment, which imparts an additional selective pressure potentially selecting for not just drug resistance but also immune escape.

The emergence of drug-resistant strains, particularly methicillin-resistant Staphylococcus aureus (MRSA), has posed a significant challenge in the treatment of staphylococcal infections. MRSA strains carry the mec gene within the Staphylococcal chromosomal cassette mec (SCCmec) region, rendering them resistant to multiple antibiotics making this resistance a global public health concern (Tong et al., 2015)

E. coli is particularly interesting in its capacity for rapid evolution through gene acquisition and genetic modification. This adaptability allows it to thrive in diverse environments and contribute to both beneficial and harmful interactions with its host organisms (Braz *et al.*, 2020).

1.3.2 Anti-inflammatory activity

Inflammation is a natural response of the animal host to exogeneous substances. The immune cells have various receptors that can detect entities like viruses, bacteria, parasites, antigens, or chemicals. When these foreign bodies are recognized, a series of pro-inflammatory pathways are triggered. This activation leads to the production of cytokines and the mobilization of immune cells such as macrophages and lymphocytes, which work together to eliminate the invaders (Al-Khayri *et al.*, 2022)

However, if the immune system is unable to eliminate these foreign substances during the initial phase, inflammation can escalate into what is known as the chronic phase. In this

stage, there is an overproduction of cytokines, chemokines, and inflammatory enzymes. The regulation of inflammation involves various receptor-mediated pathways, including Toll-like receptors, the mitogen-activated protein kinase (MAPK) pathway, and the nuclear factor kappa-light chain enhancer of activated B cells (NF-κB). NF-κB is particularly noteworthy as it governs the activity of more than fifty genes associated with inflammation. Dysregulation of these pathways can result in a variety of inflammatory disorders such as vascular proliferation, tissue damage, fibrosis, and secondary diseases such as arthritis, atherosclerosis, cardiovascular diseases, Alzheimer's disease, asthma, and cancer (Choy *et al.*, 2019).

1.3.3 Antioxidant activity

Oxidative stress arises from an imbalance that occurs between the overproduction of free radicals and a decrease in antioxidants, resulting in disrupted cellular equilibrium. Free radicals are highly reactive molecules containing unpaired electrons, making them unstable and prone to react with other molecules (Pisoschi & Pop, 2015).

The human body generates reactive oxygen species (ROS) or free radicals through various metabolic pathways, targeting carbohydrates, fats, proteins, and nucleic acids. Sources of ROS include internal factors (inflammation, phagocytosis, exercise, ischemia/reperfusion injury, free metal ions, activity of various enzymes) and external factors (cigarette smoke, industrial solvents, environmental pollutants, and UV radiation). ROS encompass several species, such as superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, nitric oxide, among others, which can inflict damage on cellular components and DNA (Pisoschi & Pop, 2015).

The demand for natural antioxidants, replacing synthetic ones, is rising, and both macroalgae and microalgae are emerging as a potential source of these antioxidants. Algae possess antioxidant defense systems to combat oxidative damage, which are categorized into enzymatic and non-enzymatic mechanisms. Although most current natural antioxidants are derived from land plants, microalgae are gaining attention from the food, cosmetic, and nutraceutical industries as a promising source of natural antioxidant compounds (Coulombier *et al.*, 2021).

1.3.4 Antiviral activity

Viral infections and outbreaks have emerged as a significant global concern, leading to substantial morbidity and mortality worldwide. Developing effective antiviral treatments and vaccines has become a formidable challenge. The search for new antiviral agents is now

considered a public health priority, with extensive global efforts dedicated to discovering safe and potent treatments for a range of viral diseases. Viruses, especially those that mutate rapidly, pose a significant pandemic risk to human health, particularly because of the limited availability of vaccines and antiviral drugs (Abookleesh *et al.*, 2022).

Nevertheless, numerous antiviral drugs have been developed in recent years, contributing significantly to managing various viral infections. Notably, in the last three decades, approximately half of all approved medicines have originated from natural products, either directly or indirectly such as various polysaccharides (Sun *et al.*, 2018), lectins (Liu *et al.*, 2020), glycosides (Mani *et al.*, 2020) and resins (Das *et al.*, 2007) originating from algae. This practice draws from a longstanding tradition of using specific plants and algae for medical purposes to treat various diseases (Newman & Cragg, 2012).

1.3.5 Bioactive compounds in seaweeds

Algae can be seen as light-driven cellular factories that produce bioactive substances. These encompass primary metabolites like lipids, amino acids, and carbohydrates, which belong to the metabolic processes of algae, as well as specialized secondary metabolites. Many of these bioactive compounds derived from algae have undergone clinical trials or are presently being assessed for their potential to address health issues. These concerns cover areas such as lipid metabolism, oxidative cellular stress, cancer, and various neurological and cardiovascular disorders (Catanesi *et al.*, 2021).

1.3.5.1 Alkaloids

Alkaloids are heterocyclic nitrogen compounds found in plants, microbes, animals, and other marine organisms (Alghazeer *et al.*, 2013). Although marine algae have relatively fewer alkaloids than terrestrial plants, they can be categorized into four groups: 2-phenylethylamine, indole, halogenated indole, and 2,7-naphthyridine derivatives. Marine algae contain mainly alkaloids of the 2-phenylethylamine and indole groups, with halogenated alkaloids, particularly those containing bromine and chloride, predominant in Chlorophyta. Indole group alkaloids are mostly found in Rhodophyta (Güven *et al.*, 2013). These alkaloids exhibit a variety of pharmacological effects, including neuromodulation, neurotransmission, growth regulation, cytotoxicity, angiogenesis, antioxidant properties, as well as antibacterial, antifungal, and larvicidal activities (Kaleağasıoğlu *et al.*, 2013).

1.3.5.2 Carbohydrates

Algal carbohydrates, such as alginates and carrageenan, are commercially used as thickening and gelling agents in various industries, including food, textiles, biotechnology, and biomedicine (Kraan *et al.*, 2012). These marine carbohydrates have recently gained attention as functional food ingredients. Seaweed-derived carbohydrates, including alginates, carrageenan, and fucoidan, exhibit a wide range of biological activities. These activities include anti-inflammatory, anticoagulant, antioxidant, antiproliferative, and immunostimulatory effects, which have been demonstrated both in vitro and in vivo assays (Garcia-Vaquero *et al.*, 2017).

1.3.5.3 Coumarins

Coumarins are a class of polyphenolic compounds belonging to the group of oxygenated heterocyclic compounds, which can be furan derivatives with four carbon atoms or pyran derivatives with five carbon atoms. While furan derivatives are not commonly found in plants, pyran derivatives are more frequent. Pyran derivatives can exist in two forms, α-pyrone and γ-pyrone. Secondary metabolites referred to as benzo-α-pyrones (coumarin) and benzo-γ-pyrones (chromone) are formed when pyrone derivatives condense with benzene in plants (Küpeli *et al.*, 2020).

In recent years, coumarins have garnered significant attention due to their diverse range of biological activities. Studies on coumarin derivatives have revealed their potential as antitumor, antibacterial and antifungal activity. This versatility in biological activities has made coumarins an intriguing subject of research and exploration for various applications (Küpeli *et al.*, 2020).

1.3.5.4 Flavonoids

Flavonoids are a group of compounds characterized by a 15-carbon skeleton consisting of two benzene rings known as A and B rings, connected by a heterocyclic pyran called the C-ring. They are classified based on structural differences and are known for their exogenous antioxidant properties. Flavonoids act as antioxidants by reducing reactive species through mechanisms such as inhibition of nitric oxide (NO) synthase, xanthine oxide synthase, and regulation of ion channels. They also modulate enzymes involved in oxidative processes (Ullah *et al.*, 2020). Additionally, flavonoid compounds found in plants have been shown to reduce ROS and enhance the activity of the enzyme glutathione S-transferase (GST) (Lago *et al.*, 2014).

1.3.5.5 Glycosides

Glycosides are compounds in which a sugar (glycone) is linked to the functional group(s) by a glycosidic bond. Glycosides can be categorized by their glycone group, glycosidic linkage, or specific properties (Evans, 2009). Biological activity depends on factors like the type of linked compound, glycone identity, and the specific glycone structure and placement. Glycosides exhibit potential for various bioactivities, including antiviral (Mani *et al.*, 2020), antiproliferative, and potential cardiovascular benefits (Rees *et al.*, 2018).

1.3.5.6 Gums and Mucilages

Gums and mucilages are biopolymers linked by O-glycosidic bonds, and are used as excipients in various industries, including medicine and cosmetics. These materials can be modified for use in drug delivery systems such as controlled release methods, film coatings, nanoparticles, ophthalmic solutions, suspensions, and implants (Prajapati *et al.*, 2014).

Gums, derived from different parts of plants such as seed epidermis, leaves, and bark, are easily soluble in water and typically are formed when plants are damaged or when subjected to unfavorable conditions. Notable examples include *Acacia tragacanth* and guar gum. Mucilages, on the other hand, are metabolic products formed within plant cells and are not easily dissolved in water. They are characterized by their thick, adhesive nature and are commonly found in different parts of plants (Deogade *et al.*, 2012).

Both fall into the category of plant hydrocolloids, which consist of amorphous monosaccharide polymers. These substances possess hydrophilic properties, allowing them to create thick or gel-like solutions when mixed with water (Anbalahan, 2017).

In the pharmaceutical industry, these natural materials offer several advantages over synthetic polymers, including biodegradability, biocompatibility, non-toxicity, improved patient tolerance with minimal side effects, absence of allergic reactions, low potential for skin and eye irritation, and cost-effectiveness (Anbalahan, 2017).

1.3.5.7 Phenolic compounds

Phenolic compounds are a highly abundant and widely distributed class of secondary metabolites found in both algae and terrestrial plants. These compounds play a crucial role in the regular growth and development of these organisms, acting as defense mechanisms against infections, injuries, and various environmental stresses. In recent years, marine macroalgae, have gained recognition as a valuable reservoir of bioactive compounds, among them phenolic compounds. Notably, some of these, such as phlorotannins, are particularly

unique to macroalgae and are not commonly found in other plant sources (Del *et al.*, 2012; Uysal *et al.*, 2018).

1.3.5.8 Phytosterols

Phytosterols are lipid compounds produced by plants and serve as a major component of plant cell membranes. While they share structurally similar to cholesterol, the differences in their C24 side chains distinguish them metabolically and functionally (Ling & Jones. 1995). For instance, brown algae (*Phaeophyta*) are primarily rich in phytosterols such as fucosterol and brassicasterol, with only a small amount of plant cholesterol, making them a promising source of phytosterols. In contrast, red algae (*Rhodophyta*) contain predominantly cholesterol as the main sterol component, with small amounts of phytosterols like sitosterol, fucosterol, chalinasterol, and desmosterol. Green algae (*Chlorophyta*) show variability in their sterol composition, which includes sterols such as ergosterol, chondrillasterol, β-sitosterol, 28-isofucosterol, cholesterol, and poriferasterol, depending on the specific species (Sánchez-Machado *et al.*, 2004).

1.3.5.9 Proteins

Functional peptides produced by macroalgae can be found stored within the cells or secreted extracellularly. These peptides show various biological activities, like antiviral, antibacterial, and antioxidant properties. For example, a peptide derived from *Ulva borealis* has been isolated and purified, demonstrating activity against the tobacco mosaic virus activity (Sun *et al.*, 2018).

1.3.5.10 Tannins

Tannins are a class of water-soluble polyphenols and belong to the subgroups of phenolic compounds that differ in their solubility. These compounds are abundantly found in plants and vegetables due to their ability to accumulate (Anne, 2017). Tannins exhibit a diverse range of properties, with diverse molecular weights, complex chemical structures, and a wide array of biological activities. Some of these activities encompass protection against both abiotic and biotic stresses, acting as a defense mechanism against pathogenic bacteria, fungi, and predators like Insects. Due to their multiple hydroxyl groups, tannins have the capacity to form complexes with proteins, metal Ions, amino acids, and polysaccharides (Al-Saif *et al.*, 2014).

1.3.5.11 Terpenes

Terpenes are a prominent group of metabolites in marine algae (Grosso *et al.*, 2011), originating from the five-carbon precursor isopentenyl pyrophosphate and are classified into various subgroups based on their carbon chain length: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), and polyterpenes (>C30) (De et al. 2009). These terpenes are biosynthesized through two primary pathways: the mevalonate (MVA) pathway and the 1-deoxyxylulose 5-phosphate/2-C-methylerithrytol 4-phosphate (DOXP/MEP) pathway. Terpenes have demonstrated significant biological potential, including anti-cancer, antioxidant, anti-inflammatory, and antimicrobial activities, among other beneficial properties (Brahmkshatriya & Brahmkshatriya, 2013).

1.3.5.12 Quinones

Quinones are naturally occurring compounds found in nature with diverse biological activities. They play a crucial role in understanding chemically induced toxicity within cells (Asche, 2005). These compounds exert their cytotoxic effects through processes that involve redox cycling. The production of ROS during the reduction of certain quinones is linked to their structural features and redox potential. Quinones can damage cancer cells by inducing ROS-related harm and can also alkylate proteins. Interestingly, some prenylated quinones have antioxidant properties, exhibiting the ability to scavenge radicals like DPPH and hydroxyl radicals. This dual nature of cytotoxicity and antioxidant activity makes them intriguing compounds for further research (Gordaliza, 2010).

1.3.5.13 Reducing sugars

Reducing sugars are a specific class of sugars that can function as reducing agents. They have an anomeric carbon that has not yet engaged in the formation of a glycosidic bond. This unbound anomeric carbon allows reducing sugars to act as reducing agents by participating in chemical reactions in which they donate electrons. When these sugars are exposed to an alkaline environment, this results in the formation of either an aldehyde or a ketone functional group. This transformation enables them to act as reducing agents, contributing with electrons during chemical reactions. Within the category of reducing sugars, can be found all monosaccharides, as well as specific disaccharides, oligosaccharides, and polysaccharides (Pratt & Cornely, 2017).

1.3.5.14 Resins

Resins are environmentally degradable long-chain molecules that originate from diverse natural sources and have sparked considerable scientific attention. They comprise proteins, nucleotides, fats, and polysaccharides, boasting properties such as biodegradability and economic value. These well-defined structural biopolymers are gaining commercial traction, particularly in fighting environmental pollution (Yaashikaa *et al.*, 2022).

Though they may possess minimal inherent bioactivity on their own, when modified in combination with other bioactive molecules, they can exhibit a wide spectrum of activities, including anticoagulant, radical scavenging, anti-HIV, antimicrobial, and antitumor activity (Das *et al.*, 2023).

1.3.5.15 Saponins

Saponins, a group of compounds found in plants, serve as a natural defense mechanism against herbivores and pathogens. They have wide applications in industries such as cosmetics, pharmaceuticals, food, and agriculture due to their versatile properties such as emulsifying and foaming properties, making them effective detergents and surfactants (Güçlü-Üstündağ & Mazza, 2007).

These plant-derived compounds have diverse biological effects, including antioxidant, anti-diabetic, anti-obesity, antibacterial, and anti-cancer activities. Researchers have explored their potential to reduce the risk of heart failure, preventing blood clot formation, relieve ulcers and inflammation, and even prevent the formation of urinary stones. Overall, saponins represent a promising class of compounds with multifaceted biological activities and practical applications across different industries (Feroz, 2018).

1.4 Importance of extraction methods

The process of extracting algal biomass usually involves three main phases: pretreatment, extraction, and formulation. Notably, due to the absence of lignin and presence of cellulose, the extraction of bioactive compounds from algal biomass is simplified. However, the substantial presence of cell wall polysaccharides presents a challenge in extracting algal metabolites (Harun *et al.*, 2014).

The pre-treatment phase entails thoroughly washing the algae sample several times with distilled water after collection to eliminate sand particles and impurities that may be

adherent. Subsequently, the collected algae are commonly dried, to reduce water content, and milled to attain a uniform sample and a higher surface-to-volume ratio (Hahn *et al.*, 2012).

The yields and composition of the resulting extracts are significantly influenced by the conditions under which the extraction takes place. Various factors, including the choice of solvent, sample-to-solvent ratio, the duration of the extraction and temperature, have been identified as key elements that affect these outcomes (Jacobsen *et al.*, 2019). Besides the extraction methods, variables such as species, geographic location, and time of harvest also have an impact on the content of the specific compounds of interest (Lee *et al.*, 2013).

1.4.1 Solid liquid extractions

The effectiveness of solid liquid extractions (SLE) is intrinsically linked to the solvent used. Typically, higher yields are achieved using colder water or aqueous mixtures of ethanol or methanol. Additionally, factors such as extraction time and temperature have an influence. SLE often requires long extraction periods due to substantial amounts of sample material involved (Heffernan *et al.*, 2014).

Opting to extract bioactive compounds from algae, rather than consuming the algae directly, can help mitigate the risk of excessive intake of potentially harmful elements (Cherry *et al.*, 2019).

The utilization of conventional solvents in the extraction of bioactive compounds has several drawbacks. These include the need for substantial amounts of environmentally harmful organic solvents, time-consuming extraction processes, challenges in achieving selectivity, and the potential for extracting undesired interfering substances along with the target compounds (Jacobsen *et al.*, 2019). The use of hazardous solvents such as methanol and chloroform for the extraction of bioactive compounds creates restrictions on their use, especially in the food and pharmaceutical sectors. This differs from water and ethanol extractions, which are more suitable for these industries due to their safer characteristics (Angela *et al.*, 2008).

1.4.2 Ultrasound - Assisted Extraction

Ultrasound-Assisted Extraction (UAE) can serve as a pretreatment method for SLE by breaking down the biomaterial, mainly the cell walls of macroalgae, enhancing the accessibility to target compounds. This often leads to higher yields in the extraction process (Barka *et al.*, 2016). This innovative approach involves the use of ultrasound waves with frequencies ranging from 20 kHz to 100 kHz. These induce the formation of microbubbles and regions with varying pressure levels (Cikoš *et al.*, 2018).

The rapid implosion of these microbubbles, resulting in cavitation in liquid interfaces, triggers the disruption of particles, enhancing mass transfer, leading to the release of bioactive compounds from a biological matrix (Kadam *et al.*, 2013).

The equipment involved in this extraction can take the form of an ultrasonic bath (indirect sonification) or an ultrasonic probe (direct sonification). The main differences between these lies in their operating conditions and how ultrasound waves interact with the sample. An ultrasonic bath involves immersing the samples in the ultrasound bath, while the ultrasonic probe is inserted directly into the sample (Flórez-Fernandez *et al.*, 2017).

