Diana Rodrigues Julião
Licenciada em Bioquímica

The Effect of Drying Process on Undervalued Brown and Red Seaweed Species: Biochemical Characterization

Dissertação para obtenção do Grau de Mestre em
Química Bioorgânica

Orientador: Carlos Manuel Lourenço Cardoso,
Investigador, Instituto Português do Mar e da Atmosfera, I.P.

Elo de Ligação: Paula Cristina de Sério Branco,
Professora Auxiliar com Agregação, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

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Presidente: Prof. Doutora Paula Cristina de Sério Branco
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Abstract

The effect of drying on two brown seaweed (*Cystoseira abies-marina, Cystoseira humilis*) and two red seaweed species (*Asparagopsis armata, Asparagopsis taxiformis*), collected from the Azores Archipelago, was evaluated through the study of their proximate and mineral composition, relevant biological activities, such as antioxidant and anti-inflammatory properties, and significant bioactives, namely polyphenols and beta-glucans. Ash and protein content ranged from 25-56 g/100g dw and 3.5-13.1 g/100g dw, respectively. Sun-dried *C. humilis* had the highest moisture decrease. Concerning insoluble dietary fibre content, the genus *Cystoseira* presented superior concentrations (43.7-53.6 g/100g dw). Contrarily, the soluble dietary fibre content is superior in the *Asparagopsis* genus (8.0-13.2 g/100g dw). For the proximate composition, no significant differences were detected concerning the drying procedure. Seaweeds from the *Cystoseira* genus showed high polyphenol levels (176-678mg GAE/100 g dw), exceeding those determined in the *Asparagopsis* genus, regardless of drying process. This was partially reflected in the antioxidant activity, which showed that extracts from the *Cystoseira* species were often more antioxidant than those from *Asparagopsis* species. The influence of the drying technique upon the antioxidant activity was limited, since in many instances there was no effect. Concerning anti-inflammatory activity, in the case of shade-dried samples, *C. humilis* had a higher activity (>30% COX-2 inhibition) but was not rendered bioaccessible. Indeed, only *A. taxiformis* displayed anti-inflammatory activity in the bioaccessible fraction, leading to bioaccessibility factors in the 90-100% range. Therefore, though bioactivities were higher in the *Cystoseira* species, *Asparagopsis* species also had a positive bioactive potential. Sun-drying produced more negative effects than shade-drying, despite not being very extensive. Regarding elemental composition, iodine was present in a considerable amount in the *Asparagopsis* genus. Iron had high concentrations in the four species. Regarding contaminants, *Cystoseira abies-marina* showed high arsenic concentrations. Iodine, bromine, magnesium, and cadmium showed the highest bioaccessibility percentages.

Keywords: *Asparagopsis*, *Cystoseira*, Bioaccessibility, Antioxidant, Anti-inflammatory

Resumo

O efeito da secagem de duas algas castanhas (*Cystoseira abies-marina*, *Cystoseira humilis*) e duas algas vermelhas (*Asparagopsis armata*, *Asparagopsis taxiformis*), coletadas no Arquipélago dos Açores, foi avaliado através do estudo da sua composição proximal e mineral, atividades biológicas relevantes, como propriedades antioxidantes e anti-inflamatórias, e compostos bioativos, nomeadamente polifenóis e beta-glucanos. Obteve-se uma variação de 25-56 g/100g dw relativamente ao teor de cinza e de 3,5-13,1 g/100g dw, relativamente ao teor de proteína. *C. humilis* seca ao sol ficou mais seca. Quanto ao teor de fibra alimentar insolúvel, o género C*ystoseira* apresentou concentrações superiores (43.7-53.6 g/100g dw). Pelo contrário, o teor de fibra alimentar solúvel foi superior no género *Asparagopsis* (8.0-13.2 g/100g dw). O método de secagem não influiu significativamente na composição proximal. As macroalgas do género *Cystoseira* apresentaram um elevado teor de polifenóis (176-678mg GAE/100g dw), superando os valores obtidos para o género *Asparagopsis*, independentemente do processo de secagem. Isso refletiu-se parcialmente na atividade antioxidante, pois os extratos de *Cystoseira* eram frequentemente mais antioxidantes do que os de *Asparagopsis*. A influência da técnica de secagem sobre a atividade antioxidante foi limitada (em muitos casos sem efeito). Em relação à atividade anti-inflamatória, nas amostras secas à sombra, *C. humilis* apresentou maior atividade (>30% de inibição da COX-2), mas não se tornou bioacessível. Apenas *A. taxiformis* exibiu atividade anti-inflamatória na fração bioacessível, com resultados no intervalo de 90-100%. Portanto, embora as bioatividades fossem maiores nas espécies *Cystoseira*, as espécies *Asparagopsis* também apresentaram um alto potencial. A secagem ao sol produziu mais efeitos negativos do que a secagem à sombra, apesar de não serem muito significativos. Relativamente à composição elementar, observou-se um elevado teor de iodo nas *Asparagopsis*. O ferro apresentou elevadas concentrações nas quatro espécies. Em relação aos contaminantes, *Cystoseira abies-marina* apresentou altas concentrações de arsénio. Iodo, bromo, magnésio e cádmio apresentaram a maior bioacessibilidade.

Palavras-chave: *Asparagopsis*, *Cystoseira*, Bioacessibilidade, Antioxidante, Anti-inflamatória

Table of Contents

Acknowledgments xi

Abstract xiii

Resumo xv

Table of Contents xvii

List of Figures xxi

List of Tables xxv

List of Abbreviations xxvii

1. Introduction 1

1.1. Marine Algae 1

1.2. Marine Macroalgae (or Seaweeds) 1

1.2.1. Brown Algae (Phaeophyta) 2

1.2.2. Red Algae (Rhodophyta) 2

1.3. Studied Species 3

1.3.1. *Asparagopsis taxiformis* 3

1.3.2. *Asparagopsis armata* 4

1.3.3. *Cystoseira abies-marina* 4

1.3.4. *Cystoseira humilis* 5

1.4. Polysaccharides 5

1.5. Dietary Fibre 7

1.6. Elemental Composition 8

1.7. Antioxidant Properties 10

1.8. Bioaccessibility and Bioavailability 12

1.9. *In vitro* digestive model as a tool to assess bioaccessibility 13

1.10. Anti-inflammatory Properties 14

1.11. Global overview of the potential of bioactivities and applications of the studied macroalgae 14

2. Objectives 17

3. Materials and Methods 19

3.1. Sample collection and preparation 19

3.2. Proximate composition 20

3.2.1. Moisture and Ash content 20
3.2.2. Protein content 21
3.2.3. Dietary Fibre content 22
3.2.3.1. Insoluble Dietary Fibre 23
3.2.3.2. Soluble Dietary Fibre 23
3.2.3.3. Total Dietary Fibre 24
3.3. Total Polyphenol content 24
3.3.1. Extract preparation 24
3.3.2. Singleton and Rossi assay 25
3.4. Antioxidant Activity 26
3.4.1. DPPH method 26
3.4.2. FRAP method 27
3.4.3. ABTS method 28
3.5. Beta-glucans (laminarin) content 29
3.6. *In vitro* Digestion Model 31
3.7. Anti-inflammatory activity 32
3.7.1. Extract preparation for *in vitro* anti-inflammatory activity 32
3.7.2. Cyclooxygenase (COX-2) inhibition method 33
3.8. Mineral Composition 35
3.8.1. Microwave-assisted acid digestion 35
3.8.2. Elemental analysis 35
3.8.3. Bromine and iodine analysis 36
3.9. Statistical analysis 36
4. Results 37
4.1. Proximate composition 37
4.2. Total polyphenol content 39
4.3. Antioxidant activity 39
4.4. Beta-glucan (laminarin) content 42
4.5. Anti-inflammatory content 43
4.6. Elemental Composition 43
4.7. Bioaccessibility 45