This technique is conducted under relatively low temperatures, which helps in retaining thermolabile compounds and avoiding extensive structural damage. This method also employs solvents like ethanol, distilled water, and methanol in minimal quantities, using various solid to solvent ratios. This approach significantly reduces extraction time, rendering UAE a swift and cost-effective alternative compared to conventional methods (Oh *et al.*, 2011).

Nevertheless, the application of this technique can face challenges. For instance, bioactive molecules like carotenoids could experience degradation due to oxidative pyrolysis caused by hydroxyl radicals generated during cavitation. Furthermore, intense frequencies have the potential to damage bioactive molecules, leading to unfavorable alterations in the extracted components (Ameer *et al.*, 2017).

1.4.3 Microwave Assisted Extraction

Microwave-Assisted Extraction (MAE) is another technique that offers an environmentally and economically sound alternative. It allows the production of cost-effective, high-quality products that meet "green" environmental standards. This is primarily due to the reduced process time and solvent usage, making it a sustainable choice (Sahin *et al.*, 2017).

This technique merges microwave and conventional solvent extraction methods, operating on the principles of ionic conduction and dipole rotation, which simultaneously affect molecules. The application of microwave heating leads to the absorption of energy by polar molecules, eliminating heat loss to the surroundings and at the same time disrupting cellular structures. The disruption of cells enhances the speed of mass transfer and diffusion from solid materials. This is further aided by the combined and synchronized action of mass and heat transfer, both working harmoniously in the same direction. In microwave extraction, microwave power is employed to carefully elevate the temperature of samples and solvents contained within high-quality vessels (Delazar *et al.*, 2012).

This technique can be executed using two primary systems: closed and open vessels. Closed vessels work under elevated temperature and pressure, whereas open vessels operate under atmospheric pressure. The open vessel system tends to be more efficient and safer,

allowing for the processing of larger sample quantities. Moreover, the process conditions in open vessels are particularly suitable for thermolabile compounds (Pérez *et al.*, 2014).

The key strengths of MAE encompass reduced consumption of organic solvent, rapid extraction, energy efficiency, affordability, and increased yields. These attributes make it a feasible alternative to conventional methods. However, certain limitations exist. It might not perform well with dried samples and requires an additional filtration step after extraction. Furthermore, the elevated temperatures involved, despite the "green" nature of the method, make it unsuitable for heat-sensitive bioactive molecules, such as proteins (Bleakley *et al.*, 2017).

1.4.4 Soxhlet extraction

Soxhlet extraction is a widely used technique for its straightforwardness and scalability. It involves an integrated setup comprising an extraction chamber, vapor duct, siphon tube, and a round bottom flask. To prevent potential blockages, a filter paper thimble or cotton plug is inserted into the extraction chamber. The process begins by placing the sample in the extraction chamber, introducing the solvent, and gently heating the flask to a boil.

The solvent, as it reaches its boiling point, transitions into vapor, rising through the vapor duct and subsequently condensing onto the sample. Simultaneously, the extraction chamber gradually accumulates solvents until surpassing the siphon tube level. At this juncture, the surplus solvent flows back into the flask through the siphon tube, sustaining an uninterrupted supply of fresh solvent. This unique attribute endows Soxhlet extraction with the status of a favored method, renowned for its thorough extraction capabilities, even finding recognition in official standards.

The advantages of Soxhlet extraction encompass higher mass transfer rates facilitated by elevated temperatures and solvent recycling, thus fostering enhanced equilibrium transfer. However, it's important to note that this technique has constraints concerning its suitability for thermostable components. Several pivotal variables impacting its effectiveness include solvent quantity, temperature, extraction duration, sample powder granularity, and the solvent-to-sample ratio (Malik & Mandal, 2022).

1.5 Gas Chromatography – Mass spectrometry

Gas chromatography (GC) is a pivotal analytical technique in modern chemistry and was first introduced by Martin and James (1952). GC employs the partitioning between a moving gas phase and a stationary liquid phase within a column for separation. The emerging gas

stream, containing the isolated components, is analyzed by a detector, with the signals serving as data inputs (Bartle *et al.*, 2002).

Central to GC is the column, influencing the quality of compound separation. Column selection primarily hinges on the interactions between the stationary phase and analyte, while considering the sample matrix and solvent. It's advisable to opt for the least polar phase yielding satisfactory results, as non-polar phases exhibit greater longevity compared to polar ones (Bartle *et al.*, 2002).

Combining GC with mass spectrometry (MS) yields one of the most powerful approaches for unravelling structural insights and selectivity within complex mixtures. Initially intricate due to the cost of MS, the emergence of affordable bench-top MS instruments over the years has triggered a surge in the technique's adoption (Bartle *et al.*, 2002). In this tandem technique (GC-MS), the mass spectrum corresponds to the structure and molecular weight of the analyte. By comparing this spectrum with libraries of known spectra, the Identification of compounds becomes feasible. This synergy between GC and MS significantly enhances the analysis of intricate mixtures (Bartle *et al.*, 2002).

Mass spectrometry is an incredibly versatile tool for investigating virtually every aspect of various molecules including proteins through the use of liquid chromatography mass spectrometry. Proteomics covers a broad range of experiments aimed at addressing a multitude of protein-related aspects, including protein sequences, quantitative abundance, subcellular distribution, functional roles, three-dimensional structures, chemical characteristics, protein-protein interactions, and more. The wide scope of proteomics showcases the comprehensive nature of this analytical technique in exploring the intricate world of proteins with other molecules, which is an essential tool to obtain a better understanding of various bioactivities that are the aim of this work (Shuken, 2023).

1.6 Objectives

The main objectives of this work are: i) to select, collect, identify and characterize macroalgae from the Portuguese coast; ii) to perform a preliminary qualitative phytochemical screening for the presence of some compounds; iii) to evaluate the antioxidant activities of selected seaweed species; iv) to assess the fatty acid profile of the selected seaweeds; v) to evaluate the antibacterial activities of the selected seaweeds; vi) to identify proteins with potential bioactivity using proteomics. Overall, the main aim of the present work is to produce information on the presence of bioactive compounds, especially those with the highest antioxidant activity and also to study the extracts with higher antibacterial activities. These goals are

achieved through a series of targeted assays for bioactive compounds using several qualitative assays, GC-MS, antibacterial assays, and proteomic analyses.

2

MATERIALS AND METHODS

2.1 Seaweed species collection

Seaweeds were collected at "Praia do Magoito" (Portugal, 38 51'40.2"N 9 27'02.2"W) on the 27th of April 2023 on the low tide, from 2pm to 3pm; Praia das Avencas-Parede (Portugal, 38 41'28.3"N 9 21'60.3"W) on the 18th of May 2023 on the low tide, from 8 am to 9 am and in early July. The time of the year chosen for the collection of algae was based on literature which indicated a potential higher concentration of molecules of interest in between the late spring and early summer in the Northern Hemisphere (Nielsen *et al.*, 2021).

The algae were collected, stored in zip lock bags, and transported in a cooler box to NOVA School of Science and Technology facilities, where they were separated from any other organisms attached and washed with milli-Q water to remove sand, salt and any other particles or impurities.

The identification was made using various guides and literature that catalogue species present on the Portuguese and European Atlantic and Mediterranean coast (Pereira & Gaspar, 2020; Fish & Fish, 2011).

The seaweed samples were stored in -45ºC until further processing.

2.2 Samples treatment

In this work, different extraction methods were tested to extract different compounds based on their hydrophobicity. Various methods were explored with different dry algae to solvent ratios and methods to obtain the best yields possible based on previous existing literature.

2.2.1 Sample preparation

Algae samples were thawed, excess of water was removed and samples were lyophilized (ScanVac CoolSafe 9L, LaboGene, Denmark) for 72 hours. The resulting dry algae were further grinded with the aid of a bladed grinder and liquid nitrogen (Air Liquide, Portugal), for finer powders a mesh sieve and pestle were used.

The resulting dry powders were stored in boxes filled with silica in the dark at room temperature.

2.2.2 Extractions

The extractions were carried out using the following species of algae: *Chondrus crispus* Stackhouse, 1797; *Codium tomentosum* Stackhouse, 1797; *Codium adhaerens* C. Agardh, 1822 *Cystoseira tamariscifolia* (Hudson) Papenfuss 1950; *Fucus spiralis* Linnaeus, 1753; *Porphyra umbilicalis* Kützing, 1843 and *Ulva intestinalis* Linnaeus, 1753.

Distilled water extraction was chosen with the aim of collecting hydrophilic molecules mostly proteins. Methanol and microwave assisted extractions were both chosen with the aim of collecting hydrophobic molecules with reported antioxidant activity with the difference between them being the method by which the plant cell wall is ruptured, by ultrasounds and microwaves energy respectively. Soxhlet extraction was chosen with the aim of extracting highly hydrophobic molecules. These extraction methods provide a high coverage of the molecules that are present in the samples.

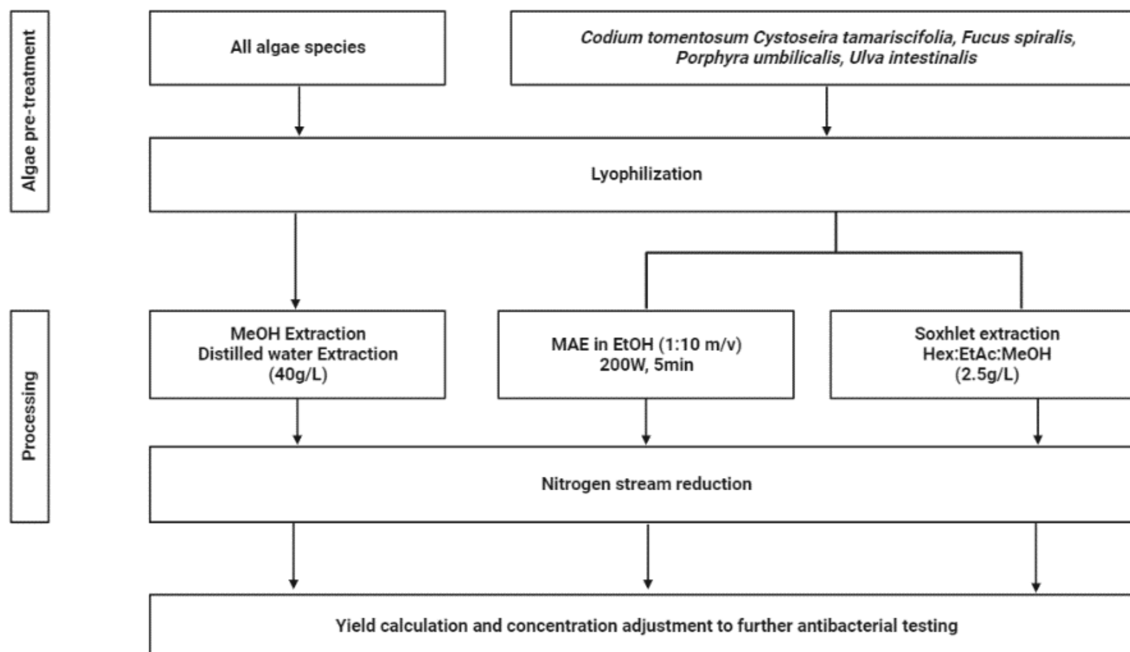

Figure 8 - Scheme representing the different extraction methods and seaweed species which went through specific extractions.

2.2.2.1 Extraction with distilled water

This extraction procedure was based on Farasat *et al.* (2014), with further modifications. In brief, 1.5 g of seaweed dry powder was extracted with 10 mL of distilled water and subjected to ultrasonication using an ultrasonic bath (J.P. Selecta, Spain) at 40 kHz for 20 min, vortexed until a homogenous mixture was obtained and then left to stand at 4 ºC, for 48 hours, in the dark.

The extracts where then centrifuged at 1,500 g for 20 minutes (CENTRIC 150, Tehtnica, Slovenia). The resulting supernatant was removed and filtered through a filter paper (Whatman No.1. Sigma-Aldrich, USA).

Following centrifugation, the remaining pellet was resuspended in 5 mL of distilled water and left for 1 hour at 4 ºC in the dark. The resulting extract was centrifuged once again at 1,500 g for 20 minutes (CENTRIC 150, Tehtnica, Slovenia), filtered through filter paper (Whatman No.1, Sigma-Aldrich, USA), combined with the volume obtained previously, and stored at -20 ºC until further analysis.

A fraction of the extract was dried, using a gentle nitrogen stream, and weighed to determine the yield.

Figure 9 - *C. crispus* extraction using distilled water.

2.2.2.2 Methanol extraction

This extraction was based on the work by Farasat *et al*. (2014) with further modifications. Thus, 200 mg of dried seaweed sample powder was extracted using 6 mL methanol (Honeywell Riedel-de Häen, Germany) in an ultrasonic bath (J.P. Selecta, Spain) at 40 kHz for 20 minutes, vortexed and left to stand at 4 ºC for 48 h.

The extract was centrifuged at 1,500 g for 10 minutes (CENTRIC 150, Tehtnica, Slovenia), filtered through filter paper (Whatman No.1, Sigma-Aldrich, USA), and stored at -20 ºC until further analysis.

A fraction of the extract was dried using a gentle nitrogen stream and weighed to determine the yield.

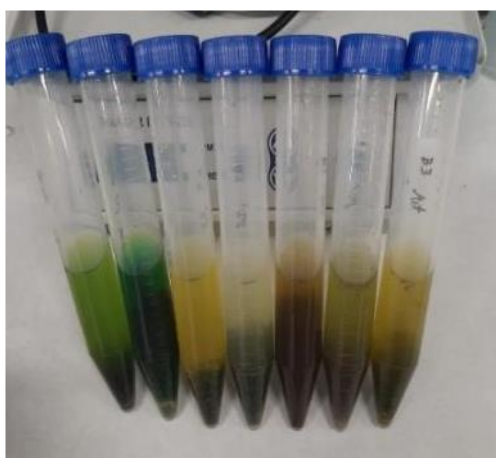

Figure 10 - Extractions using methanol before filtration.

2.2.2.3 Microwave Assisted Extraction

The extraction procedure was adapted from the method proposed by Cagalj *et al.* (2022). Thus, 1.0 g of dry algae was extracted with 10 mL of ethanol 50% using a multimode laboratory microwave reactor (MLS ETHOS 1600, MLS GmbH, Germany) for 5 minutes at 200W and a temperature of 60 ºC. The temperature was selected based on previous literature which indicated the possibility of degradation of molecules at higher temperatures (Saini and Keum, 2018). Along with these, one of the vessels was filled with 10 mL of ethanol 50% for temperature control. After extraction, the vessels were let to cool before opening.

The resulting extracts were filtered, and dried with a gentle stream of nitrogen, to determine yield and concentration adjusted to 20 mg/mL for future assays.

Figure 11 - Microwave reactor used in extractions.

2.2.2.4 Soxhlet extraction

In this method adapted from Silva *et al.* (2020), two g of dry algae powder were inserted into a paper thimble shaped cartridge, covered with hydrophilic cotton and inserted into a Soxhlet extraction apparatus. This apparatus uses a magnetic stirrer hot plate (J.P. Selecta, Spain), a 250 mL short neck round bottom flask containing a stirring magnet, a Soxhlet extractor and a condenser with flowing water kept at 12 ºC with the aid of a refrigerated bath (Haake K20, Germany).

The extraction was carried out using 200 mL of n-Hexane (Fisher Chemical, Germany) at boiling temperature for 24 hours and allowed to cool for one hour. The resulting extract was reduced via rotavapor (Büchi, Switzerland), resuspended in two mL of n-Hexane and stored

in a previously weighted flask. The same process was continued for ethyl acetate (Fisher Chemical, Mexico) and methanol (Honeywell Riedel-de Häen, Germany) in this order.

Figure 12 - Soxhlet apparatus for the extraction of selected seaweeds.

2.3 Antibacterial and biochemical assays

2.3.1 Antibacterial activity assays

For the assessment of antibacterial activity, two methods were used: growth inhibition halos and determination of the Minimum Inhibitory Concentration (MIC) value. The antibacterial activity was assessed for two bacterial species, *Staphylococcus aureus* strain COL and *Escherichia coli* strain ATCC 35218.

Staphylococcus aureus was propagated on tryptic soy agar (TSA) (Becton Dickinson and Company, France) and tryptic soy broth (TSB) (Becton Dickinson and Company, France), solid and liquid media.

Escherichia coli ATCC 35218 was propagated on lysogeny broth (LB) (Nzytech, Portugal) and lysogeny broth with 1.5% agar (LA) (Labchem, Portugal), for liquid and solid media.

The selected bacteria were inoculated on solid media, within a sterile environment and incubated overnight at 37 ºC. At the end of this period, they were checked for contaminations. The plates were then stored at 4 ºC.

2.3.1.1 Determination of Minimum Inhibitory Concentration values

Pre-inocula were prepared in 5 ml of TSB and LB for *Staphylococcus aureus* COL and *Escherichia coli* ATCC 35218 respectively, by overnight incubation at 37 ºC. The resulting culture had its optical density at 600 nm (OD_{600nm}) measured (Turner, USA) and via a dilution into 10 mL of LB was corrected to 0.005.

For this assay, a 96-well microplate was used. In the first column, the test sample was added to of the culture ($OD_{600\ nm}$ = 0.005) at a 1:20 dilution. Serial dilutions (1:2) were carried out from the first to the tenth column. In the eleventh column, solvent was added to the culture at the same proportion, as a positive growth control. In the twelfth column, sterile media with the same proportion of solvent than used in the test sample, was added as negative control.

The microplate (Thermo-Fisher, Denmark) was incubated overnight at 37 ºC and checked for growth the following day. The minimum inhibitory concentration was determined by visual inspection of the concentration that resulted in inhibition of bacterial growth.

2.3.1.2 Growth inhibition halos

Pre-inocula were prepared in 5 ml of TSB and LB for Staphylococcus aureus COL and Escherichia coli ATCC 35218 respectively, by overnight incubation at 37 ºC.

TSA and LA petri dishes were inoculated with *Staphylococcus aureus* COL and *Escherichia coli* ATCC 35218 respectively, using a cotton swab (Aptaca, Italy) that was immersed in the pre-inocula.

Sterile paper filters (Prat Dumas, France) were embedded with 10 µL of the sample to test and left to dry to remove the solvent. Papers filters embedded with the respective solvent were used as a blank.