4.7.1. Anti-inflammatory activity 45
4.7.2. Elemental Composition 45
5. Discussion 47
5.1. Proximate Composition 47
5.2. Total polyphenol content 48
5.3. Antioxidant Activity 50
5.4 Beta-glucan (laminarin) content 51
5.5. Anti-inflammatory activity 51
5.6. Elemental Composition 52
5.7. Bioaccessibility 54
5.7.1. Anti-inflammatory activity 54
5.7.2. Elemental Composition 55
6. Conclusions and Future Perspectives 57
7. Bibliography 59
8. Annexes 72
9. Additional Information 76

List of Figures

Figure 1.1. Structure of floridoside (2-O-α-D-galactopyranosylglycerol)[16]. 3
Figure 1.2. *Asparagopsis taxiformis* attached to rocks. Azores Islands, Atlantic Ocean. SeaExpert, 2019. 3
Figure 1.3. *Asparagopsis armata* attached to rocks, Azores islands, Atlantic Ocean. SeaExpert, 2019. 4
Figure 1.4. *Cystoseira abies-marina* attached to rocks, Azores islands, Atlantic Ocean. SeaExpert, 2019. 4
Figure 1.5. *Cystoseira humilis*, Azores islands, Atlantic Ocean. SeaExpert, 2019. 5
Figure 1.6. Molecular structure of laminarin (or laminaran)[66]. 6
Figure 1.7. Molecular structure of fucoidan[67]. 6
Figure 1.8. Monomeric compounds present in alginic acid[74]. 7
Figure 1.9. G-G blocks and M-M blocks (homopolymeric blocks) present in alginic acid structure. R=H[75]. 7
Figure 1.10. Chemical structure of carrageenan[78]. 7
Figure 1.11. Schematic representation of the calcium-induced gelation of alginate in accordance with the “egg-box” structure[100]. 9
Figure 1.12. Molecules of **(a)** caffeic acid and **(b)** ferulic acid[125]. 11
Figure 1.13. Structural formulas of representative phlorotannins from marine seaweeds. **(1)** phoroglucinol; **(2)** eckol; **(3)** fucodiphloroethol G; **(4)** phlorofucofuroeckol A; **(5)** 7-phloroeckol; **(6)** dieckol; **(7)** 6,6’-bieckol[121]. 12

Figure 3.1. Sun-dried and shade-dried seaweed biomass of the studied species: **(A)** Shade-dried *Cystoseira abies-marina*; **(B)** Sun-dried *Cystoseira abies-marina*; **(C)** Shade-dried *Asparagopsis armata*; **(D)** Sun-dried *Asparagopsis armata*; **(E)** Shade-dried *Cystoseira humilis*; **(F)** Sun-dried *Cystoseira humilis*; **(G)** Shade-dried *Asparagopsis taxiformis*; **(H)** Sun-dried *Asparagopsis taxiformis*. Diana Julião, October 2019. 19
Figure 3.2. Greenhouse used for the sun-drying method, where seaweeds are arranged in a horizontal position. SeaExpert, 2020. 20
Figure 3.3. Solar dryer used in the shade-drying method. SeaExpert 2020. 20
Figure 3.4. Crucibles with ash at the end of the procedure. Diana Julião, October 2019. 21
Figure 3.5. Crucibles with 1,000 ± 0,005g of duplicated samples of each macroalgae (sun-dried and shade-dried). Diana Julião, October 2019. 22
Figure 3.6. Precipitates formed after 1 hour at room temperature, for *Asparagopsis taxiformis* and *Asparagopsis armata*, sun-dried and shade-dried. Diana Julião, November 2019. 23
Figure 3.7. Precipitates formed after 1 hour at room temperature, for *Cystoseira abies-marina* and *Cystoseira humilis*, sun-dried and shade-dried. Diana Julião, November 2019. 24
Figure 3.8. Supernatant collected from the aqueous extracts, to a final volume of 25mL. From left to right: Sun-dried *Asparagopsis taxiformis*; Shade-dried *Asparagopsis taxiformis*; Sun-dried *Asparagopsis armata*; Shade-dried *Asparagopsis armata*; Sun-dried *Cystoseira humilis*; Shade-

dried *Cystoseira humilis;* Sun-dried *Cystoseira abies-marina*; Shade-dried *Cystoseira abies-marina*. Diana Julião, December 2019. 25
Figure 3.9. Supernatant collected from the ethanolic extracts, to a final volume of 25mL. From left to right: Sun-dried *Asparagopsis taxiformis*; Shade-dried *Asparagopsis taxiformis*; Sun-dried *Asparagopsis armata*; Shade-dried *Asparagopsis armata*; Sun-dried *Cystoseira humilis*; Shade-dried *Cystoseira humilis;* Sun-dried *Cystoseira abies-marina*; Shade-dried *Cystoseira abies-marina*. Diana Julião, December 2019. 25
Figure 3.10. DPPH and its stable form. In the left, 2,2-diphenyl-1-picrylhydrazyl (free radical) and in the right, 2,2-diphenyl-1-picrylhydrazine (nonradical)[177]. 26
Figure 3.11. Colour variation during the DPPH reaction. DPPH has a characterized violet colour **(A)** but when mixed with an antioxidant substance (capable of donating a hydrogen atom) gradually becomes yellow **(B)**. Diana Julião, December 2019. 27
Figure 3.12. Reduction of the ferric-tripyridyltriazine (FeIII-TPTZ) complex to the ferrous (FeII) form by an antioxidant[181]. 27
Figure 3.13. Colour variation in the FRAP reaction. The intense blue colour means that the Fe^{III}-TPTZ complex was reduced, indicating the presence of antioxidant activity. Diana Julião, January 2020. 28
Figure 3.14. One-electron oxidation of ABTS and the formation of cation radicals ($ABTS^{\bullet+}$)[183]. 29
Figure 3.15. Colour variation in the ABTS reaction. The $ABTS^{+}$ solution exhibits a blue colour **(A)** and the loss of this colour indicates the presence of antioxidant agents **(B)**. Diana Julião, January 2019. 29
Figure 3.16. (A) Coloration of the D-glucose standard after GOPOD treatment - the pink colour in the mixture indicated the presence of D-glucose, and therefore the presence of β-glucans. **(B)** Coloration of the samples after GOPOD treatment. Diana Julião, January 2020. 31
Figure 3.17. Final solutions of 1 mg/mL prepared from the supernatant collected from the aqueous extracts. From left to right: Shade-dried *Asparagopsis taxiformis*; Sun-dried *Asparagopsis taxiformis*; Shade-dried *Asparagopsis armata*; Sun-dried *Asparagopsis armata*; Shade-dried *Cystoseira humilis*; Sun-dried *Cystoseira humilis;* Shade-dried *Cystoseira abies-marina*; Sun-dried *Cystoseira abies-marina*. Diana Julião, June 2020. 32
Figure 3.18. Final solutions of 1 mg/mL prepared from the bioaccessible extract. From left to right: Shade-dried *Asparagopsis taxiformis*; Sun-dried *Asparagopsis taxiformis*; Shade-dried *Asparagopsis armata*; Sun-dried *Asparagopsis armata*; Shade-dried *Cystoseira humilis*; Sun-dried *Cystoseira humilis;* Shade-dried *Cystoseira abies-marina*; Sun-dried *Cystoseira abies-marina*. Diana Julião, June 2020. 33
Figure 3.19. Reaction catalysed by acetylcholinesterase. Cayman test kit-560131 (Cayman Chemical Company, Ann Arbor, MI, USA). 34
Figure 3.20. Anti-inflammatory activity test plate ready for prostaglandin reading. Diana Julião, June 2020. 35

Figure 4.1. Bioaccessibility of the anti-inflammatory activity in the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina* and *Cystoseira humilis*) either sun-dried or dried in the shade. 45

List of Tables

Table 4.1. Proximate crude composition (g/100g dry weight for ash, protein, moisture before and after homogenization and lyophilization, and insoluble, soluble and total dietary fibre) of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in the shade. 37

Table 4.2. Total polyphenol content (in mg GAE/100g dw) measured in aqueous (Aq.) and ethanolic (Eth.) extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade. 39

Table 4.3. Antioxidant activity as measured by DPPH (mg Ascorbic Acid Equivalent/100g dw) method in aqueous (Aq.) and ethanolic (Eth.) extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade. 40

Table 4.4. Antioxidant activity as measured by FRAP (µmol Fe^{2+} eq./g dw) method in aqueous (Aq.) and ethanolic (Eth.) extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade. 41

Table 4.5. Antioxidant activity as measured by ABTS (µmol Trolox Equivalent/g dw) method in aqueous (Aq.) and ethanolic (Eth.) extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, *Cystoseira humilis*) either sun-dried or dried in shade. 42

Table 4.6. Beta-glucan content (in %, dw) of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade. 42

Table 4.7. Anti-inflammatory activity (% inhibition of COX-2) in aqueous extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade. 43

Table 4.8. Mineral composition obtained for aqueous extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade. Concentration of sodium (Na), magnesium (Mg), phosphorus (P), sulphur (S), potassium (K), calcium (Ca), chromium (Cr), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), arsenic (As), bromine (Br), cadmium (Cd), iodine (I) and lead (Pb) are displayed. 44

Table 4.9. Elemental bioaccessibility obtained for aqueous extracts after *in vitro* digestion. Of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in the shade. 46

List of Abbreviations

AA – Ascorbic Acid

ABTS - 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

AChE – PG-acetylcholinesterase conjugate

ARA – Arachidonic Acid

AsB – Arsenobetaine

AsIII – Arsenite

AsV – Arsenate

COX-2 – Cyclooxygenase-2

Da - Dalton

DMA – Dimethylarsinic Acid

DNA - Deoxyribonucleic Acid

DPPH - α,α-Diphenyl-β-picrylhydrazyl

DW – Dry Weight

EC – European Commission

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme- Linked Immunosorbent Assay

EU – European Union

FAAS - Flame Atomic Absorption Spectroscopy

FRAP - Ferric Ion Reducing Antioxidant Power

GA – Gallic Acid

GAE – Gallic Acid Equivalents

ICP-MS - Inductively Coupled Plasma Mass Spectrometry

IL – Interleukin

LDL – Low-Density Lipoprotein

LOD – Limit Of Detection

LPS – Lipopolysaccharides

MMA – Monomethylarsonic Acid

MRL – Maximum Residue Level

MΩ.cm – Megaohms.cm

NO – Nitric Oxide

NOS - Nitric Oxide Synthase

NSAID's – Non-Steroidal Anti-Inflammatory Drugs

PG – Prostaglandin

PGE_2 – Prostaglandin E_2

RNA - Ribonucleic Acid

ROS – Reactive Oxygen Species

SAR – Structure-Activity Relationship

TNF – Tumour Necrosis Factor

TPTZ – 2,4,6-Tripyridyl-s-triazine

UV – Ultraviolet

W – Watt

WHO – World Health Organization

WW – Wet Weight

1. Introduction

1.1. Marine Algae

Algae are autotrophic organisms that live in water or in moist environments. These organisms have a wide geographical distribution, being mainly abundant in rivers, seas, and lakes. They occupy the euphotic region (i.e., the upper 200 meters of water, where the light is effective to perform photosynthesis). Some algae inhabit in adverse environments and may endure extreme conditions[1].

Algae, whose habitat is in water, may be planktonic (microalgae) – have a small dimension and be suspended in water (microscopic) - or benthic (macroalgae) – be connected to a surface (typically rock) -, and reach a larger size (macroscopic), having the capacity to reach up to 50 meters in length[1,2].

1.2. Marine Macroalgae (or Seaweeds)

Seaweeds are autotrophic organisms that belong to the Eukaryota (or Eukarya) domain. They can be classified as Phaeophyta (brown algae), Chlorophyta (green algae) or Rhodophyta (red algae). Their classification is mainly based on their coloration, that can be explained by the expression of different pigments present in their cells, but also based in genomic analysis that justify the maintenance of the phylogenetic structure established, and their nutrient and chemical composition[3,4]. Brown seaweeds belong to the *phylum* Heterokontophyta (or Ochrophyta) and their pigments are chlorophylls a, c and carotenoids (fucoxanthin's abundance accounts for their brown colour); green seaweeds belong to the *phylum* Chlorophyta, and their pigments are identical to the ones found in terrestrial plants (carotenoids and chlorophylls a, b); red seaweeds belong to the *phylum* Rhodophyta, and have photosynthetic pigments such as carotenoids (zeaxanthin, β-carotene, and lutein), chlorophyll a, and some phycobilins (R-phycoerythrin and R-phycocyanin)[1,2].

Similar to plants, seaweeds have in their composition numerous healthy compounds. The fact that they are able to produce secondary metabolites that are known for their biological activities, makes them a source of functional ingredients[3,5].

Marine algae are already used for the treatment of a range of health problems. For instance, brown algae may be used against arteriosclerosis, rheumatic processes, and hypertension; green algae may operate as anti-helmintics; and red algae are used as anticoagulants, anti-parasites, and against gastrointestinal problems. This is due to several substances in algae that influence human homeostasis[6].

There are many benefits in seaweed extracts - they yield substances such as agar-agar (E406), carrageenan (E407) and alginates (E400). Such phytochemical hydrocolloid substances find application mostly due to their rheological properties, in textile, food, paper and dairy sector. They may hold highly relevant bioactive substances[1,7,8].

1.2.1. Brown Algae (Phaeophyta)

Brown algae (or Phaeophyta) exhibit a golden-brownish colour[9]. The division Phaeophyta is deemed to encompass 13 orders (Bold & Wynne (1985))[10], yet only three orders, Laminariales, Fucales, and Dictyotales, were previously studied for their phytochemicals[11].

The colouring of brown algae can be explained by the importance of fucoxanthin, which overshines other pigments (*i.e.*, chlorophyll a and c and other xanthophylls), showing antioxidant, antidiabetic and anti-inflammatory activities[11,12]. Moreover, brown algae also have other important categories of secondary metabolites including polyphloroglucinol polyphenolic compounds[13] and non-polyphenolic, non-polar secondary metabolites such as terpenes[14], besides being rich in polysaccharides such as fucoidans and laminarins, and alginates.

Sulfur-containing carbohydrates from seaweeds may also show anti-coagulant, anti-tumour, anti-thrombotic, and anti-viral activities. These biomolecules act in the immune and inflammatory systems, shield cells from viral infection and have anti-proliferative effects on them[11].

1.2.2. Red Algae (Rhodophyta)

Red algae (or Rhodophyta) encompasses 5000–6000 species of mostly multicellular organisms. Phylogenetically they are the oldest division of lower plants[15,16]. They inhabit mainly marine environments.

A mixture of biochemical and ultrastructural features makes these algae distinct among other eukaryotic lineages. More precisely, the fact that the photosynthetic pigment is chlorophyll a, being light led to the reactive area by phycobiliproteins (phycoerythrin, allophycocyanin and phycocyanin). Pigments are involved in hemi-spherical protein complexes – phycobilisomes - attached to the surface of thylakoids[4,15,17,18].

Galactans are the major polysaccharides present in Rhodophyceae. While agar contain D- and L-galactose, carrageenan is composed only by D-galactose[19]. Agars and carrageenans are prepared industrially from algae in large quantities and are used as gelling and stabilizing agents[20,21]. Red algal galactans often show potent antiviral, antitumor and antioxidant activities[16,22,23].

Taxonomically, red algae are classified into two classes: Bangiophyceae and Florideophyceae[24]. Floridean starch isolated from the latter class, after which it is named, is a special type of starch that contains only amylopectin. Members of Bangiophyceae may, however, have both amylose and amylopectin in their starch granules[24].