The plates were divided into 2 sections to test 2 different samples per plate. The embedded filters were placed at the center of the designated section allowing space to avoid possible overlapping of the resulting halos.

The plates were incubated overnight at 37 ºC and the results were analyzed.

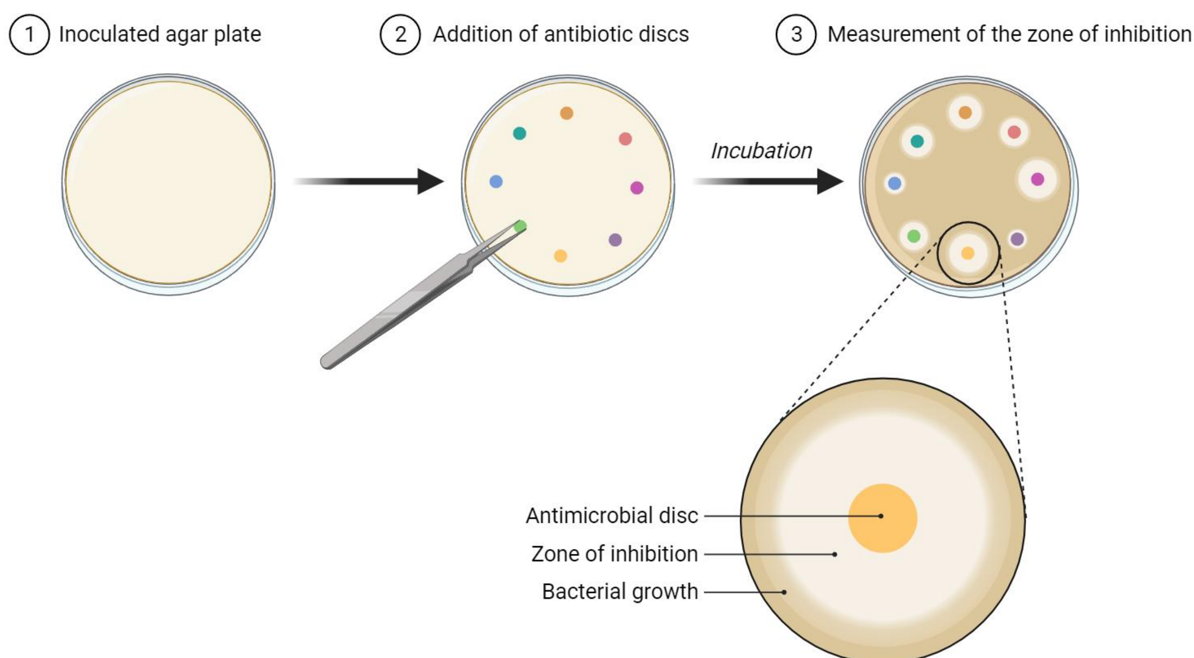

Figure 13 - Zone of inhibition test diagram (created using Biorender.com).

2.3.2 Total soluble protein

For the determination of the total protein content of each sample, two methods were used that follow the same principle of dye protein interactions but differed on the interaction they have with peptides and amino acids, these were Bradford and Lowry assays.

2.3.2.1 Bradford

This assay is based on the binding of Coomassie Brilliant Blue G-250 dye to proteins through specific amino acid residues, which are mainly arginine residues, but it is also able to bind with lower affinity to histidine, lysine, phenylalanine, tryptophane and tyrosine. This assay was first proposed by Bradford (1976) and adapted to a 96-well microplate.

First, a calibration curve was prepared using bovine serum albumin (BSA) (Nzytech, Portugal) as standard, with concentrations ranging from 4 to 0 mg/mL. Bradford´s reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 (Sigma-Aldrich, USA) in 50 mL of 96% ethanol (Fisher chemicals, United Kingdom), then adding 100 ml of 85% phosphoric acid (Panreac, Spain) and finally diluted with distilled water to a final volume of one liter and filtered to remove precipitates.

Then, to perform the assay 180 µL of Bradford reagent followed by 20 µL of sample or BSA standard were added to each well of a 96-well microplate (Greiner, Bio-one, Austria) and left to incubate for 5 minutes before reading the absorbance.

Absorbance was measured at 595 nm using a microplate reader (Synergy HTX, BioTek, USA) and the concentration of each sample was determined using the calibration curve. Results were expressed as mass of protein of mass of dry algae (mg protein/g dry algae).

2.3.2.2 Lowry

This assay is based on the oxidation of peptides that occurs with Folin's reagent which can be catalyzed by copper as proposed by Lowry et al. (1951). This oxidation is sensitive to peptide bonds and tyrosine and tryptophane amino acids.

Thus, 2% Na_2CO_3 (Sigma, Germany) in 0,1M NaOH (Laborspirit, Portugal), 1% $CuSO_45H_2O$ (Sigma, Germany) and 2% Potassium Sodium tartrate (Sigma, Germany) were prepared, these solutions will be referred forward as solutions A, B1 and B2 respectively. Solution B was prepared by mixing 1.0 mL of B1 and B2. Solution C was prepared by mixing 4,.9 mL of solution A with 0,1 mL of solution B. Solution D was prepared by diluting 1.0 mL of Folin-Ciocalteau reagent (Sigma-Aldrich, Switzerland) in 5 mL of H_2O. A calibration curve was also prepared using bovine serum albumin (Nzytech, Portugal) with concentrations ranging from 4 to 0 mg/mL.

Then, 40 µL of sample or standards were added to 200 µL of solution C and were left to rest for 10 minutes at room temperature, followed by 40 µL of solution D a 30-minute incubation. After the reaction time has passed, 140 µL were added to the wells of a 96-well microplate (Greiner, Bio-one, Austria) , meaning each microtube will fill two wells.

The absorbance was read at 680 nm using a microplate reader (Synergy HTX, BioTek, USA) and the concentration of each sample was determined using the calibration curve. Results were expressed as mass of protein of mass of dry algae (mg protein/g dry algae).

2.3.3 Phytochemical assays

For a preliminary identification of the algae content in a qualitative manner, a series of qualitative tests were performed to assess the presence of various molecules with pharmaceutical and industrial interest these being: Alkaloids, carbohydrates, coumarins, flavonoids, glycosides, gums, phenolic compounds, phytosterols, proteins, tannins, triterpenes, quinones, reducing sugars, resins and saponins.

2.3.3.1 Alkaloids

Qualitative analysis for alkaloid presence was conducted using Mayer's test as described by Shaikh & Patil (2020) adapted from Auwal *et al.* (2014), in which to 1.0 mL of aqueous acidified extract two drops of Mayer's reagent (6:1 mixture of 18.1 g/L mercuric chloride (Merck, Germany) aqueous solution and 200 g/L potassium iodide (Pronalab, Portugal) aqueous solution) were added to the side of the test tube. A positive result is indicated by the formation of a creamy white or yellow precipitate.

2.3.3.2 Carbohydrates

For the qualitative assessment of the presence of carbohydrates was used a test for the presence of starch as described by Shaikh & Patil (2020) adapted from Audu *et al.* (2007), in which 5 mL of a 5% KOH (Labchem, Portugal) solution was added to 1.0 mL of an aqueous algae extract. A positive result is confirmed by a light shade of yellow.

2.3.3.3 Coumarins

To perform a qualitative analysis for the detection of coumarins, a test for its presence was performed as described by Shaikh & Patil (2020) adapted from Singh & Kumar (2017). In this, test, also referred as NaOH test, to a 1.0 mL of aqueous extract, 1.0 mL of a 10% NaOH (Labchem, Portugal) solution and 1.0 mL of chloroform (Honeywell Riedel-de Häen, Germany) were mixed. A positive result is indicated by a yellow color.

2.3.3.4 Flavonoids

To qualitatively ascertain the presence of flavonoids, an alkaline reagent test was performed as described by Shaikh & Patil (2020) adapted from Singh & Kumar (2017). Thus, to 1.0 mL of an aqueous extract, 2.0 mL of a 2%NaOH (Laborspirit, Portugal) solution were added followed by a few drops of HCl (Honeywell Flucka, Australia). A positive result is evidenced by an intense yellow color which after the addition of acid then becomes colorless.

2.3.3.5 Glycosides

Qualitative analysis for the presence of glycosides was conducted using concentrated H_2SO_4 as described by Shaikh & Patil (2020) adapted from Sheel *et al.* (2014). Thus, to 2.5 mL of aqueous plant extract, 1.0 mL of glacial acetic acid (Honeywell Flucka, Germany) were

added followed by a few drops of 5% $FeCl_3$ (Alfa Aesar, Germany) and concentrated H_2SO_4 (Honeywell Riedel-de Häen, Germany). A positive result is indicated by the presence of a brown ring at the surface of the resulting mixture.

2.3.3.6 Gums and Mucilages

To assess the presence of gums and mucilage, an alcohol test was performed adapted from Shaikh & Patil (2020) adapted from Raaman (2006). To 1.0 mL of aqueous extract, 2.5mL of methanol (Honeywell Riedel-de Häen, Germany) was added with constant stirring. A positive result is indicated by a white or cloudy precipitate.

2.3.3.7 Phenolic molecules

The qualitative assessment of the presence of phenolic molecules is based on a test for carotenoids, as described by Shaikh and Patil (2020) adapted from Tyagi (2017). Thus, to 1mL of a 10% (m/v) chloroform algae extract, 1.0 mL of H_2SO_4 (Honeywell Riedel-de Häen, Germany) are added. A positive result is indicated by a blue color formed at the interface.

2.3.3.8 Phytosterols

The presence of Phytosterols was qualitatively determined using the Hesse's response method, as described by Shaikh and Patil (2020) adapted from Kumar & Jat (2018). Thus, to 2.5 mL of an aqueous extract, 1.0 mL of chloroform Honeywell Riedel-de Häen, Germany) and H_2SO_4 (Honeywell Riedel-de Häen, Germany) were added. A positive result is indicated by a pinkish red ring in the lower chloroform layer.

2.3.3.9 Proteins

The qualitative determination of the presence of proteins was carried out using the Bradford assay as described by Bradford (1976). Thus, to 0.5 mL of aqueous plant extract an equal amount of Bradford reagent (described in section 2.3.2.1) is added. A positive result is evident by the formation of a blue color.

2.3.3.10 Quinones

The qualitative evaluation for the presence of quinones entailed the use of the concentrated HCl test adapted from Shaikh and Patil (2020) adapted from Basumatary (2016). Thus,

to a small amount of raw dry algae, 0.2 mL of HCl (Honeywell Flucka, Australia) was added. A green color in the supernatant indicates a positive result.

2.3.3.11 Reducing Sugars

A qualitative assay for the detection of reducing sugars was performed using the Benedict's test as described by Shaikh and Patil (2020) adapted from Singh & Kumar (2017). To 0.5 mL of an aqueous extract was combined to 0.5 mL of Benedict's reagent (8:1 mix of a 0,216 g/mL sodium citrate (Sigma-Aldrich, Germany) 0.125 g/mL sodium carbonate (Sigma, Germany) aqueous solution with a 21.6 g/mL copper sulphate (Merck, Germany) aqueous solution) and boiled for 2 minutes. A positive result is indicated by the change into a green, yellow or red color.

2.3.3.12 Resins

To perform a qualitative assessment for the detection of resins, we employed a turbidity test as described by Shaikh and Patil (2020) adapted from Santhi and Sengottuvel (2016). To 1mL of aqueous extract, 2 mL of 4% HCl were added. A positive result is shown by the appearance of turbidity in the mixture.

2.3.3.13 Saponins

The presence of saponins was qualitatively determined using a foam test described by Shaikh and Patil (2020) adapted from Tiwari *et al.* (2011). To 0.5 g of dry algae, 2.0 mL of distilled water were added and vigorously shaken. A positive result is indicated by a persistent foam that can last up to 10 minutes.

2.3.3.14 Tannins

In order to assess the qualitative presence of tannins, the 10% NaOH test was used as described by Shaikh and Patil (2020) adapted from Singh and Kumar (2017). Thus, to 0.4mL of aqueous plant extract, 4.0 mL of a 10% NaOH (LabChem Portugal) solution were added, and the resulting mixture was continuously shaken. A positive result was indicated by the formation of emulsions.

2.3.3.15 Triterpenes

A qualitative assessment for the detection of triterpenes was conducted using the Salkowski test as described by Shaikh and Patil (2020) adapted from Singh and Kumar (2017). To 1.0 mL of a chloroform algae extract, a few drops of H_2SO_4 (Honeywell Riedel-de Häen, Germany) were added. A positive result is indicated by the formation of a golden yellow layer at the bottom.

2.3.4 Antioxidant assays

In this work, various methods for detecting and measuring antioxidant activity were used. These varied on the target molecule or interaction with which compound is being analyzed. This provides us not only information regarding the antioxidant activity but can also elucidate the pathway by which this activity occurs.

2.3.4.1 ABTS

This assay is based on the reduction reaction of a radical ABTS solution, which will turn from a blueish tone to colorless as described in Re *et al.* (1999) and adapted to a microplate.

An $ABTS^+$ solution was prepared by mixing ABTS 7 mM (Alfa Aesar, India) with potassium persulphate solution 2.45 mM (Carlo Erba, France) in a 1:1 ratio and left overnight protected from light. This solution was then diluted to perform the assay (Honeywell Riedel-de Häen, Germany), it was considered sufficiently diluted when it reached an absorbance of 0.7 at 734 nm. A standard curve was prepared using Trolox as standard (Sigma-Aldrich, Russian Federation) with concentrations ranging from 15 to 0 µg/mL.

Then, 100 µL of the standard or sample and 100 µL of $ABTS^+$ solution was added to each well of a 96-well microplate (Greiner, Bio-one, Austria), being left to incubate for 6 minutes at room temperature in the dark before the absorbance being measured at 734 nm in a microplate reader (Synergy HTX, BioTek, USA) and the results were expressed as a percentage of inhibition according to equation 1.

$$\%\,inibition = (Abs_{control} - (Abs_{sample} - Abs_{blank})/Abs_{control}) \times 100$$

Equation 1 - ABTS Inhibition determination

2.3.4.2 DPPH

This assay is based on the reaction between an antioxidant molecule with a stable free radical DPPH●, leading to a loss of color which can be monitored by checking the absorption at near 517 nm. This assay is based on the assay previously described by Blois *et al.* (1958) and adapted to 96-well microplate.

First, a 24 μg/mL DPPH solution was prepared by dissolving 2.4 mg of DPPH (Sigma, Germany) in 100 mL of methanol (Honeywell Riedel-de Häen, Germany) along with a standard curve, which was prepared using Trolox as standard (Sigma-Aldrich, Russian Federation) with concentrations ranging from 0.5 to 0.0156 mg/mL.

Then, 10 μL of sample or standard curve along with 190 μL of DPPH solution was added into the wells (in triplicate) of a 96-well microplate (Greiner, Bio-one, AustrIwhich was left to incubate for 30 minutes at room temperature covered with aluminum foil and the absorbance was then read at 517 nm in a microplate reader (Synergy HTX, BioTek, USA).

The antioxidant activity of the sample was expressed as Trolox equivalents (TE) in relation to the dry algae weight (mgTE/g DW).

2.3.4.3 Ferric Reducing Antioxidant Power (FRAP) assay

This assay is based on the principle of the reduction of ferric tripyridyl triazine complexes to the ferrous form at low pH which will create a blue color which can be monitored by measuring changes in absorption at 593 nm, first described by Benzie *et al.*, (1996) and being further adapted for a 96-well microplate.

The FRAP reagent solution was prepared by mixing 50 mL of 0.3 M sodium acetate buffer (pH 3.6), 5 mL of 10 mM 2,4,6-tri(2-piridil)-s-triazine solution (TPTZ, Sigma-Aldrich, Switzerland) and 5 mL of 20 mM ferric chloride solution (Alfa Aesar, Germany). A standard curve was prepared using Trolox as standard (Sigma-Aldrich, Russian Federation), ranging from 0 To 50 μg/mL.

First, 15 μL of ultrapure water were added to each well of a 96-well microplate (Greiner, Bio-one, Austria) followed by 20 μL of sample, standard or methanol for blanks and then 265 μL of FRAP reagent. For sample blanks, 280 μL ultrapure water and 20 μL of sample were added to each well. Afterwards, the microplate was incubated for 30 min at 37 ºC (Labnet, USA), in the dark.

The absorbance was measured at 595 nm in a microplate reader (Synergy HTX, BioTek, USA) and the results were expressed as milligrams of Trolox equivalents per gram of dry weight (mg TE/g DW).

2.3.4.4 Total Phenolic Content

Total phenolic content was determined using the Folin-Ciocalteau method as described in Singleton *et al.*, (1965) and adapted to a 96-well microplate as described by Müller *et al.*, (2010) with some modifications. This assay is based on the reduction of phosphor-molybdate heteropoly acids Mo(VI) center in the heteropoly complex to Mo(V), resulting in a blue coloration which is measured spectrophotometrically at around 750 nm.

For this assay, a calibration curve was prepared using gallic acid (Alfa Aesar, Germany) as standard, with concentrations ranging from 0 to 2.0 mg/mL prepared by serial dilutions. A solution of 10% (v/v) Folin-Ciocalteau reagent (Sigma-Aldrich, Switzerland) and a 10 mg/mL sodium carbonate aqueous solution (Sigma-Aldrich, USA) were also prepared.

Then, to perform the assay, 20 µL sample or standard and 100 µL Folin-Ciocalteau reagent (Sigma-Aldrich, Switzerland) 10% were added to a 96-well microplate and left to incubate in the dark at room temperature for 5 minutes. Afterwards, 80 µL of sodium carbonate 10 mg/mL were added, placed in a plate shaker (Comecta-Ivymen, Spain) for one minute and left to incubate in the dark at room temperature for 30 minutes.

Absorbance was read at 750 nm in a microplate reader (Synergy HTX, BioTek, USA) and the results expressed as mM gallic acid equivalent per gram of dry algae material (mg GAE/g DW).

2.3.4.5 Total Tannin Content

Total tannin content was determined via the determination of condensed tannins using acidified vanillin, this assay is based on the formation of a tannin-vanillin complex under acidic conditions. This assay is based on the methods proposed by Broadhurst *et al.* (1978) and adapted to a 96-well microplate with further modifications by Zhong *et al.* (2020).

A calibration curve was prepared by preparing methanolic (Honeywell Riedel-de Häen, Germany) catechin (Sigma-Aldrich, France) as standard solution ranging from 0 to 1.0 g/L.

Then, 25 µL of sample or standard, 150 µL 4% (w/v) methanolic (Honeywell Riedel-de Häen, Germany) vanillin (Sigma-Aldrich, Germany) solution and 25 µL 32% (v/v) methanolic (Honeywell Riedel-de Häen, Germany) sulfuric acid (Honeywell Riedel-de Häen, Germany) solution were mixed in a 96-well plate and incubated at room temperature for 15 min.

The absorbance was measured at 500 nm in a microplate reader (Synergy HTX, BioTek, USA). The results are presented as microgram equivalents of catechin per gram of dry algae (µg CE/g DW).

2.3.5 GC-MS

Samples were also analyzed by GC-MS using a gas chromatography mass spectrometer (6850 coupled with 5975C VL MSD, Agilent Technologies, USA), equipped with an autosampler (G45134A, Agilent Technologies), using a BB-5ms fused silica capillary column, 30 m x 0.25 mm x 0.25 µm (Phenomenex, USA). Helium (99.9% purity) was used as the carrier gas with a column flow rate of 1.0 ml/min.

Oven temperature program: initial temperature of 80 ºC, raised at a rate of 4 ºC/min till 200 ºC, then raised at 1 ºC/min up to 215 ºC for 15 minutes and finally raised at 12 ºC/min up to 308 ºC for 7.75 minutes. The injection volume was 1 µL. The resulting peaks were identified using the MSD Chemstation Data Analysis F.01.03.2357 (Agilent Technologies, Inc., USA) with the use of an available library.

The samples analyzed were pretreated to detect fatty acid methyl esters as described in 2.3.5.1 and it was also possible to detect in samples from Soxhlet extraction different molecules with relevance to the present work.

2.3.5.1 FAMEs

In this assay, fatty acids were converted to their fatty acid methyl esters by direct transesterification within tissues and without initial lipid isolation. This method was first described by Lepage *et al.* (1986) and further modified by Kumari *et al.* (2011).

Thus, 500 mg of dried sample powder were added to 5.0 mL of a methanolic (Honeywell Riedel-de Häen, Germany) acetyl chloride (Sigma-Aldrich, Germany) solution in a ratio of 1:19 (v/v) and it was then spiked with 10 µL of an internal standard solution (1.0 mg/mL C13:0 TAG (Sigma-Aldrich, USA) in n-Hexane (Fisher Chemical, Germany). The solution was esterified in a dry bath (Labnet, USA) at 80 ºC for one hour and then left to cool. 1.0 mL of water and 2.0 mL of n-Hexane (Fisher Chemical, Germany) were added to the mixture which was then vortexed and centrifuged (CENTRIC 150, Itnica, Slovenia) at 2,057 x g for 5 minutes.

The organic phase was collected in 200 µL GC-MS vials (Alwsci, China), later dried and combined under a gentle flow of nitrogen and solubilized with 10 µL of n-Hexane (Fisher Chemical, Germany) for GC-MS analysis.

2.3.6 Short length fatty acids

For the fatty acid analysis HPLC equipment DIONEX Summit (Sunnyvale, CA, USA) was used, VFA and NVFA were separated with an Aminex HPX-87H cation exchange column (300 x 7.8 mm, particle size 9 µm) for organic acid analysis which was protected by a guard

column. The sample amount was 20 µl. The fatty acids were detected at 210 nm with the column temperature maintained at 35 ºC. The chromatography with a mobile phase composed of 10 mN H_2SO_4 with a flow rate of 0.6 mL/min.

The data collected was treated using Chromeleon I Dionex 1996-2006 Version 6.80 DU10c Build 2859 (179491).

2.3.7 Lipidic content (gravimetric method)

In this assay, lipids are extracted and quantified by a simple two-step extraction method using biological material such as algae (Breil *et al.*, 2017). This method was first described by Bligh *et al.*, (1959) with further modifications to a total volume of 50 mL.

Therefore, in a 50 mL falcon, 2 g of lyophilized algae sample, 16 mL of methanol, 16 mL of dichloromethane and 6.4 mL of phosphate buffer were subjected to an ultrasonic bath (J.P. Selecta, Spain) for 10 minutes at 40 kHz.

The resulting mixture was then shaken using a plate shaker (Comecta-Ivymen, Spain) for 20 minutes and then left to rest overnight. Next, the resulting phases were separated and filtered using filter paper (Whatman, nº1, Sigma-Aldrich, USA)

A fraction of the organic phase was removed and separated into three different 2 mL microtubes pre-weighted and dried by a gentle nitrogen stream. The resulting dry extract was weighed and considered to contain 100% lipidic content.

2.3.8 Proteomics

In order to increase our understanding of the protein composition within our algae specimens, a proteomic analysis was also performed with the close aid of the Proteomic Services at Bioscope Lab at FCT NOVA, which provided the protein content as well as the identity of proteins present in the selected algae species.

2.3.8.1 Pre-analytical sample preparation

Each algae sample, weighing 100 mg, was mixed in 5 mL of the reducing Tris-SDS extraction solution (0.1M Tris-HCl (Sigma–Aldrich) with a pH of 8.0, 1% SDS (Thermo Fisher Scientific, USA) (m/v), containing 10mM tris(2-carboxyethyl) phosphine (TCEP). Samples were sonicated for 4 minutes (30 s on, 10 s off) at 50% Ultrasonic Amplitude, and 40 kHz Ultrasonic Frequency (Branson SLPe digital sonifier, USA) followed by incubation at 95 ºC in a water bath for 5 min. After cooling to room temperature, algae lysates were clarified by centrifugation at 10,000 g for 20 min. The clear supernatants were transferred to new tubes and stored at -80 ºC until further use.

2.3.8.2 Protein digestion

Protein digestion was executed employing the Filter-aided sample preparation method (FASP) as described in Winiewski (2019) with modifications as described in Carvalho *et al.* (2020). Briefly, 0.4 mL of protein extract was loaded into a Vivaspin 500 centrifugal concentrator 30,000 Da MWCO (Sartorious, Germany), followed by centrifugation at 14,000 x g for 20 min at 22ºC. The previous procedure was repeated to secure the proteome from a total volume of 0.8 mL of the algae extract within the FASP membrane.

The proteins were reduced and alkylated by adding 200 µL of reducing/alkylation solution containing 10 mM TCEP, and 40 mM chloroacetamide prepared in 8M urea, 0.1M Tris-HCl (Sigma–Aldrich) pH 8.0.

Samples were incubated for 30 minutes at 37 ºC. Afterwards, samples were centrifuged at 14,000 g for 10 minutes at 22 ºC. Then samples were washed 3 times with 200 µL of 8 M Urea, 0.1 M Tris-HCl (Sigma–Aldrich) pH 8.0, followed by two washes with 70 mM Triethylammonium bicarbonate buffer (TEAB). Proteome digestion was performed by the addition of 100 µL of 0.01 µg/µL Trypsin/Lysine-C in 70 mM TEAB buffer, followed by overnight incubation at 37 ºC. Finally, peptides were recovered with 2 x 100 µL of 3% (v/v) Acetonitrile (I) in 0.1% aqueous Formic Acid (FA) by centrifugation at 14000 x g for 10 min at 22ºC. Peptides were dried and stored at -20 ºC until further analysis by Nano-LC-MS/MS.

2.3.8.3 Total peptide quantification

Before quantification, peptides were resuspended in 100 µL of 3% (v/v) I, containing 0.1% (v/v) aqueous FA. Then, samples were homogenized for 5 min on vortex followed by 10 min on an ultrasonic bath (Branson SLPe digital sonifier, USA) at 100% Ultrasonic Amplitude, 35 kHz ultrasonic frequency. Total peptide quantification was performed via the Pierce™ Quantitative Colorimetric Peptide Assay (480 nm).

The calibration curve was generated using the Peptide Digest Assay Standard provided in the kit (0 to 500 µg/mL). The analysis was performed using a CLARIOstar® High-Performance Monochromator Multimode (BMG LABTECH, Germany). Each calibration curve standard and samples were measured in duplicates.

2.3.8.4 LC-MS/MS data acquisition

The analysis was carried out using an UltiMate™ 3000 RSLCnano System coupled to an Impact HD (Bruker Daltonics, USA). This configuration featured a CaptiveSpray nano-Booster, utilizing acetonitrile as dopant (Carvalho et al., 2020). The ionization source

parameters were set as follows: a capillary voltage of 1300 V, nanoBooster pressure at 0.2 bar, dry gas flow rate at 3.5 L/min, and a temperature of 150 ºC.

Digests were adjusted to a concentration of 350 ng/µL by diluting with 3% (v/v) I in 0.1% (v/v) aqueous FA. Subsequently, 1.0 µL of this diluted sample was loaded onto a µPAC™ Trapping column (PharmaFluidics, Belgium). Peptides were desalted for 4 min at a flow rate of 10µL/min using 1% CAN containing 0.1% (v/v) aqueous Trifluoracetic Acid. Subsequent peptide separation was achieved on a 2 m µPACTM (PharmaFluidics, Belgium) analytical column.

MS acquisition was set to cycles of MS (2.0 Hz), followed by MS/MS (8-32 Hz), cycle time 3 seconds, with active exclusion (precursors were excluded from precursor selection for 0.5 min after the acquisition of one MS/MS spectrum, intensity threshold for fragmentation of 2500 counts). Together with active exclusion set to one, reconsider precursor if the intensity of a precursor increases by a factor of 3, this mass was taken from temporarily exclusion list and fragmented again, ensuring that fragment spectra were taken near to the peak maximum. All spectra were acquired in the range 150-2200 m/z.

2.3.8.5 Data analysis

All MS/MS spectra were exported into Mascot Generic Files (.mgf) files for data analysis by applying an internal script from Data Analysis 4.2 (Bruker, USA). Data analysis was performed using Mascot 2.3.02 (Matrix Science, United Kingdom) integrated into ProteinScape 4.0 (Bruker, USA). Search parameters were as follows: NCBI Taxonomy browser downloaded FASTA files Porphyra (genus, 16,818 proteins), Sargassaceae (family, 14,310 proteins) and Ulvaceae (family, 12,603 proteins), trypsin/P was selected as the digestion enzyme, and two missed cleavages were allowed. Monoisotopic peptide masses were searched with 20 ppm peptide mass tolerance and 0.05 Da fragment mass tolerance. Carbamidomethyl on Cysteine was selected as a fixed modification, and oxidation on Methionine, and Acetylation of protein N-terminal as the variable modifications. False Discovery Rate (FDR) adjusted to 1% (Jorge *et al.*, 2019).

Data were also analyzed using String (V12.0) to search for protein-protein interactions, including direct (physical) and indirect (functional) associations.

2.4 Statistical analysis

Results were analyzed using a one-way ANOVA, after checking the statistical assumptions. In cases where these assumptions were not met, the data was subjected to a logarithmic

transformation before the ANOVA analysis. Tukey's post-hoc test was used to assess differences between samples.

Statistical analysis was carried out with a 5% significance level using Statistica 8.0 software (StatSoft Inc., USA).

All data was expressed as mean ± standard deviation (mean ± SD).

3

RESULTS

3.1 Extraction yield

The extraction yield was determined using three replicas of one mL samples, in pre-weighted microtubes, of each resulting liquid extract and subjecting them to a gentle nitrogen stream in order to evaporate all the solvent content. The remaining dry extracts were weighted, and their total yield determined.

The results of the extraction yields (% total dry algae) for each extraction method assayed are presented in table 1. The results show a great variability between the extraction methods. The highest extraction yields were determined in methanolic extractions using *P. umbilicallis* (75.5 ± 5.4 %) followed by two *U. intestinalis* extractions, 45.5 ± 3.2 % and 30.6 ± 2.6 % for ethanolic MAE and methanolic extraction respectively.

Extraction yields are expressed as a percentage relative to the mass of algae used (mean ± standard deviation).

Table 1 - Extraction determined for the different extraction methods tested on seaweed species expressed as percentage relative to the mass of algae used (mean ± standard deviation).

				Soxhlet			
Species	Aqueous	Methanolic	MAE	Hexane	Ethyl acetate	Methanol	Total
Chondrus crispus	14.1 ± 1.2	28.7 ± 0.6					
Codium adhaerens	19.6 ± 2.4	8.9 ± 0.8					
Codium tomentosum	5.6 ± 0.4	15.4 ± 1.1	12.9 ± 1	1.9 ± 0.2	0.5 ± 0.1	3.8 ± 0.4	6.1 ± 0.4
Cystoseira tamariscifolia	7.9 ± 1	9.9 ± 1.2	10.9 ± 0.2	1.9 ± 0.1	3 ± 0.2	15.3 ± 1.1	20.1 ± 1.1
Fucus spiralis	4.0 ± 0.2	16.2 ± 2.4	10.1 ± 0.2	4.7 ± 0.1	2.3 ± 0.1	13.5 ± 0.3	20.4 ± 0.3
Porphyra umbilicalis	20.6 ± 2.6	75.5 ± 5.4	2.7 ± 0.4	1,7 ± 0.1	1.4 ± 0.1	14.6 ± 0.7	17.6 ± 0.7
Ulva intestinalis	8.3 ± 0.2	30.6 ± 2.6	45.5 ± 3.2	2,4 ± 0.1	2.5 ± 0.3	15.6 ± 1.7	20.5 ± 1.8

n = 3

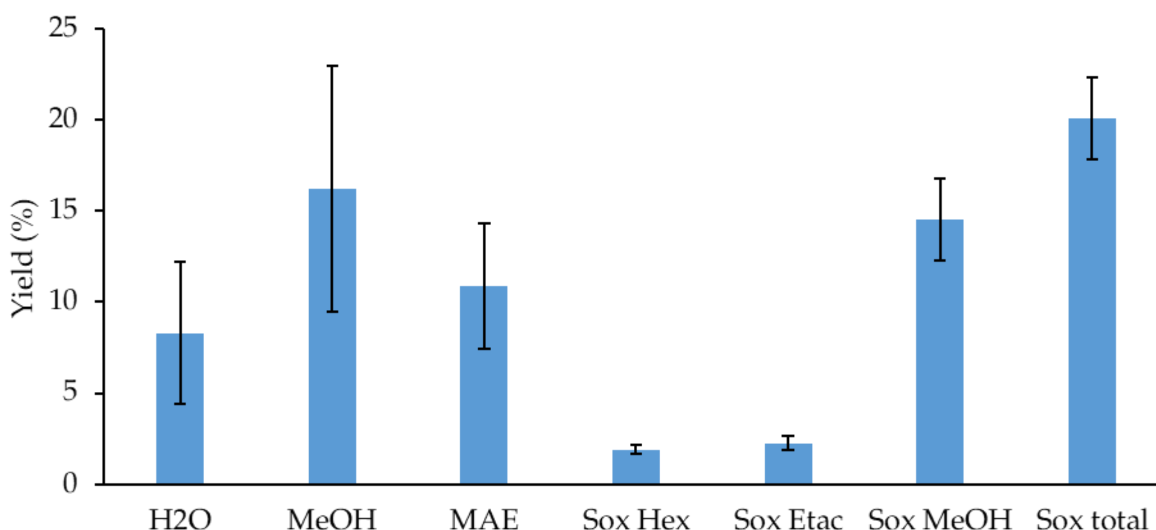

Figure 14 - Average extraction yields of the extraction methods assayed (H_2O: aqueous extracts, MeOH: methanolic extracts, MAE: MAE extracts; Sox Hex: hexane extracts through Soxhlet, Sox Etac: ethyl acetate extracts through Soxhlet, Sox MeOH: methanol extracts through Soxhlet, Sox total: sum of all Soxhlet extracts.)

Regarding yield, in the aqueous extracts, there is a significant difference ($p < 0.05$) between the yield of *C. adhaerens* and *P. umbilicalis* compared to the other species analyzed. In methanolic extracts, there was a significant difference ($p < 0.05$) between *P. umbilicalis* and all other seaweed species. In MAE extracts, *Ulva intestinalis* stands out showing significant differences ($p < 0.05$) compared to the other species analyzed and an almost four times higher yield compared to the second highest result for this extraction method.

For the Soxhlet extractions, the highest total yields were observed in the methanol extracts, showing significant differences ($p < 0.05$) compared to extraction with the other solvents. However, most of the species analyzed showed similar yields, with no significant differences ($p > 0.05$) between the highest yields; *C. tomentosum* and *P. umbilicalis* showed significantly ($p < 0.05$) lower yields.

Considering the average of all extraction methods, the combined Soxhlet and methanolic extracts showed a higher yield, with no significant difference between the two extraction methods.

3.2 Antibacterial assays

The results for zone of inhibition assays are shown in table 2. Antibacterial activity was only observed in extract samples obtained through Soxhlet extraction. In all species that showed antibacterial activity, ethyl acetate extracts were one of the extracts to present antibacterial activity. Only *C. tamariscifolia* methanolic and ethyl acetate extracts showed

antibacterial activity for both target pathogens (*E. coli* and *S. aureus*). No positive results were obtained using MIC assay.

Table 2 - Qualitative results of antibacterial assays in samples that showed at least one positive result.

	Extracts	a	b	c	d	e
Target pathogen	*E. coli*	✓	✓	X	✓	X
	S. aureus	✓	✓	✓	X	✓

✓: present, X: undetected; a) *C. tamariscifolia* extracted with ethyl acetate; b) *Cystoseira tamariscifolia* extracted with methanol; c) *U. intestinalis* extracted with ethyl acetate; d) *F. Spiralis* extracted with ethyl acetate; e) *C. tomentosum* extracted with ethyl acetate; n = 1

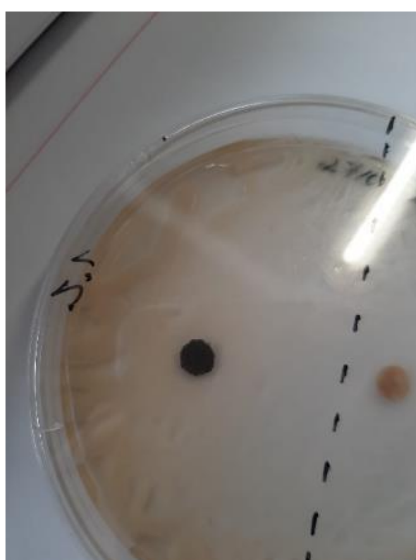

Figure 15 - Example of extract presenting a small hallo surrounding the sample imbued in paper filter.