Floridean starch hold several low-molecular glycosides, that act not only as primary photosynthetic reserve products, but also as osmoregulators. Floridoside (2-*O*-α-D-galactopyranosylglycerol) (Figure 1.1) may be considered the most commonly distributed substance of this group[16,25].

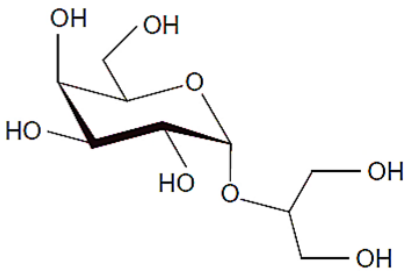

Figure 1.1. Structure of floridoside (2-O-α-D-galactopyranosylglycerol)[16].

As to neutral structural polysaccharides, red algae is composed by cellulose, a linear (4)-linked-D-glucan, found in the cell walls in moderate amounts (usually less than 10%), mannans and xylans[16,26].

1.3. Studied Species

1.3.1. *Asparagopsis taxiformis*

Asparagopsis taxiformis (Delile) Trevisan de Saint-Léon, also known as Supreme Limu (Figure 1.2)[27], is a red seaweed, from the *phylum* Rhodophyta, *subphylum* Eurhodophytina, *class* Florideophyceae, *subclass* Rhodymeniophycidae, *order* Bonnemaisoniales and *family* Bonnemaiosoniaceae[28]. The colour of this seaweed can vary from yellowish red to dark red. Its structure possesses a base with rigid erect branches covered in numerous soft and fuzzy branchlets that get shorter near the top. *Asparagopsis* is salted and may be eaten cooked or raw – displaying a spicy flavour and an intense odour[29].

Asparagopsis inhabits reefs at one metre (and more) depth. It has a wide distribution across temperate and tropical marine coastal ecosystems[30] – they have populations spread in Atlantic, Mediterranean, and Indo-Pacific regions[31].

Asparagopsis species show strong antimicrobial features[31-32]. This genus produces a variety of biomolecules, such as acrylates and ketones, that may be bioactives[31,32,33]. Their production is linked to the capacity of algae to avoid autotoxicity by storing them in specialized storage structures[31].

Figure 1.2. *Asparagopsis taxiformis* attached to rocks. Azores Islands, Atlantic Ocean. SeaExpert, 2019.

1.3.2. *Asparagopsis armata*

Asparagopsis armata Harvey (Figure 1.3)[2], is a red seaweed from the *phylum* Rhodophyta, *subphylum* Eurhodophytina, *class* Florideophyceae, *subclass* Rhodymeniophycidae, *order* Bonnemaisoniales and *family* Bonnemaisoniaceae[28]. *A. armata* is native to the Southern Hemisphere (Australia and New Zealand) but is also present in Northern Hemisphere. Nowadays, it has expanded almost to all marine environments. It is described as a gametophyte plant that occurs in June-September, with a pale purplish-red colour that changes to orange when the seaweed is removed from water.

A. armata extract shows antioxidant and antibacterial activities. It is used in many cosmeceuticals, presenting anti-cancer activity[1,34,35], and it is collected from the wild or cultivated for the extraction of hydrocolloids[1,36]. *Asparagopsis* species are present in the Atlantic and the Pacific area, where they find dermo-cosmetical and pharmaceutical applications[37]. The extracts of this seaweed may contain anti-parasite[38], antiviral[39,40], antimycotic[34], and antimicrobial[34,41,42] compounds.

Figure 1.3. *Asparagopsis armata* attached to rocks, Azores islands, Atlantic Ocean. SeaExpert, 2019.

1.3.3. *Cystoseira abies-marina*

Cystoseira abies-marina (S.G. Gmelin) (Figure 1.4) is a brown seaweed from the *phylum* Ochrophyta, *class* Phaeophyceae, *subclass* Fucophycidae, *order* Fucales and *family* Sargassaceae[28].

This species has a characteristic cylindrical axle[43]. *C. abies-marina* is mostly present in Macaronesia in areas subjected to wave energy. Its extracts display antioxidant[44,45,46], antimicrobial[1], and cytotoxic[44] activity.

Figure 1.4. *Cystoseira abies-marina* attached to rocks, Azores islands, Atlantic Ocean. SeaExpert, 2019.

1.3.4. *Cystoseira humilis*

Cystoseira humilis Schousboe ex Kutzing (Figure 1.5) is a brown seaweed from the *phylum* Ochrophyta, *class* Phaeophyceae, *subclass* Fucophycidae, *order* Fucales and *family* Sargassaceae[28]. *C. humilis* is distinguished by the existence of air-vesicles.

Within the *order* Fucales, this seaweed is distinguished by its broad dispersion. It is found in the three main oceans. *C. humilis* extracts have antibacterial, antioxidant and cytotoxic activity[47-48,149]. *C. humilis* is considered rich in biomolecules for medical, cosmeceutical and food uses[50].

Figure 1.5. *Cystoseira humilis*, Azores islands, Atlantic Ocean. SeaExpert, 2019.

1.4. Polysaccharides

Polysaccharides are broadly distributed in nature, where they have a role in structure-forming skeletal compounds (for instance, cellulose, hemicellulose and pectin in algae and plants); storage compounds (for example, starch and dextrins in plants) and water-binding compounds (such as agar, pectin and alginate in plants)[51].

Marine algae have in their constitution storage and cell wall polysaccharides. The cell wall polysaccharides mostly consist of cellulose, hemicelluloses and neutral polysaccharides[7]. Both cell wall and storage polysaccharides are species specific: brown algae contain alginic acid, laminarin (β-1,3 glucan), sargassan and fucoidan (sulphated fucose); and red algae have xylans, agars, carrageenans, floridean starch (amylopectin-like glucan) and water-soluble sulphated galactan[7,6,8].

Polysaccharides may demonstrate anti-tumour, antiviral and antioxidant activities. Besides that, they also show anticoagulant properties, prevent obesity and diabetes, and hypocholesterolemic action[6,52-53]. These molecules are crucial in the food industry, regarding food texturing, and are responsible for characteristics such as viscosity and consistency, having commercial applications in food processing, both in natural and modified forms, as thickeners, stabilisers for emulsions and dispersions, emulsifiers and as coating substances to protect sensitive food from undesired change[8,54].

Among the great number of polysaccharides present in seaweeds, laminarin, fucoidan, alginic acid and carrageenan have stood out for their numerous applications.

Laminarin (or laminaran) (Figure 1.6) is defined as a linear water-soluble polysaccharide encompassing 20 to 25 glucose units, consisting of (1,3)-β-D-glucan with randomly intra-chain β(1,6) branching[55]. Two kinds of laminarin chains have been described: M or G. M chains terminate with a mannitol residue, while G chains terminate with a glucose residue[11,56]. Most laminarins are considered dietary fibres[56]. These polysaccharides show antibacterial, anti-tumour, anticoagulant, anticancer, and immunomodulatory activities[57].

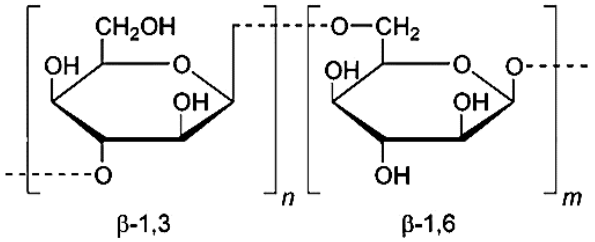

Figure 1.6. Molecular structure of laminarin (or laminaran)[58].

Fucoidans (Figure 1.7), mostly found in brown seaweeds, are fucose-based sulfur-containing polysaccharides, displaying different proportions of mannose, galactose, and glucose. Their constituents are linked by α(1→2)-bonds[59]. Fucoidans are mainly found in *Laminariales* and *Fucales* species and constitute 25–30% of seaweed dry weight (dw)[60].

Possibly, this sulfated polysaccharide is not found in the other two main seaweed groupings. They have shown antioxidant, anticoagulant, antiallergic, anticancer, antiviral, and other activities[61].

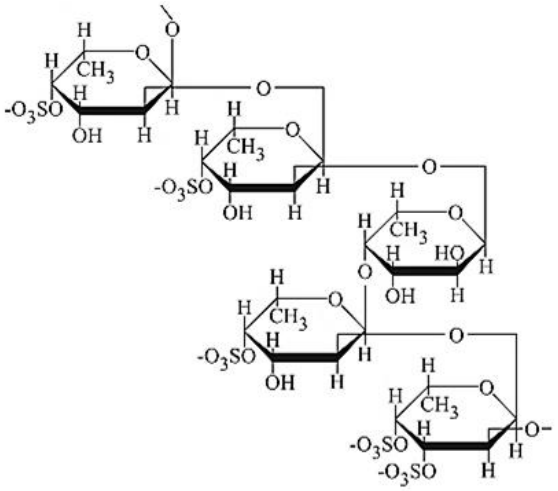

Figure 1.7. Molecular structure of fucoidan[59].

Alginic acid (or alginate) comprises a group of polysaccharides containing 1,4-linked β-D-mannuronic (i.e. M block) and α-L-guluronic acid (i.e. G block) residues (Figure 1.8). These residues are grouped at variable proportions in G-G blocks and M-M blocks (homopolymeric blocks) (Figure 1.9) and M-G blocks (heteropolymeric blocks)[62]. Mannuronic acid forms β(1→4) linkages, while guluronic acid forms α(1→4) linkages. These linkages tend to create a sterical hindrance around the carboxylic acid groups[63]. As a result, M blocks create linear domains, whereas G blocks are responsible for a more inflexible structure (folded regions)[64,65].

Alginate from brown seaweeds has the capacity to chelate metal ions, thereby generating viscous solutions, that are used in the food and pharmaceutical industries[11].

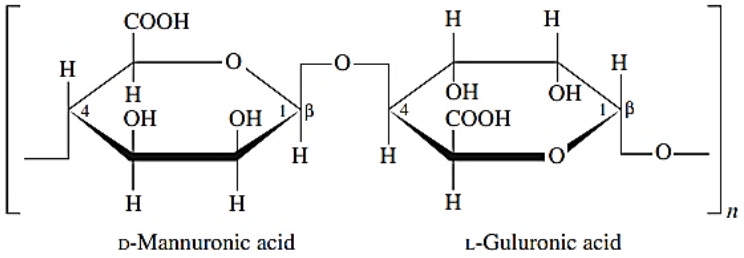

Figure 1.8. Monomeric compounds present in alginic acid[66].

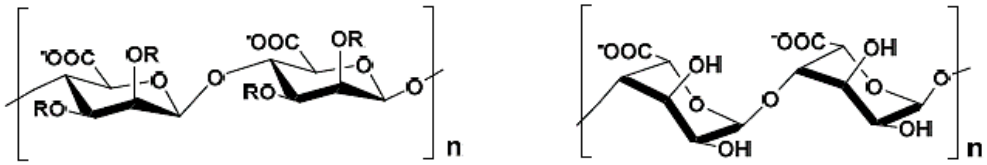

Figure 1.9. G-G blocks and M-M blocks (homopolymeric blocks) present in alginic acid structure. R=H[67].

Carrageenan (Figure 1.10) comprises sulfur-containing linear galactans, present in some species of marine red algae[21]. These compounds consist of dimers of an α(1→4)-linked D-galactopyranose residue (D) or 3,6-anhydro-D-galactopyranose residue (DA) and a β(1→3)-linked D-galactopyranose residue (G)[68].

They are used in the pharmaceutical and food industries, as viscosity-building, texturizing, and gel-forming ingredients[69].

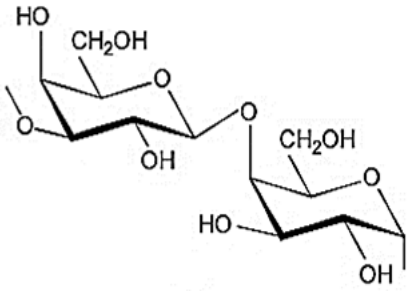

Figure 1.10. Chemical structure of carrageenan[70].

1.5. Dietary Fibre

According to the European Union regulation (EU) No 1169/2011, “fibre” can be defined as carbohydrates with >3 monomeric units, that remain undigested or unabsorbed at the small intestine. Dietary fibres can have beneficial physiological effects[71].

Non-digestible polysaccharides are called dietary fibre, which represents an extremely heterogeneous group of compounds. Dietary fibres have diverse formula components and structures as well as properties and bioactivities. All the compounds that belong to the dietary fibre group, except for lignin, are polysaccharides that belong to the carbohydrate group, such as

pectins, hemicelluloses, mucilages, cellulose and, in some cases, also and resistant starch and oligosaccharides[51].

Usual seaweed carbohydrates remain undigested after passing through the various digestive compartment and, therefore, belong to the group of dietary fibres[72].

Seaweeds are richer in fibre than most terrestrial vegetable foods. Seaweed fibre consumption is advisable, since it: (1) contributes to the development of the beneficial intestinal microflora[73-74]; (2) acts as an anti-hyperglycemic agent[75]; and (3) decreases the risk of colon cancer[76]. Edible seaweeds contain 33–62% dietary fibre (dw), being largely soluble[8,77].

1.6. Elemental Composition

Minerals can be defined as the constituents that remain as ash, after the combustion of plant and animal tissues.

Macroalgae are capable of accumulating essential minerals such as sodium (Na), magnesium (Mg), selenium (Se), copper (Cu), cobalt (Co), phosphorous (P), molybdenum (Mo), iodine (I), manganese (Mn), potassium (K), iron (Fe), calcium (Ca) and zinc (Zn)[78]. All these minerals, that are essential for human nutrition, are available in dietary seaweeds – they can offer minerals frequently absent from freshwater and plants/vegetables that grow in soils with a low mineral content.

As for human health maintenance, I as a role in growth and development, since it is involved in the production of thyroid hormones[79]. Fe and Mn are part of various metabolic processes[80]. Similarly, Cu is vital for skin strength, and for upkeep of blood vessels, epithelial and connective tissues, as it is a part of many enzymes. Besides that, it is involved in haemoglobin, myelin and melanin synthesis[81,82]. Br has important functions in the formation of collagen IV and in the activation of α-amylase in saliva. Zn is also vital for growth and development, besides regulating immune response[80,81].

As for plant and algal cells, Fe is important in plant metabolism as it is required in photosynthesis and chlorophyll synthesis[83]. Cu is required for photosynthetic electron transport, mitochondrial respiration and oxidative stress responses[84,85]. Zn is an enzyme cofactor and as an important role in proteins that control DNA expression[86].

The mineral uptake process of macroalgae is dependent upon the content and/or type of polysaccharides in the cell wall. Brown algae has a high capacity for mineral uptake[87]. This as to do with the fact that the polysaccharides in their cell wall are mostly composed of alginates and sulphated polysaccharides. Alginates gel if in presence of multivalent cations. The propensity of alginates to bind divalent cations - Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, Ca^{2+} - augments with the guluronic acid level (G content)[88]. The ‘‘zigzag’’ structure[89] – “egg-box” structure (Figure 1.11) – explains the enhanced affinity of polyguluronic acid residues toward divalent ions. This structure has the capacity to accommodate Ca^{2+} (and other cations) more easily.