3.3 Protein quantification

The results of protein quantification are shown in table 3. In the Bradford assay, the highest value, 1.465 ± 0.005 mg protein/g DW was measured in the methanolic extract of *C. tamariscifolia*, followed by 1.114 ± 0.078 mg protein/g DW and 1.033 ± 0.181 mg protein/g DW in the MAE extracts of *U. intestinalis* and in the methanolic extracts of *C. tomentosum,* respectively.

In the Lowry assay, the highest value, 72.253 ± 7.218 mg protein/g DW was measured in *Porphyra umbilicalis* aqueous extract, followed by 59.124 ± 3.033 mg protein/g DW and

42.623 ± 2.178 mg protein/g DW in MAE extracts from *C. tamariscifolia* and *F. spiralis* respectively.

The Lowry assay showed a statistically higher ($p < 0.05$) soluble protein when compared to the Bradford assay.

Protein quantification carried out using the Bradford method showed significantly higher protein levels ($p < 0.05$) in methanolic extracts of both *Codium sp.*, *C. tamariscifolia* and *F. spiralis* than in aqueous extracts. Protein quantification performed using the Lowry method obtained similar results. However, in the Lowry method, the highest protein concentrations were determined in the MAE extracts in most species evaluated, all showing significant differences compared to the other extracts tested, except those from *P. umbilicalis*. In both tests (Bradford and Lowry), Soxhlet extracts showed low protein content, except for the methanolic extracts obtained by this method, which presented concentrations significantly ($p < 0.05$) higher than those using other solvents.

Table 3 - Results of protein quantification in the seven species of macroalgae analyzed, expressed as mg soluble protein /g DW (mean ± standard deviation).

Species			Bradford	Lowry
Chondrus crispus	H_2O		0.16 ± 0.02	11.39 ± 0.41
	MeOH		0.19 ± 0.02	28.61 ± 1.39
Codium adhaerens	H_2O		0.04 ± 0.02	3.98 ± 0.69
	MEOH		0.2 ± 0.05	31.14 ± 5.78
Codium tomentosum	H_2O		0.07 ± 0.02	10.83 ± 2.17
	MEOH		1.03 ± 0.18	10.51 ± 1.18
	MAE		n.d.	36.5 ± 4.5
	Soxhlet	Hex	n.d.	0.86 ± 0.12
		EtAc	n.d.	0.75 ± 0.11
		MeOH	n.d.	1.28 ± 0.21
Cystoseira tamariscifolia	H_2O		0.11 ± 0.02	14.9 ± 0.6
	MeOH		1.47 ± 0.01	14.3 ± 0.7
	MAE		0.08 ± 0.01	59.12 ± 3.03
	Soxhlet	Hex	0.03 ± 0	0.4 ± 0.03
		EtAc	n.d.	0.49 ± 0.04
		MeOH	2.05 ± 0.1	24.57 ± 2.16
Fucus spiralis	H_2O		0.05 ± 0.01	4.61 ± 0.17
	MeOH		0.6 ± 0.01	14.72 ± 0.72
	MAE		0.32 ± 0.05	42.62 ± 2.18
	Soxhlet	Hex	0.06 ± 0.01	1.73 ± 0.11
		EtAc	0.07 ± 0.01	0.59 ± 0.05
		MeOH	n.d.	4.06 ± 0.36
Porphyra umbilicalis	H_2O		0.68 ± 0.05	72.25 ± 7.22
	MeOH		0.04 ± 0.01	3.38 ± 0.35
	MAE		0.78 ± 0.1	14.65 ± 1.67
	Soxhlet	Hex	0.16 ± 0.02	1.88 ± 0.24
		EtAc	n.d.	1.28 ± 0.18
		MeOH	0.22 ± 0.04	3.71 ± 0.56
Ulva intestinalis	H_2O		n.d.	11.7 ± 1.9
	MeOH		n.d.	8.03 ± 1.41
	MAE		1.11 ± 0.08	42.28 ± 7.93
	Soxhlet	Hex	n.d.	2.33 ± 0.47
		EtAc	n.d.	1.79 ± 0.18
		MeOH	0.22 ± 0.01	2.59 ± 0.06

n.d. = not detected; n = 3

3.4 Phytochemical assays

The results of the phytochemical screening of the selected seaweed species are presented in table 4. For all classes of target molecules tested, only alkaloids, glycosides and proteins were present in all algae species analyzed and the tannin class of molecules was not detected in any of the seaweed species studied. *C. tamariscifolia* and *F. spiralis* showed the greatest number of positive results across all species, while *C. crispus* showed the lowest number of positive results.

Table 4 - Results of phytochemical qualitative assay results in seven analyzed species of macroalgae selected for analysis.

	a	b	c	d	e	f	g
Alkaloids	✓	✓	✓	✓	✓	✓	✓
Carbohydrates	X	✓	✓	✓	✓	✓	✓
Coumarins	✓	✓	X	✓	✓	✓	✓
Flavonoids	X	X	X	✓	✓	X	X
Glycosides	✓	✓	✓	✓	✓	✓	✓
Gums and Mucilages	X	X	✓	✓	✓	X	✓
Phenolic	✓	X	✓	X	✓	X	✓
Phytosterols	X	X	X	✓	X	✓	X
Proteins	✓	✓	✓	✓	✓	✓	✓
Tannins	X	X	X	X	X	X	X
Tritrepenes	X	✓	X	X	X	✓	X
Quinones	X	✓	✓	X	✓	X	✓
Reducing Sugars	X	X	X	✓	✓	X	X
Resins	✓	✓	X	✓	✓	X	X
Saponins	✓	✓	✓	✓	X	✓	✓

✓: present, X: not detected; a) *C. crispus*; b) *C. adhaerens*; c) *C. tomentosum*; d) *C. tamariscifolia*; e) *F. spiralis*; f) *P. umbilicalis*; g) *U. intestinalis.*

3.5 ABTS assay

The results of the ABTS assay are shown in table 5. The highest value, 77.7 ± 0.3 (% inhibition) was measured in the aqueous extract of *U. intestinalis*, followed by 77.3 ± 0.3 (% inhibition) and 76.8 ± 0.5 (% inhibition) in the aqueous extracts of *P. umbilicalis* and *C. tomentosum,* respectively.

Table 5 - Results of ABTS assay in the selected seaweed species analyzed and expressed as percentage (%) of inhibition (mean ± SD).

Species	Aqueous	Methanolic	MAE	Soxhlet Hexane	Soxhlet Ethyl acetate	Soxhlet Methanol
Chondrus crispus	62.3 ± 8.9	n.d.				
Codium adhaerens	75.8 ± 1.1	67.5 ± 1.4				
Codium tomentosum	76.8 ± 0.5	n.d.	28 ± 4.3	n.d.	n.d.	n.d.
Cystoseira tamariscifolia	47.2 ± 0.1	28.6 ± 3	65.7 ± 13.4	n.d.	n.d.	n.d.
Fucus spiralis	67.8 ± 0.5	n.d.	n.d.	n.d.	n.d.	n.d.
Porphyra umbilicalis	77.3 ± 0.3	64.6 ± 0.9	n.d.	n.d.	n.d.	n.d.
Ulva intestinalis	77.7 ± 0.3	n.d.	30.2 ± 2.5	n.d.	n.d.	n.d.

n.d. = not detected; n = 3

All extracts obtained through Soxhlet didn't present detectable inhibition, whereas the aqueous extracts showed a remarkable inhibition of some samples, higher than 75%.

Within the aqueous extracts, *C. adhaerens*, *C. tomentosum*, *P. umbilicalis*, and *U. intestinalis* showed very similar inhibition values with no statistically significant difference ($p > 0.05$). Of those species that didn't show such high activity, *Fucus spiralis* showed a significant difference ($p < 0.05$) to all species with exceptions for both *Codium* species and *C. Crispus*.

Within the methanolic extracts, once more *C. adhaerens* and *P. umbilicalis* showed a statistically higher ($p < 0.05$) inhibition when in comparison with *C. tamariscifolia* but these showed no statistically significant differences between each other ($p > 0.05$).

C. tamariscifolia showed significant higher inhibition rates ($p < 0.05$) compared to the other species and to its methanolic extracts obtained by MAE. However, there were no significant differences ($p > 0.05$) when compared to its aqueous extract.

3.6 DPPH

The results of the DPPH radical scavenging ability assay are shown in table 6. The highest value, 49.2 ± 0.3 (% oxidation inhibition), was measured in the *U. Intestinalis* extract obtained through microwave assisted extraction (MAE), followed by 11 ± 0.1 (% inhibition) and 10.3 ± 1.9 (% inhibition) measured in *C. tamariscifolia* after extraction with hexane obtained through Soxhlet method and the aqueous extracts obtained from *P. umbilicalis,* respectively.

Table 6 - Results of DPPH assay in the selected species of seaweed analyzed and expressed as percentage (%) of inhibition (mean ± SD).

				Soxhlet		
Species	Aqueous	Methanolic	MAE	Hexane	Ethyl acetate	Methanol
Chondrus crispus	n.d.	n.d.				
Codium adhaerens	n.d.	n.d.				
Codium tomentosum	n.d.	n.d.	n.d.	1.2 ± 0.1	n.d.	n.d.
Cystoseira tamariscifolia	n.d.	n.d.	5.1 ± 0.5	11 ± 0.1	2.6 ± 1	3.9 ± 0.3
Fucus spiralis	n.d.	n.d.	n.d.	n.d.	3.2 ± 1.8	n.d.
Porphyra umbilicalis	10.3 ± 1.9	n.d.	n.d.	n.d.	n.d.	7.7 ± 0.3
Ulva intestinalis	0.6 ± 0.1	n.d.	49.2 ± 0.3	n.d.	n.d.	4.1 ± 0.3

n.d. = not detected; n = 3

In this test, the results for most of the samples analyzed were unable to be detected.

Regarding the aqueous extracts, only two showed significant radical scavenging activity (% inhibition): *P. umbilicalis* and *U. intestinalis*, which was also observed in the other extraction methods carried out. As mentioned above, *U. intestinalis* showed a significant ($p< 0.05$)

higher radical scavenging activity in MAE extraction compared to the other extraction methods that showed detectable inhibition, while *P. umbilicalis* only showed any detectable inhibition in methanolic extracts obtained through Soxhlet method.

C. tamariscifolia showed its greatest inhibition in the hexane extraction obtained by Soxhlet, with a significant increase ($p < 0.05$) compared to the other extraction methods tested.

3.7 FRAP assay

The results of the FRAP assay are shown in table 7. The highest value, 1.17 ± 0.14 mg TE/g DW was measured in the methanolic extract of *C. tamariscifolia*, followed by 0.91 ± 0.07 mg TE/g DW and 0.28 ± 0.01 mg TE/g DW measured in the methanolic extracts of *C. adhaerens* and *P. umbilicalis,* respectively.

Table 7 - Results of FRAP assay determined in the selected seaweed species analyzed and expressed as mg TE/g DW (mean ± SD).

				Soxhlet		
Species	Aqueous	Methanolic	MAE	Hexane	Ethyl acetate	Methanol
Chondrus crispus	0.04 ± 0.01	0.14 ± 0.01				
Codium adhaerens	0.05 ± 0.01	0.91 ± 0.07				
Codium tomentosum	0.02 ± 0.01	0.16 ± 0.03	n.d.	0.04 ± 0.01	0.04 ± 0.01	0.1 ± 0.01
Cystoseira tamariscifolia	0.09 ± 0.01	1.17 ± 0.14	0.08 ± 0.01	0.18 ± 0.1	0.21 ± 0.04	0.27 ± 0.07
Fucus spiralis	0.06 ± 0.02	0.27 ± 0.01	0.13 ± 0.01	0.07 ± 0.01	0.11 ± 0.03	0.22 ± 0.01
Porphyra umbilicalis	0.07 ± 0.01	0.28 ± 0.01	n.d.	0.02 ± 0.01	0.03 ± 0.01	0.13 ± 0.01
Ulva intestinalis	0.01 ± 0.01	0.18 ± 0.01	0.14 ± 0.02	0.01 ± 0.01	0.09 ± 0.02	0.15 ± 0.01

n.d. = not detected; n = 3

C. tamariscifolia showed a significant higher reducing power ($p < 0.05$) than all the other species in the methanolic extracts and ethyl acetate extracts obtained by Soxhlet extraction, except for the hexane extracts of *F. Spiralis*, obtained by Soxhlet, and the aqueous extracts of *P. umbilicalis*.

3.8 Total Phenolic Content (TPC) assay

The TPC assay results are shown in table 8. The highest value, 0.88 ± 0.06 mg GAE/ g DW was measured in the methanolic extract of *C. tamariscifolia*, followed by 0.78 ± 0.15 mg GAE/ g DW and 0.55 ± 0.11 mg GAE/ g DW in the methanolic extract of *C. tamariscifolia* obtained by extraction with Soxhlet and the methanolic extract of *C. adhaerens.*

Table 8 - Results of TPC assay in the seaweed species s analyzed and expressed as mg GAE/g DW (mean ± SD).

Species	Aqueous	Methanolic	MAE	Soxhlet		
				Hexane	Ethylacetate	Methanol
Chondrus Crispus	0.04 ± 0.01	0.38 ± 0.02				
Codium adhaerens	0.06 ± 0.02	0.55 ± 0.11				
Codium tomentosum	0.05 ± 0.01	0.35 ± 0.06	0.51 ± 0.08	0.03 ± 0.01	0.01 ± 0.01	0.06 ± 0.01
Cystoseira tamariscifolia	0.23 ± 0.02	0.88 ± 0.06	0.03 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.78 ± 0.15
Fucus Spiralis	0.05 ± 0.01	0.38 ± 0.02	0.09 ± 0.01	0.04± 0.01	0.1 ± 0.02	0.1 ± 0.01
Porphyra Umbilicalis	0.03 ± 0.01	0.14 ± 0.01	0.01 ± 0.01	0.04 ± 0.01	n.d.	0.1 ± 0.01
Ulva Intestinalis	n.d.	0.09 ± 0.01	0.03 ± 0.01	0.08 ± 0.01	n.d.	0.15 ± 0.03

n.d. = not detected; n = 3

Across all species assessed, there is a significant ($p < 0.05$) increase in the phenolic content between methanolic and aqueous extracts. In most of the species analyzed, extraction with methanol resulted in greater extraction efficiency of phenolic compounds. However, in *C. tomentosum*, MAE extraction significantly ($p <0.05$) increased its phenolic content compared to simple methanol extraction.

3.9 Total tannin content (TTC) assay

The results for TTC assay are shown in table 9. The highest value, 2.51 ± 0.34 µg CE/g DW was measured in the methanolic extract of *Fucus Spiralis* obtained through Soxhlet extraction, followed by 2.36 ± 0.16 µg CE/g DW and 1.28 ± 0.09 µg CE/g DW measured in the methanolic extract of *C. tamariscifolia* obtained through Soxhlet extraction and in the methanolic extracts of *C. tamariscifolia*.

Table 9 - Results of TTC assay in the selected seaweed species analyzed and expressed as µg CE/g DW (mean ± SD).

Species	Aqueous	Methanolic	MAE	Soxhlet		
				Hexane	Ethylacetate	Methanol
Chondrus crispus	0.02 ± 0.01	0.01 ± 0.01				
Codium adhaerens	n.d.	n.d.				
Codium tomentosum	n.d.	n.d.	0.79 ± 0.03	0.45 ± 0.02	1.08 ± 0.04	1.08 ± 0.04
Cystoseira tamariscifolia	0.4 ± 0.02	1.28 ± 0.09	n.d.	0.38 ± 0.01	0.35 ± 0.01	2.36 ± 0.16
Fucus spiralis	0.01 ± 0.01	0.59 ± 0.11	0.26 ± 0.01	1.15 ± 0.09	0.91 ± 0.01	2.51 ± 0.34
Porphyra umbilicalis	0.07 ± 0.01	n.d.	n.d.	0.06 ± 0.01	n.d.	0.08 ± 0.01
Ulva intestinalis	n.d.	0.08 ± 0.03	0.11 ± 0.01	0.02± 0.01	0.02 ± 0.01	0.16 ± 0.02

n.d. = not detected; n = 3

The methanolic extracts of *C. tamariscifolia* and *Fucus spiralis* showed higher content ($p < 0.05$) in tannins in comparison to aqueous extracts which is confirmed by Soxhlet extractions where methanolic extracts presented higher content ($p < 0.05$) in tannin content compared to

the other extraction methods tested in all seaweed species with exception to *C. tomentosum* where the extraction with ethyl acetate showed no significant differences ($p > 0.05$).

3.10 GC-MS

The molecules identified by GC-MS are shown in table 10. along with possible bioactivities reported in the literature.

All species subjected to this analysis (*C. tamariscifolia*, *P. umbilicalis* and *U. intestinalis*) showed twelve different molecules each and across all three species, 26 different molecules with six showing antioxidant activity, 8 showing antimicrobial or antibacterial activity, two showing anti-inflammatory, two showing anticancer and other types of activity. The species with the higher number of molecules with reported antioxidant properties was *P. umbilicalis* with 4 molecules, the hexane extract the main contributor containing three molecules.

Of the 26 molecules identified, seven steroids, five fatty acids, four diterpenes, three alkenes, two alcohols, one alkane, one FAME, one triol, one monosaccharide and one triterpene were found. Of the six molecules identified with antioxidant activity, three were fatty acids, one alkene, one sterol and one triterpene. Of the eight that presented antibacterial or antimicrobial activity, two were alkenes, two diterpenes, one alcohol, one alkane, one fatty acid and one sterol.

Table 10 - Results of GC-MS in the three selected species of macroalgae analyzed.