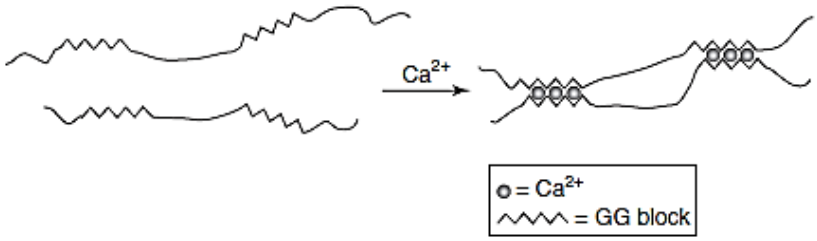

Figure 1.11. Schematic representation of the calcium-induced gelation of alginate in accordance with the "egg-box" structure[90].

It is considered that alginates create a well-organised solution network, in the presence of Ca^{2+}, or other cations, through the dimerization of the polyguluronic sequences (GG blocks). This polyguluronic sections allow for the connection of chains, on coordination sites, that form cavities, where the cations can be accommodated[91,92]. Since different algae species have in their structure alginates with different M and G contents, the physico-chemical properties of alginates can vary significantly. Alginates with a higher GG concentration have the capacity to form stronger gels. The specific affinity for divalent metal ions and the respective gelling ability have been claimed to augment as follows: MM block<MG block<GG block[59,63,90].

Likewise, agar and carrageenan, found in the cell walls of red algae, can establish a connection with cations through their hydroxyl and sulfate anionic groups and therefore accumulate them, though not so effective as alginates and other polysaccharides[93].

Due to macroalgae strong bioadsorptive abilities, elemental concentrations can reach values 10-100 times higher than those of terrestrial plants. There is a wider range in elemental content, not observed in edible terrestrial plants, that may be linked to variables such as seaweed phylum, locations, time of harvest, and environmental variability[94].

Regarding Na and K, most algae present higher concentrations when compared to vegetables. Despite that, they usually show low Na/K ratios – this low ratios are of great importance regarding heart and cardiovascular system, because low Na/K ratios are recognized as reductors of blood pressure[95]. However, very substantial concentrations of Na are still considered a major shortcoming regarding macroalgae as food, since Na intake is already high in many developed and developing countries[82,96]. Ca and P, alongside Mg, are also present in high concentrations in seaweed, surpassing values obtained for fruits and vegetables. P is found at the same levels in the major seaweed groupings, presenting contents that are in the 0.5-7 g/kg dw range[82]. Seaweeds, especially brown algae, can also accumulate high levels of I. The introduction of macroalgae in diet may be a major route to guarantee the recommended I intake every day. An upper limit for I intake of 600 µg/day in the case of adults and of 200 µg/day for children of 1-3 years has been set[97]. The seaweed consumption must follow the limit recommendations, since the consumption of more than the upper limit of 600 µg/day (adults) and 200 µg/day (small infants) may cause poisoning effects that have been related to the health complications such as thyroid cancer, hyperthyroidism or hypothyroidism[80].

In addition, macroalgae may also store hazardous elements in their tissues, namely, arsenic (As) and antimony (Sb), cadmium (Cd), mercury (Hg), lead (Pb), tin (Sn), aluminium (Al), and strontium (Sr).

In European countries, according to the European Commission Regulation (EC) No 396/2005, for Hg, a maximum residue level (MRL) for seaweed is set at 0.01 mg/kg[97]. The Regulation EC No 629/2008 of the European Commission, which was established for food supplements whose formulation is only or mainly constituted by dried macroalgae or macroalgae-derived products, set a maximum level of Cd allowed of less than 3 mg/kg dw[97]. This authority (EC No. 744/2012) has also set the maximum arsenic content in complementary feed and/or a feed meal at 40 and 10 mg/kg (presupposing 12% humidity), respectively, and the maximum Pb levels of 15 mg/kg (12% humidity) for phosphates and calcareous marine algae[98].

As and Sb constitute a major contamination problem in macroalgae. Even though antimony, when compared to arsenic, is less abundant in nature, their chemistry and toxicity are similar[99]. Arsenic can occur in four oxidation states - As(V), As(III), As(0), and As(-III) -, that are organic or inorganic. In seaweed, organic As is mainly found as arsenosugars, but also as methyl forms [monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and arsenobetaine (AsB)], whereas the inorganic As is mainly found as arsenate (As(V)) and arsenite (As(III))[100]. Organic As shows almost no toxicity, whereas inorganic As is hazardous. It is linked to various diseases, encompassing the cardiovascular system and cancer[100,101]. The toxicity of arsenic compounds is shown to be reliant on factors such as oxidation number, particle size physical state, and rate of absorption into cells[102]. Usually a high toxicity is associated with the low oxidation number while high methylation is associated with a low toxicity. The decreasing toxicity order can therefore be presented as: arsenite > arsenate > monomethylarsonic acid > dimethylarsinic acid[100]. Cd, Hg, and Pb are also metals known for their harmful effects upon human health. According to the World Health Organization (WHO), Cd exposure may disturb calcium metabolism and lead to cancer[103]. Hg exposure can disturb the neuronal function, leading to numerous diseases at the immune, gastrointestinal, and nervous level[104]. Pb exposure is not as harmful as Cd and Hg, despite binding easily to proteins and can consequently lead to enzymatic breakdown and also disturb neuronal function, mostly of small children[82].

1.7. Antioxidant Properties

An antioxidant is a compound that is able to give an electron to a highly reactive radical and eliminate it, therefore limiting its ability to cause cellular harm. These antioxidants are capable of interacting with free radicals, thus terminating their chain reactions (free radicals scavenging property) and protecting vital molecules. Some antioxidants result from the organism's metabolic activity, such as glutathione and ubiquinol, while other antioxidants, for instance, ascorbic acid and β-carotene, can be attained from the diet[105,106].

Antioxidant properties are determined by the capacity of a bioactive molecule to protect an organism from damage caused by free radicals. The fact that free radicals are able to operate as oxidants and reductants makes them very reactive and unstable[105]. Oxygen-based free radicals – Reactive Oxygen Species (ROS) – comprise the superoxide radical ($O_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($ROO^{\bullet}$), and nitric oxide radical ($NO^{\bullet}$)[107,108]. Consequently, too much ROS may trigger negative effects as their toxicity makes them capable of oxidizing biologically

relevant molecules (*i.e.*, lipids, proteins, enzymes, DNA and RNA), causing an extensive oxidative damage on cells and tissues[105,108]. This oxidative damage is connected with a variety of chronic diseases in humans, related to aging, carcinogenesis and atherosclerosis, including myocardial infarction, cancer, diabetes, as well as Alzheimer's and Parkinson's diseases[108,109].

It should be noted that seaweeds hardly show serious photodynamic damage during metabolism despite growing in harsh environments, under sunlight and high oxygen levels that may generate ROS, this proves that defence systems and substances are available to seaweeds – they can produce bioactive compounds to defend themselves against external factors[109-110].

Phenolic compounds impart the most antioxidant properties to plants, including seaweeds, as their antioxidant activity is mainly manifested through a sub-class of this phenolic compounds, denominated phlorotannins[111]. The capacity of these compounds to inactivate free radicals is justified by its structure-activity relationship (SAR). Hence, there are differences between the antioxidant properties of different phenolic molecules that are ascribable to their specific SAR[112]. This group of compounds is central in plant and algae defence as they are involved in systems that fight bacteria and stresses, for instance, ultraviolet (UV) radiation[3].

Phenolic acids have high antioxidant capacity and are easily digested and absorbed by the human organism. Caffeic acid (Figure 1.12 (a)) and ferulic acid (Figure 1.12 (b)) – phenolic acids usually found in the diet – circulate as derived molecules, namely in sulfate and glucuronate forms, after their absorption from the gastrointestinal tract[113].

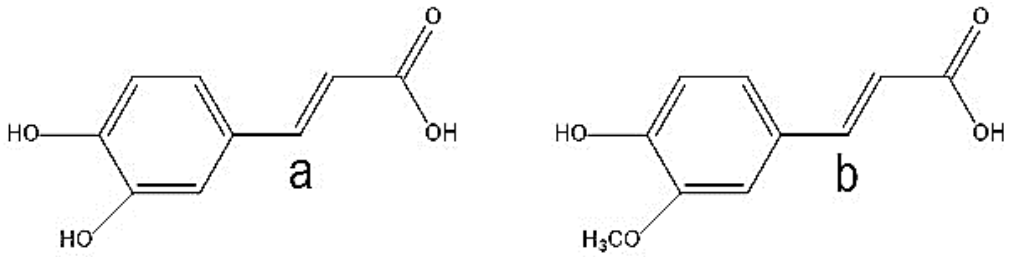

Figure 1.12. Molecules of **(a)** caffeic acid and **(b)** ferulic acid[114].

Phlorotannins exist mainly in seaweed, which usually have up to approximately 15% per algae dw[115]. Besides being produced by algae secondary metabolism, these molecules are structurally important for cell walls[116]. They result from the polymerization of phoroglucinol (Figure 1.13 (1)) (1,3,5-trihydroxybenzene) molecules and are synthetized by the polyketide pathway in seaweed[110]. These compounds are very hydrophilic and their molecular size is variable[117]. Brown seaweeds contain a varied range of phloroglucinol-derived phenolic substances. According to the bonds formed, phlorotannins may be termed: fucols (containing a phenyl bond); fucophloroethols (ether and phenyl bonds); fuhalols and phlorethols (ether bond); and eckols (dibenzodioxin bond). Compounds such as eckol (Figure 1.13 (2)), fucodiphloroethol G (Figure 1.13 (3)), phlorofucofuroeckol A (Figure 1.13 (4)), 7-phloroeckol (Figure 1.12 (5)), dieckol (Figure 1.13 (6)), and 6,6'-bieckol (Figure 1.13 (7)) belong to the group identified phlorotannins in brown seaweed[110,117]. Phlorotannins from brown algae present higher antioxidant activity when compared to other polyphenols derived from terrestrial plants[3].

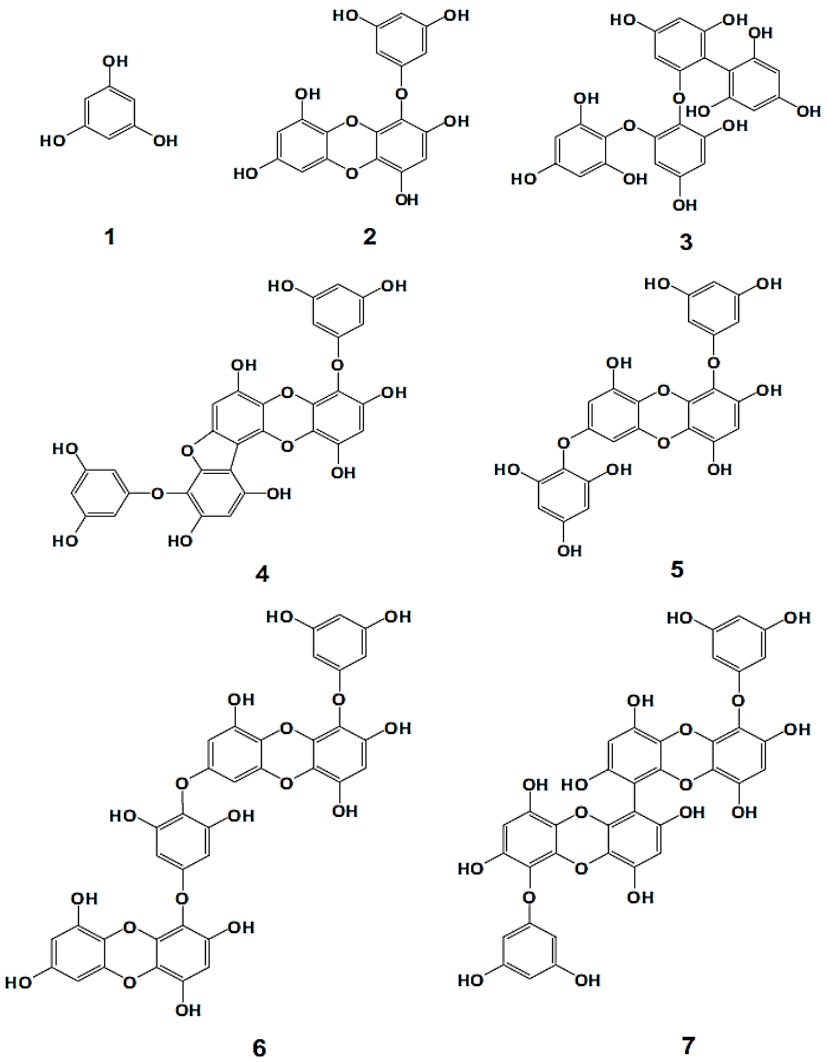

Figure 1.13. Structural formulas of representative phlorotannins from marine seaweeds. **(1)** phoroglucinol; **(2)** eckol; **(3)** fucodiphloroethol G; **(4)** phlorofucofuroeckol A; **(5)** 7-phloroeckol; **(6)** dieckol; **(7)** 6,6'-bieckol[110].

1.8. Bioaccessibility and Bioavailability

Bioaccessibility and bioavailability are concepts that correlate with food digestion and nutrient absorption[118]. After ingestion, food is exposed to different processes that can transform compounds then released into the systemic circulation[82]. When assessing if a certain compound is valuable for human health, its concentration is not enough, as its total amount does not mirror its bioaccessibility[119].

The term bioaccessibility refers to the appropriate release of nutrients and other components, including bioactives, from the food matrix, because of gastrointestinal tract conditions (oral cavity, gastric environment, and intestinal lumen)[118,120]. The bioaccessibility of a compound depends on the consumer physiological conditions (e.g., age and health) and the composition of food (matricial aspect)[120]. The compounds that are available in the human body may differ from the pre-digestion ones, as only the bioaccessible fraction (compounds freed after digestion) becomes accessible for function and/or accumulation. Therefore, the compounds that remain in the human body can be quite different from ingested amount[119].

Bioaccessibility can be determined with an *in vitro* digestion system which simulates the human digestive function. Despite being less exact than *in vivo* methodologies, this model is rapid, easy to use and inexpensive[120,121].

Bioavailability correlates with bioaccessibility in the way that for a molecule to be bioavailable, it as to be bioaccessible, - it was to be freed from the food matrix in the digestion process, along with absorption by the intestinal cells and transport to target tissue(s) [122]. Still, the fact that a molecule is bioaccessible does not guarantee its bioavailability, that is, its absorbance and transport in the systemic circulation[82].

The term bioavailability combines bioactivity and bioaccessibility, in which bioaccessibility relates to the food matrix release, digestion modifications, absorption trough intestinal wall and pre-systemic metabolism, while bioactivity involves transport, assimilation and metabolism to and by the target tissue, and processes related to biomolecular interactions[123,124]. Reported studies show that the food product, processing or preparation can change the oral bioaccessibility of compounds[125]. The bioaccessibility and bioavailability assessment of seaweeds is an important step to help define its nutritional values[123].

The determination of the bioaccessibility and bioavailability of macroalgae mineral content is of great importance, mainly due to the possible presence of toxic elements, but also because of the many advantages of nutritionally important minerals, trace elements and bioactive elements.

The bioaccessibility of elements depends on its chemical form[126], as well as particularities in the food matrix[121]. It represents the fraction of the element, which is freed from the food matrix and is considered as an index of maximal oral bioavailability[121]. The bioavailability, as above mentioned, represents the fraction of elements that is incorporated into systemic circulation and is distributed within the target sites[125]. The elements' bioavailability may be compromised due to their interaction with dietary fibres, that are not absorbable. They interact with negatively charged polysaccharides, namely alginates and carrageenan, forming non-soluble adducts, consequently reducing bioavailability[127].

1.9. *In vitro* digestive model as a tool to assess bioaccessibility

Digestion is a complex process that generates novel compounds that are absorbed by the human body. These nutrients are needed for energy, growth and cell repair[123].