Algae sp.	Solvent	Match	Prob (%)	Formula	Class	Name	Activity	Source
Cystoseira tamariscifolia	Hexane	837	65.9	$C_{16}H_{32}O_2$	fatty acid	n-Hexadecanoic acid (palmitic acid)	Antioxidant	Sheela & Uthayakumari, 2013
		865	58.6	$C_{17}H_{34}O_2$	fatty acid	Hexadecanoic acid, methyl ester	Antioxidant	Sheela & Uthayakumari, 2013
		889	36.1	$C_{20}H_{38}$	diterpene	Neophytadiene	Antimicrobial	Ceyhan-Gьvensen & Keskin, 2016
		831	32.6	$C_{20}H_{40}O$	diterpene	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Anti-inflammatory, antithrombotic, antimicrobial and antitumor	Ko *et al.*, 2018
		802	13.4	$C_{28}H_{45}ClO_2$	sterol	Cholest-5-en-3-ol (3β)-, carbonochloridate		
		821	23.0	$C_{28}H_{46}O$	sterol	Ergosta-5,24(28)-dien-3-ol, (3β)-		
		891	39.5	$C_{29}H_{48}O$	sterol	Stigmasta-5,24(28)-dien-3-ol, (3β,24Z)-		
		830	14.7	$C_{30}H_{50}$	triterpene	Squalene	Anticancer and antioxidant	Kim & Karadeniz, 2012
		808	18.1	$C_{30}H_{50}O$	sterol	26,26-Dimethyl-5,23-ergostadien-3β-ol	Antimicrobial and cardio protective	Aditya *et al.*, 2020
	EtAc	926	63.6	$C_{20}H_{38}$	diterpene	Neophytadiene	Antimicrobial	Ceyhan-Gьvensen & Keskin, 2016
		810	17.4	$C_{20}H_{40}$	diterpene	3,7,11,15-Tetramethylhexadec-2-ene		
		892	51.7	$C_{20}H_{40}O$	diterpene	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Anti-inflammatory, antithrombotic, antimicrobial and antitumor	Ko *et al.*, 2018
		844	56.7	$C_{27}H_{44}O$	sterol	Desmosterol		
	MeOH	811	4.85	$C_{16}H_{32}$	akene	Cetene	Antimicrobial and antioxidant	Mou *et al.*, 2013
		876	33.9	$C_{20}H_{38}$	diterpene	Neophytadiene	Antimicrobial	Ceyhan-Gьvensen & Keskin, 2016
		827	38.9	$C_{20}H_{40}O$	diterpene	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Anti-inflammatory, antithrombotic, antimicrobial and antitumor	Ko *et al.*, 2018
Porphyra umbilicalis	Hexane	863	73.5	$C_{16}H_{32}O_2$	fatty acid	n-Hexadecanoic acid (palmitic acid)	Antioxidant	Sheela & Uthayakumari, 2013
		849	26.9	$C_{20}H_{30}O_2$	fatty acid methyl ester	5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)-		
		864	30.4	$C_{20}H_{40}O$	diterpene	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Anti-inflammatory, antithrombotic, antimicrobial and antitumor	Ko *et al.*, 2018
		837	33.9	$C_{28}H_{46}O$	steroid	Ergosta-5,24(28)-dien-3-ol, (3β)-		
		871	31.2	$C_{30}H_{50}O$	steroid	Cholest-5-en-3-ol, 24-propylidene-, (3β)-		
		806	20.1	$C_{37}H_{76}O$	alcohol	1-Heptatriacotanol	Antimicrobial	Ganesh & Mohankumar, 2017
	EtAc	873	33.0	$C_{20}H_{40}O$	diterpene	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Anti-inflammatory, antithrombotic, antimicrobial and antitumor	Ko *et al.*, 2018
		817	23.7	$C_{30}H_{50}O$	steroid	Cholest-5-en-3-ol, 24-propylidene-, (3β)-		
	MeOH	879	91.4	$C_3H_8O_3$	triol	Glycerin		
		817	33.6	$C_6H_{12}O_6$	monosaccharide	D-Allose		
		883	75.0	$C_{16}H_{32}O_2$	fatty acid	n-Hexadecanoic acid (palmitic acid)	Antioxidant	Sheela & Uthayakumari, 2013
		809	11.6	$C_{18}H_{34}O_2$	fatty acid	cis-Vaccenic acid	Antibacterial	Qadir *et al.*, 2020
		800	40.7	$C_{19}H_{38}O_4$	fatty acid	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Antioxidant, anti-inflammatory and anthelmintic	Kumar & Prasher, 2023
		870	21.6	$C_{20}H_{30}O_2$	fatty acid	cis-5,8,11,14,17-Eicosapentaenoic acid		
		807	42.6	$C_{29}H_{50}O$	sterol	β-Sitosterol	Antioxidant and antidiabetic	Babu & Jayaraman, 2020
		858	30.2	$C_{30}H_{50}O$	steroid	Cholest-5-en-3-ol, 24-propylidene-, (3β)-		

Algae sp.	Solvent	Match	Prob (%)	Formula	Class	Name	Activity	Source
Ulva intestinalis	HEX	807	24.5	$C_{20}H_{38}$	diterpene	Neophytadiene	Antimicrobial	Ceyhan-Gьvensen & Keskin, 2016
		813	34.3	$C_{29}H_{50}O$	sterol	β-Sitosterol	Antioxidant and antidiabetic	Babu & Jayaraman, 2020
		857	32.0	$C_{30}H_{50}O$	sterol	Cholest-5-en-3-ol, 24-propylidene-, (3β)-		
	ACET	872	38.0	$C_{20}H_{38}$	diterpene	Neophytadiene	Antimicrobial	Ceyhan-Gьvensen & Keskin, 2016
		815	48.6	$C_{20}H_{40}O$	diterpene	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	Anti-inflammatory, antithrombotic, antimicrobial and antitumor	Ko *et al.*, 2018
		879	32.6	$C_{29}H_{48}O$	steroid	Stigmasta-5,24(28)-dien-3-ol, (3β,24Z)-		
	MEOH	800	7.10	$C_{14}H_{28}$	alkene	1-Tetradecene	Antimicrobial	Lammers *et al.*, 2021
		866	18.5	$C_{14}H_{30}$	alkane	Tetradecane	Antibacterial and antifungal	Nasr *et al.*, 2022
		843	69.0	$C_{16}H_{32}O_2$	fatty acid	n-Hexadecanoic acid (palmitic acid)	Antioxidant	Sheela & Uthayakumari, 2013
		833	6.66	$C_{16}H_{34}O$	alcohol	1-Hexadecanol		Amudha *et al.*, 2018
		847	18.8	$C_{17}H_{34}$	alkene	8-Heptadecene	Insecticide	Nazzi *et al.*, 2002
		864	34.6	$C_{20}H_{38}$	diterpene	Neophytadiene	Antimicrobial	Ceyhan-Gьvensen & Keskin, 2016
		920	48.1	$C_{20}H_{40}O$	diterpene	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	Anti-inflammatory, antithrombotic, antimicrobial and antitumor	Ko *et al.*, 2018
		863	35.4	$C_{30}H_{50}O$	sterol	Cholest-5-en-3-ol, 24-propylidene-, (3β)-		

3.11FAMEs

The results of the Fatty acids profiles are shown in table 11. along with the possible bio-activities reported in the literature, with the main focus on antioxidant activity. In *C. tamaris-cifolia,* 29 different molecules were found; in *P. umbilicalis*, 18 different molecules; in *U. intes-tinalis* 23 different molecules and across all species assessed 42 different molecules. Of these, 3 showed antimicrobial or antibacterial activity, 7 anti-inflammatory activity, 6 antioxidant activity and 3 anti-cancer or tumoral activity. The species with the most antioxidant molecules was *U. intestinalis.*

Table 11 - Results of FAMEs in the three selected species of macroalgae analyzed.

Algae sp.	Match	Prob (%)	Formula	Class	Name	Activity	Source
Cystoseira tamariscifolia	881	49.0	$C_9H_{12}O_6$	fatty acid methyl ester	1-Propene-1,2,3-tricarboxylic acid, trimethyl ester, (E)-		
	836	49.5	$C_{14}H_{28}O_2$	fatty acid methyl ester	Tridecanoic acid, methyl ester	Anthelminthic, anti-inflammatory, antimicrobial and anticancer	Chowdhury *et al.*, 2021
	811	41.4	$C_{15}H_{30}O_2$	fatty acid methyl ester	Methyl tetradecanoate	Antimicrobial	Mazumder *et al.*, 2020
	839	41.3	$C_{15}H_{28}O_2$	fatty acid methyl ester	Methyl myristoleate	Antimicrobial	Clément *et al.*, 2007
	906	55.7	$C_{16}H_{32}O_2$	fatty acid methyl ester	Pentadecanoic acid, methyl ester	Antifugal and antibacterial	Karthikeyan *et al.*, 2021
	812	33.4	$C_{17}H_{32}O_2$	fatty acid methyl ester	7-Hexadecenoic acid, methyl ester, (Z)-	Antioxidant	Reza *et al.*, 2021
	942	24.5	$C_{17}H_{32}O_2$	fatty acid methyl ester	9-Hexadecenoic acid, methyl ester, (Z)-	Antioxidant	Reza *et al.*, 2021
	939	21.6	$C_{17}H_{32}O_2$	fatty acid methyl ester	Methyl hexadec-9-enoate		
	920	28.0	$C_{17}H_{32}O_2$	fatty acid methyl ester	(Z)-Methyl hexadec-11-enoate		
	927	71.1	$C_{17}H_{34}O_2$	fatty acid methyl ester	Hexadecanoic acid, methyl ester	Antioxidant	Sheela & Uthayakumari, 2013
	858	17.3	$C_{18}H_{34}O_2$	fatty acid methyl ester	cis-10-Heptadecenoic acid, methyl ester		
	857	16.7	$C_{18}H_{34}O_2$	fatty acid methyl ester	Methyl 9-heptadecenoate or 9-17:1		
	914	40.9	$C_{19}H_{30}O_2$	fatty acid methyl ester	methyl stearidonate (6Z,9Z,12Z,15Z)-Methyl octadeca-6,9,12,15-tetraenoate	Antibacterial	Park *et al.*, 2013
	852	25.8	$C_{19}H_{32}O_2$	fatty acid methyl ester	Methyl γ-linolenate	Anticancer	Jubie *et al.*, 2015
	923	17.6	$C_{19}H_{34}O_2$	fatty acid methyl ester	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Analgesic, anti-inflammatory and ulcerogenic	Hadi *et al.*, 2016
	811	26.5	$C_{19}H_{36}O_2$	fatty acid methyl ester	9-Octadecenoic acid (Z)-, methyl ester	Anti-inflammatory	Krishnamoorthy & Subramaniam, 2014
	942	21.1	$C_{19}H_{36}O_2$	fatty acid methyl ester	Oleic acid, methyl ester (9-Octadecenoic acid (Z)-, methyl ester)	Anti-inflammatory	Krishnamoorthy & Subramaniam, 2015
	938	71.3	$C_{19}H_{38}O_2$	fatty acid methyl ester	Methyl stearate	Antimicrobial	El-Chaghaby *et al.*, 2022
	932	37.9	$C_{21}H_{32}O_2$	fatty acid methyl ester	5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)-		
	843	20.5	$C_{21}H_{34}O_2$	fatty acid methyl ester	Methyl (Z)-5,11,14,17-eicosatetraenoate		
	931	64.8	$C_{21}H_{34}O_2$	fatty acid methyl ester	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-		
	868	21.1	$C_{21}H_{34}O_2$	fatty acid methyl ester	ω-3 Arachidonic Acid methyl ester	Antitumor, antinflamatory	Tallima & El, 2017
	878	25.7	$C_{21}H_{36}O_2$	fatty acid methyl ester	8,11,14-Eicosatrienoic acid, methyl ester, (all-Z)-		
	881	26.8	$C_{21}H_{36}O_2$	fatty acid methyl ester	8,11,14-Eicosatrienoic acid, methyl ester, (Z,Z,Z)-		
	839	15.0	$C_{21}H_{38}O_2$	fatty acid methyl ester	cis-11,14-Eicosadienoic acid, methyl ester		
	890	68.5	$C_{21}H_{42}O_2$	fatty acid methyl ester	Eicosanoic acid, methyl ester		
	901	41.8	$C_{23}H_{34}O_2$	fatty acid methyl ester	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-		
	830	59.9	$C_{23}H_{46}O_2$	fatty acid methyl ester	Docosanoic acid, methyl ester		
	854	71.1	$C_{25}H_{50}O_2$	fatty acid methyl ester	Tetracosanoic acid, methyl ester		

Algae sp.	Match	Prob (%)	Formula	Class	Name	Activity	Source
Porphyra umbilicalis	874	62.3	$C_{14}H_{28}O_2$	fatty acid methyl ester	Tridecanoic acid, methyl ester	Antibacterial	Jin *et al.*, 2021
	937	67.5	$C_{15}H_{30}O_2$	fatty acid methyl ester	Methyl tetradecanoate	Antimicrobial	Mazumder *et al.*, 2020
	873	52.8	$C_{16}H_{32}O_2$	fatty acid methyl ester	Pentadecanoic acid, methyl ester	Antifugal and antibacterial	Karthikeyan *et al.*, 2021
	806	25.7	$C_{17}H_{32}O_2$	fatty acid methyl ester	7-Hexadecenoic acid, methyl ester, (Z)-	Antioxidant	Reza *et al.*, 2021
	944	71.3	$C_{17}H_{34}O_2$	fatty acid methyl ester	Hexadecanoic acid, methyl ester	Antioxidant	Sheela & Uthayakumari, 2013
	846	16.4	$C_{19}H_{30}O_2$	fatty acid methyl ester	(6Z,9Z,12Z,15Z)-Methyl octadeca-6,9,12,15-tetraenoate		
	853	27.1	$C_{19}H_{32}O_2$	fatty acid methyl ester	Methyl γ-linolenate	Anticancer	Jubie *et al.*, 2015
	840	30.7	$C_{19}H_{34}O_2$	fatty acid methyl ester	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Analgesic, anti-inflammatory and ulcerogenic	Hadi *et al.*, 2016
	895	27.1	$C_{19}H_{36}O_2$	fatty acid methyl ester	9-Octadecenoic acid (Z)-, methyl ester	Anti-inflammatory	Krishnamoorthy & Subramaniam, 2014
	924	70.7	$C_{19}H_{38}O_2$	fatty acid methyl ester	Methyl stearate	Antimicrobial	El-Chaghaby *et al.*, 2022
	907	26.7	$C_{21}H_{32}O_2$	fatty acid methyl ester	5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)-		
	866	37.2	$C_{21}H_{34}O_2$	fatty acid methyl ester	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-		
	888	28.9	$C_{21}H_{34}O_2$	fatty acid methyl ester	ω-3 Arachidonic Acid methyl ester	Antitumor and anti-inflamatory	Tallima & El Ridi, 2017
	913	57.0	$C_{21}H_{36}O_2$	fatty acid methyl ester	8,11,14-Eicosatrienoic acid, methyl ester, (Z,Z,Z)-		
	888	23.7	$C_{21}H_{38}O_2$	fatty acid methyl ester	cis-11,14-Eicosadienoic acid, methyl ester		
	899	28.2	$C_{21}H_{40}O_2$	fatty acid methyl ester	cis-Methyl 11-eicosenoate	Immune system stimulant	Alqarni *et al.*, 2019
	852	31.5	$C_{23}H_{44}O_2$	fatty acid methyl ester	Methyl 11-docosenoate		
	805	22.6	$C_{25}H_{48}O_2$	fatty acid methyl ester	15-Tetracosenoic acid, methyl ester, (Z)-	Neuroprotective	Moni *et al.*, 2021

Algae sp.	Match	Prob (%)	Formula	Name	Activity	Source
Ulva intestinalis	818	48.8	$C_{13}H_{26}O_2$	Dodecanoic acid, methyl ester	Antimicrobial	Matsue *et al.*, 2019
	835	50.2	$C_{14}H_{28}O_2$	Tridecanoic acid, methyl ester	Anthelminthic, anti-inflammatory, antimicrobial and anticancer	Chowdhury *et al.*, 2021
	940	71.4	$C_{15}H_{30}O_2$	Methyl tetradecanoate	Antimicrobial	Mazumder *et al.*, 2020
	882	46.6	$C_{16}H_{32}O_2$	Pentadecanoic acid, methyl ester	Antifugal and antibacterial	Karthikeyan *et al.*, 2021
	888	38.0	$C_{17}H_{26}O_2$	Methyl 4,7,10,13-hexadecatetraenoate		
	875	23.9	$C_{17}H_{28}O_2$	7,10,13-Hexadecatrienoic acid, methyl ester		
	910	45.3	$C_{17}H_{30}O_2$	7,10-Hexadecadienoic acid, methyl ester		
	845	35.8	$C_{17}H_{32}O_2$	7-Hexadecenoic acid, methyl ester, (Z)-	Antioxidant	Reza *et al.*, 2021
	941	57.9	$C_{19}H_{30}O_2$	(6Z,9Z,12Z,15Z)-Methyl octadeca-6,9,12,15-tetraenoate		
	880	35.7	$C_{19}H_{32}O2$	Methyl γ-linolenate	Anticancer	Jubie *et al.*, 2015
	931	48.6	$C_{19}H_{32}O_2$	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	Antimicrobial	Rahman *et al.*, 2014
	877	19.6	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid (E,E)-, methyl ester,	Antioxidant	Mazumder *et al.*, 2020
	914	15.1	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Analgesic, anti-inflammatory and ulcerogenic	Hadi *et al.*, 2016
	905	27.9	$C_{19}H_{36}O_2$	Oleic acid, methyl ester (9-Octadecenoic acid (Z)-, methyl ester)	Anti-inflammatory	Krishnamoorthy & Subramaniam, 2014
	947	13.3	$C_{19}H_{36}O_2$	11-Octadecenoic acid, methyl ester	Antioxidant and antimicrobial	Mazumder *et al.*, 2020
	919	39.2	$C_{21}H_{32}O_2$	5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)-		
	896	49.5	$C_{21}H_{34}O_2$	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-		
	899	43.1	$C_{21}H_{34}O_2$	ω-3 Arachidonic Acid methyl ester	Antitumor and anti-inflamatory	Tallima & El, 2017
	887	35.7	$C_{21}H_{36}O_2$	8,11,14-Eicosatrienoic acid, methyl ester, (Z,Z,Z)-		
	904	39.4	$C_{23}H_{36}O_2$	Docosapentaenoic Acid methyl ester	Anti-inflamatory	Calder, 2021
	882	44.7	$C_{23}H_{38}O_2$	cis-7,10,13,16-Docosatetraenoic acid, methyl ester		
	882	68.8	$C_{23}H_{46}O_2$	Docosanoic acid, methyl ester		
	808	58.1	$C_{25}H_{50}O_2$	Tetracosanoic acid, methyl ester		

3.12 Short chain fatty acids

The results of this analysis showed the presence of several short chain fatty acids, however, very few were identified. Eleven different molecules were detected in *C. tamariscofia*, however, only one was possible to be identified: porionic acid. Seventeen were detected in *P. umbilicalis*, but none was identified unequivocally. While 23 were detected in *U. intestinalis*¸ but only one was identified: the formic acid.

3.13 Lipid content

Lipid content results are shown in table 12. The highest value, 22.4 ± 7.2 % of DW was measured in *Codium tomentosum*, followed by 19.62 ± 5.4% of DW and 18.62 ± 8.7% of DW determined in *C. tamariscifolia* and *P. umbilicalis* respectively. Notably, *C. tomentosum* showed almost three times more lipid content than *C. adhaerens*. However, statistics showed, no significant differences (p> 0.05) among the different species analyzed.

Table 12 - Lipid content determined in the seven species of macroalgae analyzed and expressed percentage of dry weight (% of DW) (mean ± SD)

Species	Lipidic content
Chondrus crispus	15.4 ± 2.7
Codium adhaerens	8.2 ± 1.5
Codium tomentosum	22.4 ± 7.2
Cystoseira tamariscifolia	19.6 ± 5.4
Fucus spiralis	10.9 ± 3.8
Porphyra umbilicalis	18.6 ± 8.7
Ulva intestinalis	14.7 ± 3.1

n = 3

3.14 Proteomics

3.14.1 *Porphyra umbilicalis*

Proteomic data obtained for *P. umbilicalis* was analyzed with String software (V.12) using *Cyanidioschyzon merolae*, a red seaweed (best match) for the analyses in the absence of *Porphyra sp.* in the search parameters.

Results are presented in table 13 and show that most proteins are involved in the process of protein translation and biosynthetic processes. The biological process, molecular function and other relevant Information were obtained from UniProt (www.uniprot.org). Regarding the molecular function, the identified proteins are related with structural constituents of ribosomes and with RNA binding. Figure 16 shows the protein interactions found for this seaweed. In addition, String analyses also revealed that most of the proteins identified are located in the seaweed chloroplasts.

Table 13 - Biological processes and molecular functions of proteins identified in *P. umbilicalis*.

Biological process (Gene Ontology)				
Accession	Description	Count in network	Strength	False discovery rate
GO:0006412	Translation	7 of 276	1.2	2.59×10^{-5}
GO:0044249	Cellular biosynthetic process	8 of 836	0.78	0.00016
GO:1901576	Organic substance biosynthetic process	8 of 859	0.76	0.00017
Molecular Function (Gene Ontology)				
Accession	Description	Count in network	Strength	False discovery rate
GO:0003735	Structural constituent of ribosome	6 of 151	1.41	2.29×10^{-5}
GO:0019843	rRNA binding	4 of 53	1.67	0.00034
GO:0003723	RNA binding	6 of 373	1.00	0.0011

Legend: Count in Network -The first number indicates how many proteins in the network are annotated with a particular term. The second number indicates how many proteins in total (in the network and in the background) have this term assigned; Strength - Log_{10}(observed / expected). This measure describes how large the enrichment effect is. It's the ratio between i) the number of proteins in the network that are annotated with a term and ii) the number of proteins that we expect to be annotated with this term in a random network of the same size; False Discovery Rate - This measure describes how significant the enrichment is. Shown are p-values corrected for multiple testing within each category using the Benjamini–Hochberg procedure.

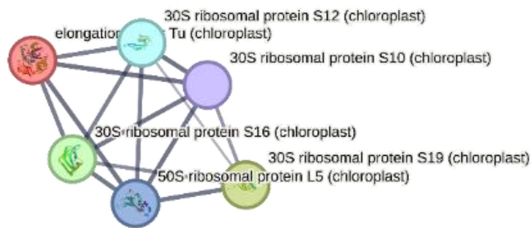

Figure 16 - String network showing protein interactions found for *P. umbilicalis*.

Legend: Network nodes represent proteins; colored nodes: query proteins and first shell of interactors; filled nodes: a 3D structure is known or predicted; Edges represent protein-protein associations and line thickness indicates the strength of data support.

String analyses revealed that proteins represented in the network nodes are constituted by:30S ribosomal protein S19, chloroplastic, a protein S19 which forms a complex with S13 that binds strongly to the 16S ribosomal RNA. (94 aa); 30S ribosomal protein S16, chloroplast. (75 aa); 50S ribosomal protein L5 (chloroplast), which binds 5S rRNA and forms part of the central protuberance of the 50S subunit. (177 aa); 30S ribosomal protein S10 (chloroplast), involved in the binding of tRNA to the ribosomes. (98 aa); 30S ribosomal protein S18 (chloroplast) and 30S ribosomal protein S6, chloroplastic, which binds together with S18 to 16S ribosomal RNA. Belongs to the bacterial ribosomal protein bS6 family. (99 aa); diphosphate Geranylgeranyl diphosphate synthase, chloroplast, belonging to the FPP/GGPP synthase family. (388 aa); elongation factor Tu (chloroplast), which is a protein that promotes the GTP-dependent binding of aminoacyl- tRNA to the A-site of ribosomes during protein biosynthesis, it belongs to the TRAFAC class translation factor GTPase superfamily. Classic translation factor GTPase family. EF-Tu/EF-1A subfamily. (410 aa); 30S ribosomal protein S12 (chloroplast) and 30S ribosomal protein S5, chloroplastic, with S4 and S12 plays an important role in translational accuracy and belongs to the universal ribosomal protein uS5 family. (153 aa).

3.14.2 *Ulva intestinalis*

Proteomic data obtained for *U. intestinalis* was analyzed with String software using *Bathycoccus prasinos*, a green seaweed (best match) for the analyses in the absence of *Ulva sp.* in the search parameters.

Results are shown in table 14 and show that most proteins are involved in the reductive pentose phosphate cycle, translation, metabolic and biosynthetic processes, and cellular processes. Regarding molecular function, the proteins identified in this species are mostly related

with structural components, binding and noteworthy with antioxidant activity. Figure 17 show protein interactions found for this seaweed. In addition, String analyses also revealed that most of the proteins identified are located in seaweed chloroplasts and cytoplasm.

Table 14 - Biological processes and molecular functions of proteins identified in *U. intestinalis*.

Biological process (Gene Ontology)

Accession	Description	Count in network	Strength	False discovery rate
GO:0019253	Reductive pentose-phosphate cycle	3 of 14	1.83	0.00372
GO:0006412	Translation	11 of 326	1.03	1.77×10^{-6}
GO:0006518	Peptide metabolic process	12 of 360	1.02	8.35×10^{-7}
GO:0044271	Cellular nitrogen compound biosynthetic process	12 of 684	0.74	9.92×10^{-5}
GO:1901566	Organonitrogen compound biosynthetic process	13 of 818	0.7	9.62×10^{-5}
GO:1901564	Organonitrogen compound metabolic process	14 of 1787	0.39	0.0400
GO:0044237	Cellular metabolic process	21 of 2937	0.35	0.00050
GO:0071704	Organic substance metabolic process	19 of 3191	0.27	0.0407
GO:0009987	Cellular process	24 of 4873	0.19	0.0158

Molecular Function (Gene Ontology)

Accession	Description	Count in network	Strength	False discovery rate
GO:0019843	rRNA binding	8 of 93	1.43	2.15×10^{-7}
GO:0016209	Antioxidant activity	3 of 36	1.42	0.0395
GO:0003735	Structural constituent of ribosome	10 of 186	1.23	1.11×10^{-7}
GO:0005198	Structural molecule activity	11 of 241	1.16	1.11 x 10-7
GO:0003723	RNA binding	9 of 655	0.64	0.0223
GO:1901363	Heterocyclic compound binding	20 of 2121	0.47	1.69e-05
GO:0097159	Organic cyclic compound binding	20 of 2128	0.47	1.69e-05
GO:0005488	Binding	22 of 3570	0.29	0.0026

Legend: Count in Network -The first number indicates how many proteins in the network are annotated with a particular term. The second number indicates how many proteins in total (in the network and in the background) have this term assigned; Strength - Log_{10}(observed / expected). This measure describes how large the enrichment effect is. It's the ratio between i) the number of proteins in the network that are annotated with a term and ii) the number of proteins that we expect to be annotated with this term in a random network of the same size; False Discovery Rate - This measure describes how significant the enrichment is. Shown are p-values corrected for multiple testing within each category using the Benjamini–Hochberg procedure.

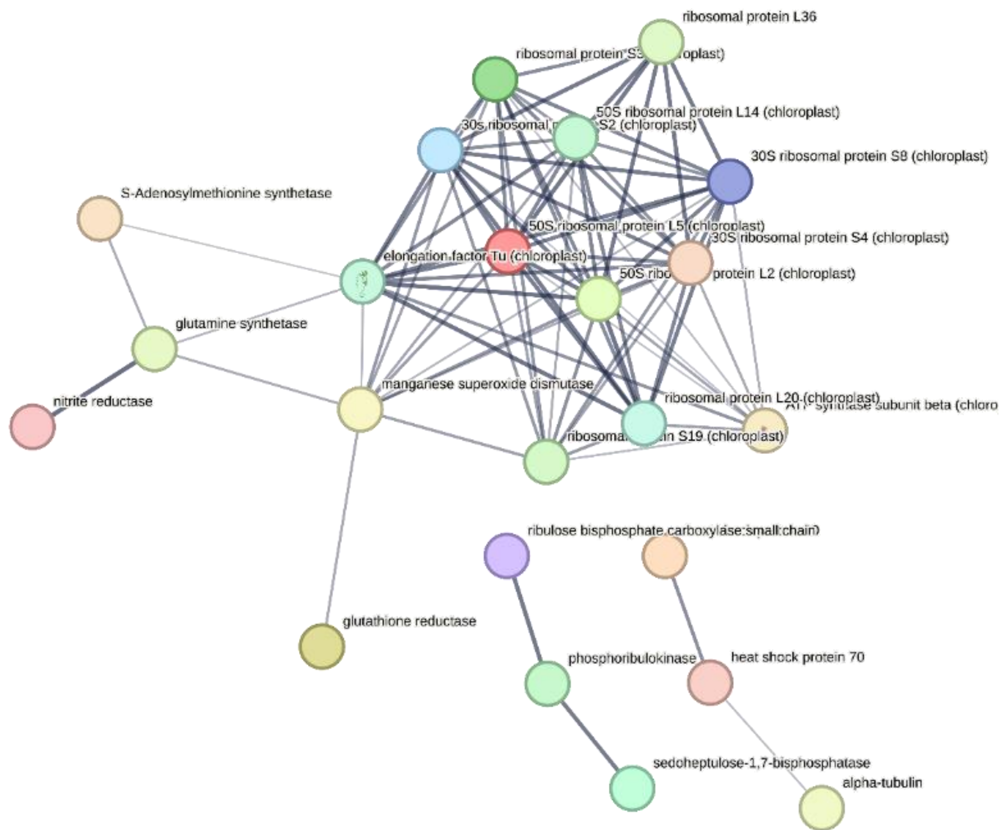

Figure 17 - String network showing protein interactions found for *U. intestinalis.*

Legend: Network nodes represent proteins; colored nodes: query proteins and first shell of interactors; filled nodes: a 3D structure is known or predicted; Edges represent protein-protein associations and line thickness indicates the strength of data support.

String analyses also revealed that proteins represented in the network nodes are: 50S ribosomal protein L5 (chloroplast), which binds 5S rRNA, forms part of the central protuberance of the 50S subunit. (184 aa); Heat shock protein 90 a molecular chaperone involved in the stability and function of a diverse of conditionally activated and/or expressed signaling proteins. (780 aa); Glutathione reductase, which helps to protect plant cells from ROS and also maintains high levels of reduced glutathione. (528 aa); 50S ribosomal protein L2, chloroplastic, that belongs to the universal ribosomal protein uL2 family. (277 aa); 30S ribosomal protein S3, chloroplastic, which belongs to the universal ribosomal protein uS3 family. (211 aa); sedoheptulose-1,7-bisphosphatase - Chloroplast, involved in the Calvin cycle. (378 aa); 30S ribosomal protein S2 - chloroplastic, a dedicated translation machinery responsible for the synthesis of chloroplast genome-encoded proteins. (227 aa); 30S ribosomal protein S8 (chloroplast). one of the primary rRNA binding proteins, it binds directly to 16S rRNA central domain where it helps coordinate assembly of the platform of the 30S subunit. (122 aa); ribulose bisphosphate carboxylase small chain - RuBisCO catalyzes two reactions: the carboxylation of D- ribulose 1,5-bisphosphate, the primary event in carbon dioxide fixation, as well as the oxidative

fragmentation of the pentose substrate. Both reactions occur simultaneously and in competition at the same active site; Belongs to the RuBisCO small chain family. (170 aa); nitrite reductase, Involved In the nitrite reduction. (1060 aa); Heat shock protein 70 family, a chaperone that help cells avoid the adverse effects of physiological stress. (571 aa); 30S ribosomal protein S4 (chloroplast), one of the primary rRNA binding proteins, it binds directly to 16S rRNA where it nucleates assembly of the body of the 30S subunit. Belongs to the universal ribosomal protein uS4 family. (196 aa); S-adenosylmethionine synthase, which catalyzes the formation of S-adenosylmethionine from methionine and ATP. (392 aa); ATP synthase subunit beta, chloroplastic, produces ATP from ADP in the presence of a proton gradient across the membrane and belongs to the ATPase alpha/beta chains family. (480 aa); manganese superoxide dismutase, a mitochondrial manganese superoxide dismutase. (404 aa); Tubulin alpha chain, major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain. (415 aa); Glutamine synthetase catalytic region, which belongs to the glutamine synthetase family. (725 aa); 60S ribosomal protein L36 that belongs to the eukaryotic ribosomal protein eL36 family. (100 aa); 30S ribosomal protein S19, chloroplastic, a protein that forms a complex with S13 that binds strongly to the 16S ribosomal RNA. (92 aa); Ascorbate peroxidase, belonging to the peroxidase family. (366 aa); Phosphoribulokinase. a photosynthetic enzyme that catalyzes the phosphorylation of ribulose 5-phosphate into ribulose 1,5-bisphosphate (381 aa); 50S ribosomal protein L14 (chloroplast), which binds to 23S rRNA. (119 aa); Elongation factor Tu, chloroplastic, a protein that promotes the GTP-dependent binding of aminoacyl- tRNA to the A-site of ribosomes during protein biosynthesis and belongs to the TRAFAC class translation factor GTPase superfamily. (411 aa); 50S ribosomal protein L20, chloroplastic, which binds directly to 23S ribosomal RNA and is necessary for the in vitro assembly process of the 50S ribosomal subunit. It is not involved in the protein synthesizing functions of that subunit. (114 aa).

3.14.3 *Cystoseira tamariscifolia*

Proteomic data obtained for *C. tamariscifolia* was analyzed with String software using *Ectocarpus siliculosus*, a brown seaweed (best match), for the analyses in the absence of *Cystoseira sp.* in the search parameters.

Results are shown in table 15 and show that most proteins are involved in translation, metabolic processes, tricarboxylic acid cycle and generation of precursor metabolites and energy. Regarding molecular function, the proteins identified in this species are mostly related with structural constituent of ribosome and RNA binding. Figure 18 show protein interactions found for this seaweed. In addition, String analyses also revealed that most of the proteins identified belong to the cytoplasm.

Table 15 - Biological processes and molecular functions of proteins identified in *C. tamariscifolia*.

Biological process (Gene Ontology)

Accession	Description	Count in network	Strength	False discovery rate
GO:0006412	Translation	7 of 321	1.47	2.32e-06
GO:0044260	Cellular macromolecule metabolic process	8 of 1302	0.92	0.00018
GO:0044237	Cellular metabolic process	11 of 3841	0.59	0.00027
GO:0044238	Primary metabolic process	11 of 4039	0.57	0.00043
GO:0071704	Organic substance metabolic process	11 of 4380	0.53	0.00087
GO:1901564	Organonitrogen compound metabolic process	9 of 2479	0.69	0.0011
GO:0019538	Protein metabolic process	8 of 1868	0.77	0.0015
GO:0034641	Cellular nitrogen compound metabolic process	8 of 1851	0.77	0.0015
GO:0006099	Tricarboxylic acid cycle	2 of 21	2.11	0.0146
GO:0006091	Generation of precursor metabolites and energy	3 of 172	1.38	0.0268

Molecular Function (Gene Ontology)

Accession	Description	Count in network	Strength	False discovery rate
GO:0003735	Structural constituent of ribosome	7 of 157	1.78	8.90e-09
GO:0019843	rRNA binding	5 of 44	2.19	6.64e-08
GO:0003723	RNA binding	6 of 628	1.11	0.00082

Legend: Count in Network -The first number indicates how many proteins in the network are annotated with a particular term. The second number indicates how many proteins in total (in the network and in the background) have this term assigned; Strength - Log10(observed / expected). This measure describes how large the enrichment effect is. It's the ratio between i) the number of proteins in the network that are annotated with a term and ii) the number of proteins that we expect to be annotated with this term in a random network of the same size; False Discovery Rate - This measure describes how significant the enrichment is. Shown are p-values corrected for multiple testing within each category using the Benjamini–Hochberg procedure.

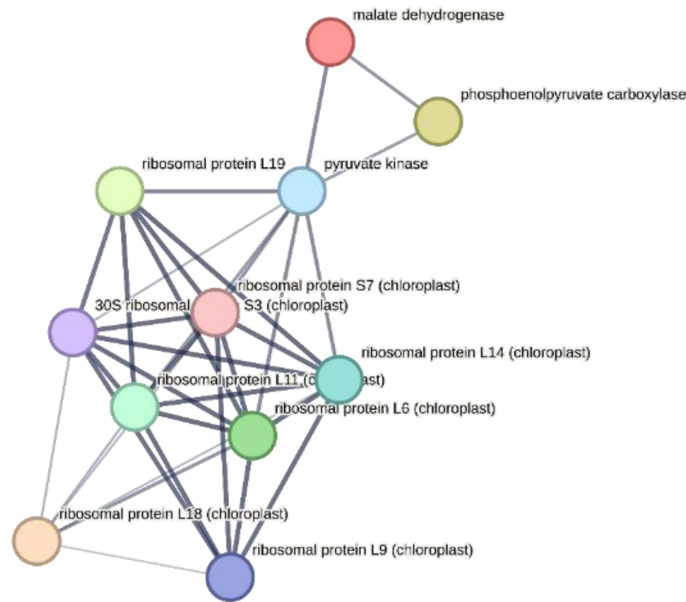

Figure 18 - String network showing protein interactions found for *C. tamariscifolia*.

Legend: Network nodes represent proteins; colored nodes: query proteins and first shell of interactors; filled nodes: a 3D structure is known or predicted; Edges represent protein-protein associations and line thickness indicates the strength of data support.

String analyses also revealed that proteins represented in the network nodes are: Malate dehydrogenase. (276 aa), an enzyme that reversibly catalyzes the oxidation of malate to oxaloacetate using the reduction of NAD to NADH; 50S ribosomal protein L18, chloroplastic, which binds - Binds 5S rRNA and, forms part of the central protuberance of the 50S subunit. (109 aa); Phosphoenolpyruvate carboxylase, which is found in plants and some bacteria and catalyzes the addition of bicarbonate to phosphoenolpyruvate to form oxaloacetate and inorganic phosphate. (949 aa); Ribosomal protein L19, which belongs to the eukaryotic ribosomal protein eL19 family. (190 aa); 50S ribosomal protein L6, chloroplastic, which Binds 23S rRNA. (178 aa); 50S ribosomal protein L11, chloroplastic, which forms part of the ribosomal stalk which helps the ribosome interact with GTP-bound translation factors. (141 aa); 50S ribosomal protein L14, chloroplastic, which binds to 23S rRNA. (121 aa); Pyruvate kinase that - belongs to the pyruvate kinase family. (499 aa); 50S ribosomal protein L9, chloroplastic, which binds to the 23S rRNA and belongs to the bacterial ribosomal protein bL9 family. (155 aa); 30S ribosomal protein S3, chloroplastic, which belongs to the universal ribosomal protein uS3 family. (222 aa); 30S ribosomal protein S7, chloroplastic, one of the primary rRNA binding proteins, it binds directly to 16S rRNA where it nucleates assembly of the head domain of the 30S subunit. (156 aa).

4

DISCUSSION

Since medieval times seaweeds have been used as food throughout Asia and in Europe due to their high nutritional value. Recently, with increasing research on seaweed growth conditions, composition, and bioactive compounds, many potential biotechnological applications in various industries (food, pharmaceutical, cosmetic, bioremediation, etc.) have been investigated and Id. However, there is a gap in the understanding of mechanisms behind many bioactivities exhibited by algae species, mainly due to the fact that its composition and expression of metabolites are heavily influenced by factors such as temperature, salinity, location, water depth and geographic location.

The present study reports the results of chemical and biochemical characterization of seven selected seaweed species, *C. crispus, C. adhaerens, C. tomentosum, C. tamariscifolia, F. spiralis, P. umbilicalis* and *U. intestinalis* in order to explore their potential as a source of antibacterial and antioxidant metabolites. For this purpose, the characterization included: the qualitative and quantitative identification of some bioactive compounds, the evaluation of antioxidant and antibacterial potential and the assessment of fatty acids and volatile organic compounds present in the seaweeds.

The extraction process is a vital aspect of the study of bioactive molecules, with their application being at times detrimental for the stability of some molecules and thus inadequate. Considering this, the macroalgae were subjected to different extraction methods, described in literature as adequate for the extraction of bioactive compounds with antioxidant and antibacterial activities. These extraction methods were solvent extraction using methanol and distilled water, microwave assisted extraction (MAE) and Soxhlet extraction using hexane, ethyl acetate and methanol. The first two extraction methods were carried out using all collected species, and the resulting extracts were assessed for both target bioactivities (antioxidant and antibacterial activity) through various assays. Species that showed high potential (*C. tomentosum*, *C. tamariscifolia*, *F. spiralis*, *P. umbilicalis* and *U. intestinalis*) were subjected to further

extractions (Soxhlet extraction using hexane, ethyl acetate and methanol and MAE) and their molecular profile elucidated using GC-MS.

Phytochemical screening revealed that ubiquitous molecules present in most lifeforms such as proteins and glycosides were present along with alkaloids across all studied species. However, no tannins were detected using these qualitative assays.

Tannins are usually present in seaweeds, mainly in brown seaweeds such as *C. tamariscifolia* and *F. spiralis* (Ford *et al.*, 2020). The assay employed for the detection of tannins was aimed at hydrolysable tannins, therefore it may not be representative for various classes of tannins.

Phytosterols are a class of plant sterols similar in structure and function to cholesterol and are responsible for the stability and structure of the biological membranes in plants. Their absence in most of the species analyzed is therefore unexpected. The assay is not reported to be specific for a specific class of phytosterols and has been used in other studies to reliably determine the presence of phytosterols.

However, contrasting information can be found in the literature. In *C. crispus*¸ Alkhalaf, (2021) described through HPLC, molecules such as flavonoids and tannins in algae collected in the red sea, however in the present study we were not able to detect these compounds. Vitorino *et al.* (2020) found a quinone in algae species collected in the northern Portuguese coast. These differences may be due to the low amount of these molecules in our samples. In fact, the other studies used more precise and sensitive techniques (HPLC), to identify these molecules. Another possibility is that the quantity of dry algae was not enough to detect these compounds by our methods.

In *C. adhaerens*, Sudha *et al.* (2014) used phytochemical assays and found flavonoids, phenolic molecules and tannins in seaweeds collected in the south of the Indian subcontinent. Alghazeer *et al.* (2022) performed phytochemical studies on seaweed species collected from the Libyan coast of the Mediterranean that were also studied in the present work: *C. tomentosum* and *U. intestinalis*. In *C. tomentosum* their work reported the presence of molecules which we were not able to detect or were absent in our samples such as coumarins, flavonoids and tannins. Nevertheless, in our work we detected quinones which were not detected in their work. Regarding *U. intestinalis*, compounds such as flavonoids, tannins and terpenes were found by the same authors in contrast to our work. These differences can be due to low quantity of algae used in our assays. Nonetheless, great differences can be found throughout scientific literature.

In this work, to assess the antioxidant activity, three different assays were employed: ABTS assay, DPPH assay and FRAP assay. These assays use different mechanisms for the assessment of antioxidant activity which are key factors in understanding the different results obtained by these methods.

The ABTS assay results showed that the aqueous extracts of *C. adhaerens*, *C. tomentosum*, *P. umbilicalis*, and *U. intestinalis* had remarkably similar inhibition percentages, with no significant differences ($p > 0.05$), which may indicate that this inhibition mechanism may be present or is similar in several species. Extracts obtained through Soxhlet extraction showed inhibition bellow the detection limit of the method as well as both *F. spiralis* methanolic and MAE extracts, indicating that the results of this test depend on the presence of hydrophilic molecules whose recovery through these methods may be lower when compared to aqueous extraction.

The results obtained with DPPH assay for most of the samples analyzed were below the detection limit of the method, with special focus on the methanolic extracts for which all the were undetected. This may be due to an inefficient extraction procedure or to a low quantity of algae used in the extraction process.

The results obtained with the FRAP assay indicated that the methanolic and some Soxhlet extracts had higher antioxidant activity when compared to the aqueous extracts, while the results obtained by ABTS and DPPH methods showed hydrophilic extracts showed higher activity than hydrophobic ones. Jiménez (2013) showed that DPPH assay showed a greater performance using aqueous extracts. Nwachukwu *et al.*, (2021) further elaborates on the importance of solvent in these assays, ABTS being the most adequate for both hydrophobic and hydrophilic solvents while FRAP assay showing a variety of interference from amino acids, slow-reacting compounds (such as polyphenols) and redox potential lower that of Fe^{3+}/Fe^{2+} redox pair.

In these three antioxidants assays, three species stand out from the others and were then submitted to further analysis by GC-MS and proteomics: *C. tamariscofia*, *P. umbilicalis* and *U. intestinalis.*

C. tamariscofia showed higher activity mainly in hydrophilic solvent extracts and sometimes the highest in all seaweed species analyzed across different extraction methods. This observation, outlined to analyze its composition in hydrophobic molecules such as fatty acids, FAMEs and other volatile organic compounds (VOCs).

In contrast, *P. umbilicalis* and *U. intestinalis* showed higher antioxidant activity in Iqueous extracts by DPPH and ABT assays, requiring a different approach to *C. tamariscofia* in elucidation of molecules with antioxidant activity. For this, a proteomic analysis was chosen to gain further insight.

Growth Inhibition halo assays showed antibacterial activity against at least one of the pathogens studied (*E. coli* and *S. aureus*) in ethyl acetate extracts (Soxhlet) in all algae species analyzed. The methanolic extract of *C. tamariscifolia* also showed antibacterial activity against both pathogens. However, MIC assays showed no antibacterial activity even when testing the same concentrations as previously reported in the literature for *C. tamariscofia*. For example,

Cagalj *et al.* (2022), in a study with seaweeds collected in the Adriatic Sea, showed that not only this species showed antibacterial activity against the bacterial pathogens employed in our study (*E. coli* and *S. aureus*) but also highlighted that changes that may occur with seasonal sampling could in fact be favorable due to the season of collection coincides with the period of expressing compounds with highest antibacterial activity. This may be due to differences In the nutrient content that seaweed exhibit between the different geographical regions where they are sampled, which may lead to changes in the metabolism and produced metabolites, since in this study, samples collected in the Adriatic sea.

Bradford and Lowry protein quantification methods yielded different results, which can be attributed to their varying sensitivities. The Bradford assay primarily relies on the binding of the dye to proteins through specific amino acid residues, notably arginine (Compton & Jones, 1985). In contrast, the Lowry assay detects peptide bonds and amino acids like tyrosine and tryptophan (Legler *et al.*, 1985). Considering that seaweeds do not possess a predominance of any of these amino acids (Vieira *et al.*, 2018) it can be inferred that the Lowry method Is better suited for quantifying the seaweed protein content. This preference for the Lowry method aligns with previous findings reported by other researchers (Vieira *et al.*, 2018).

The Lowry protein quantification method yielded higher results compared to the Bradford method but still delivered lower values than those reported in prior studies (Domingo *et al.*, 2023; Mansur *et al.*, 2020; Paiva *et al.*, 2018; Seenivasan *et al.,* 2012; Wahlstörm *et al.*, 2018). This discrepancy may be attributed to incomplete protein extraction during cell lysis, the absence of an acid or an alkaline step in the extraction process or insufficient starting amount of sample. The sonication of sample through MAE improved protein yield, which is in accordance with the literature (Kadam *et al.*, 2018). In contrast, the Soxhlet extraction drastically decreased the protein yield, which is also in accordance with the literature (Pirzada & Altintas, 2021).

The phenolic content of methanolic extracts was higher than of the aqueous extracts, as previously described (Babbar *et al.*, 2014). In most species the content decreased with the sonification of the samples which is in agreement with the literature that shows that sonification can be detrimental to the stability of phenolic compounds (Annegowda *et al.*, 2010). In Soxhlet extraction a slight decrease was observed in some species (*C. tomentosum*, *C. tamariscifolia* and *F. spiralis*), which can be associated to the thermostability of phenolic compounds and the affinity to certain solvents employed in this extraction method (Esparza *et al.*, 2020). The results showed that phenolic compounds determined in *C. crispus* were higher than those reported in the literature (Alkhalaf, 2021). On the other hand, in *C. adhaerens*, *C. tomentosum*, *C. tamariscifolia*, *F. spiralis, P. umbilicalis* and *U. intestinalis* phenols were lower than those reported in the literature (Radman *et al.*, 2021; Hafez *et al.*, 2022; Moussa *et al.*, 2020; Francisco *et al.*¸2020; Garcimartín *et al.*, 2015; Farasat *et al.*, 2014). However, once again, this variability

can be due to the climatic and geological characteristics where seaweed grow giving them different compositions.

The tannin content was higher in methanolic extracts than in aqueous extracts, which was confirmed by Soxhlet extractions, where methanolic extracts presented higher tannin content ($p < 0.05$) compared to the other extraction methods. This is in accordance with the literature, which reports that tannins show their highest solubility in solvents with limited polarity, such as methanol (Fraga-Corral *et al.*, 2020). The results of Soxhlet extracts we can see further supported of this fact, since the methanol extracts presenting a much higher tannin content than other solvents, with notable exception of *C. tomentosum,* which showed no significant differences ($p > 0.05$) between ethyl acetate and methanol extracts using this technique. When comparing the results obtained to those of the literature we find that they were mostly bellow the reported levels for *C. chrispus* (Alkhalaf *et al.*, 2021) *C. tomentosum*, (Abd *et al.*, 2022) *C. tamariscifolia* (Cagali *et al.*, 2022) and *F. spiralis* (Almeida *et al.*, 2021) which is in accordance with the results of phytochemical assays.

The lipid content determined in the seaweed species assessed was far higher than it was reported in the literature, with *C. crispus* showing more than nine times higher content (Guerreiro *et al.*, 2019), *C. adhaerens* showing almost seven fold higher content (Seenivasan *et al.*, 2012), *C. tomentosum* and *C. tamariscifolia* showing twice as much (Rey *et al.*, 2020; Vizetto-Duarte *et al.*, 2016), *F. spiralis* showing threefold as much (Francisco *et al.*, 2020), *Porphyra umbilicalis* eight fold higher (Campos *et al.*, 2022) and in *U. intestinalis* nine fold higher content (da Costa *et al.*, 2020). An increase in lipidic content has been associated to stress conditions such as high salinity, harsh exposure to light and nutrient limitation (Sun *et al.*, 2018), which can be responsible for this increase. However, the present seaweed species were collected in their natural environment but in different locations and in different seasons from the previous studies. These differences in results may also be associated with experimental errors, to avoid these, an internal standard should be used in order to validate these results and the conditions in which these were collected should be recorded.

The GC-MS analyses identified 23 FAMEs in *U. intestinalis*, 29 in *C. tamariscifolia* and 18 in *P. umbilicalis*. Additionally, Soxhlet extracts allowed to putatively identify several other compounds, some of them related to antioxidant and antibacterial activity but also with anti-inflammatory or anticancer activity, among others. However, these cannot be correlated directly with the activity expressed in other assays. This is due to the fact that this analysis was merely qualitative making it impossible to outline a dose-response relationship between activity across the different assays and concentration of the various molecules. For further elucidation of the role that each molecule could have, further extractions and fractionalization should be performed for the compounds present in each seaweed sample. The FAMEs

identified have also been identified in studies in the same species (Harryssson *et al.*, 2021; Silva *et al.*, 2013).

The results obtained in the analysis of volatile organic compounds showed a much less diversity of molecules when compared to the literature such as Kulkarni *et al.* (2021) in which the analysis of *Ulva intestinalis* Soxhlet extracts yielded a greater diversity of molecules. This can be due to the fact that in this study the Soxhlet extraction time was only 6 hours whereas in the present work the sample was subjected to 24 hours long extraction period. This could lead to degradation of thermal unstable molecules which would generate fragments with much lower molas masses, being unable to be detected with the equipment used.

The proteomic analysis was carried out with the closest algae species present in the databases, since the selected species studied are not represented in the databases. Additionally, the proteomic analysis generated a series of hypothetical proteins, meaning that they are predicted to be expressed from an open reading frame, but for which there is no experimental evidence of translation (Ljaq *et al.*, 2019). In the case of *P. umbilicalis*, for instance, there were 1277 hypothetical proteins. On the other hand, most of the proteins identified are related to regulation, structural functions, metabolism, biosynthetic processes, and other cellular processes. However, some proteins that were Identified in all seaweed species analyzed are related to chaperoning and/or thermal stress (e.g. GroEL, HSPs) assisting in protein folding, while others as allophycocyanin and phycocyanin (identified in *P. umbilicalis*) are related with strong antioxidant and antibacterial activities. Other proteins identified are related to antioxidant activity, as catalase, glutathione reductase and superoxide dismutase identified in *U. Intestinalis*. The expression of these proteins associated with antioxidant responses (Rajput *et al.*, 2021) may be because the seaweeds were collected at low tide and were therefore exposed to air and solar radiation, and as such the expression of these proteins constitutes a defense mechanism against stress. Moreover, calmodulin identified in *P. umbilicalis*, is a calcium-binding protein, with an important role in both regulating plant growth and development, but also in the resistance mechanisms to several biotic and abiotic stresses (Shi and Du, 2020). Another protein reported to have antioxidant properties is cytochrome c, which was identified in all species analyzed (Pereverzev *et al.*, 2003). In fact, these proteins help may help seaweed facing the abiotic stress as terrestrial plants which have a complex antioxidant defense system with numerous enzymatic components that play a crucial role in overcoming various stress conditions. (Rajput *et al.*, 2021). Additionally, a polysaccharide binding protein was identified in *P. umbilicalis*. Natural polysaccharides have been related to antioxidant activities since the antioxidant mechanisms of natural polysaccharides mainly contain the regulation of signal transduction pathways, the activation of enzymes and the scavenging of free radicals (Bai *et al.*, 2022). Another protein identified in *P. umbilicalis* is thioredoxin, which is a key antioxidant system in defense against oxidative stress (Lu *et al.* 2014; Liu, 2023). Phytoene synthetase was

identified in *C. tamariscifolia* and is involved in the biosynthesis of carotenoids which have antioxidant activity (Krinsky, 1989; Stra *et al.*, 2023). Whereas for *U. intestinalis*, ascorbate peroxidase was identified which is known as a scavenging enzyme of the antioxidant defense mechanisms (Gomes et al., 2022). A cyclophilin was identified in *U. Intestinalis*. This protein has chaperoning function (Hill *et al.*, 2022) but was reported to enhance antioxidant response by binding to peroxiredoxins and activating its peroxidase activity (Lee *et al.*, 2001). In fact, according to Singh *et al.*, (2020) cyclophilins are highly versatile proteins with multiple functions, regulating a wide range of growth and developmental processes, from hormone signaling to stress responses. Interestingly, Lee *et al.* (2007), reported a cyclophilin with antifungal activity in cabbage.

5 Conclusions and future prospects

The present work provides relevant information regarding the presence of bioactive compounds in seaweeds from the Portuguese coast. Most of the compounds and proteins identified were related to antioxidant activity, however, few compounds or proteins identified could be related to antibacterial activity. However, more studies are needed for further clarification. The results obtained allowed us to select *C. tamariscofia*, *P. umbilicalis* and *U. intestinalis* as the most promising to carry out future work in terms of elucidating and purifying compounds of interest to the pharmaceutical or food industry and to obtain information that could aid to better understanding the mechanisms behind the activities presented, not only by identifying the molecules present in the seaweed species but also by exploring the potential presented by the different extracts obtained..

Subsequent separation of the molecular content of the samples, through HPLC, for example, and other tests could make it possible to unequivocal correlate bioactivity with molecules present in samples.

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AFONSO MARQUES

SEARCH FOR BIOACTIVE COMPOUNDS WITH ANTIOXIDANT AND ANTI-BACTERIAL ACTIVITY IN SEAWEED SPECIES FROM THE PORTUGUESE COAST

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