Enzymes have a critical role in the breakdown of ingested nutrients. Main enzymes are α-amylase in the mouth, pepsin in the stomach, and trypsin, α-chymotrypsin, pancreatin (mixture of proteases, amylase, and lipase) and lipase, alongside bile (constituted by phospholipids, acids and salts, cholesterol, and electrolytic components that ensure a pH of 5-6), in the small intestine[123,128,129]. Various methodologies have been developed for this purpose - *in vivo* and *in vitro* techniques are the major groups of techniques for bioaccessibility determination[130].

In vivo digestion models can be performed using two approaches, either executing overall balance studies or quantifying the concentration of the focal substances[130,131]. Despite being the most accurate models, both strategies rely on human and animal tests with the goal of attaining

their bioaccessibility assessments, reason why ethical issues arise, as potentially harmful substances may be used in the model, besides being technically difficult and costly[129].

In vitro digestion models make it possible to closely simulate the biotransformation occurring in the gastrointestinal tract, using a laboratory controlled environment under reproducible and accurate conditions[132]. These models include numerous aspects, namely, enzyme levels, pH values, duration, and sodium chloride concentrations[129].

1.10. Anti-inflammatory Properties

Inflammation happens when damaging stimuli, such as tissue damage, pathogens, or destructive chemicals occur. It can be included as a part of a non-specific and defensive response[133]. The inflammation purpose is to protect the tissues against the damaging stimuli previous mentioned by removing the trigger of inflammation and stimulating the cell restoration mechanisms[134]. Inflammation may be acute or chronic[135,136]. In the inflammatory mechanism, various defence cells release high quantities of nitric oxide (NO), cytokines, such as interleukin (IL)-1β, IL-6, prostaglandin E_2 (PGE_2) and tumour necrosis factor (TNF)-α. These compounds cause some harm to the tissues, while activating macrophages in auto-immune diseases, etc[137-138]. PGE_2 is a vital inflammatory mediator that is generated through the catalysis of cyclooxygenase-2 (COX-2)[139]. Macrophages are pivotal during inflammation controlling various immunopathological effects, for instance, the enhanced formation of inflammatory mediators, namely, IL-6, IL-1IS, COX-2 and TNF-α, and cytokines[140].

The inflammatory response is vital when opposing infection. However, the effects caused by inflammation, especially in the case of the chronic phase can cause health issues, such as rheumatoid arthritis and atherosclerosis[141-142]. Consequently, anti-inflammatory compounds have an important role for the mitigation of inflammatory disorders. Consumption of food containing anti-inflammatory substances has a long tradition[143]. Anti-inflammatory drugs, as most commercialized drugs, can have side effects – as an example: Aspirin can cause stomach bleeding; COX-2 inhibitor Vioxx® and Celebrex® can cause heart problems; and non-steroidal anti-inflammatory drugs (NSAID's) were reported as a cause of many deaths yearly[144-145]. Hence, the discovery of new anti-inflammatory drugs from marine algae could bring developments to the sector of biomedical research and industry.

The fact that algae bioactive compounds have the capacity to counteract inflammatory harmful effects and, therefore, replace the synthetic drugs in use, makes them, nowadays, a major focal area of medicinal research.

1.11. Global overview of the potential of bioactivities and applications of the studied macroalgae

The macroalgae studied in this work - *Asparagopsis taxiformis*, *Asparagopsis armata*, *Cystoseira abies-marina* and *Cystoseira humilis* – have shown, since their first studies, a number of properties that are beneficial for human health and use/application. Nowadays, these algae are

used for their nutraceutical compounds that find various applications in the pharmaceutical, cosmetic, and food industries.

When referring to *Cystoseira abies-marina*, this macroalgae is used directly for food and as a fertilizer[1]. Both *C. abies-marina* and *C. humilis* extracts show antibacterial, antifungal, antimicrobial and antioxidant properties, given the existence of bioactive molecules, such as phlorotannins[44,50,146,147].

Asparagopsis armata extract shows powerful antioxidant and antibacterial activities. Their bioactives have anti-cancer properties, besides holding antifungal, antimicrobial and antiviral activities[1,50]. *Asparagopsis taxiformis* is characterized by their anticoagulant, anticyanobacterial, antifouling, antifungal and antimicrobial activities[1,148,149,150]. This macroalgae distinguishes itself from other algae for their anti-methanogenic activity – the brominated compounds (in particular, the compound bromoform), present in the genus *Asparagopsis* display an effective ability to inhibit archaebacterial metabolism. According to Roque *et al.* (2019)[151], methane production in the cattle rumen, and consequent emission, can be reduced by approximately 90% by incorporating 5% *A. taxiformis* in cattle feed. The inclusion of *A. taxiformis* in feed may help in solutions against climate change, as methane from cattle contributes significantly to the planetary methane emissions and this gas may be a more harmful greenhouse gas than CO_2[152].

The characteristics known and listed above make for the importance of studying and deepening the knowledge about these macroalgae.

2. Objectives

Despite seaweeds still being undervalued marine resources, these organisms have been receiving a growing attention over the years by the scientific community due to reported studies that confirm their nutraceutical and biotechnological potential. Many seaweed species, however, are still lacking in-depth studies to obtain information about their bioactive compounds, as factors such as geographical origin, seaweed phylum, and environmental, seasonal, and physiological variations can affect their composition and properties.

The objective of this work was to perform a bioprospection study on *Asparagopsis taxiformis*, *Asparagopsis armata*, *Cystoseira abies-marina* and *Cystoseira humilis*, four undervalued macroalgae, from the Azorean Archipelago. This involved their chemical characterization through the determination of their proximate composition. Besides that, an assessment of their biological activity (including bioaccessibility) was carried out, performing the determination of antioxidant and anti-inflammatory activities, but also involved analysis of total phenolic content and β-glucans (laminarin) levels. Lastly, the elemental composition was analysed, as well as the mineral bioaccessibility.

This study was also designed to evaluate the effect of the drying process (sun-drying *vs* shade-drying) on the biological activities and bioactive contents of aqueous and ethanolic extracts.

3. Materials and Methods

3.1. Sample collection and preparation

The studied species were the red seaweeds *Asparagopsis taxiformis* and *Asparagopsis armata*, and the brown seaweeds *Cystoseira abies-marina* and *Cystoseira humilis*. All seaweeds were dried, both in the sun and in the shade (Figure 3.1).

They were harvested from the Azores Archipelago (Portugal), located in the Atlantic Ocean, more specifically on the Faial island - *Asparagopsis taxiformis* was harvested from the wild in Pasteleiro (Faial), in which the sun- and shade-dried portions were collected on April 2019; *Asparagopsis armata* was collected in Castelo Branco (Faial), where the sun-dried portion was collected on May 2019 and the shade-dried portion was collected on June 2019; *Cystoseira abies-marina* and *Cystoseira humilis* were collected in Lajinha (Faial), where the shade-dried and sun-dried portions of both seaweeds where collected on June 2019.

Figure 3.1. Sun-dried and shade-dried seaweed biomass of the studied species: **(A)** Shade-dried *Cystoseira abies-marina*; **(B)** Sun-dried *Cystoseira abies-marina*; **(C)** Shade-dried *Asparagopsis armata*; **(D)** Sun-dried *Asparagopsis armata*; **(E)** Shade-dried *Cystoseira humilis*; **(F)** Sun-dried *Cystoseira humilis*; **(G)** Shade-dried *Asparagopsis taxiformis*; **(H)** Sun-dried *Asparagopsis taxiformis*. Diana Julião, October 2019.

The sun-drying method is performed in a greenhouse (Figure 3.2), where the macroalgae are arranged in a horizontal position. The greenhouse daytime temperature ranges from 28ºC to 35ºC and in the night the temperature ranges from 18ºC to 22ºC. Regarding the moisture content, it is of about 90% in the first drying day, while in the subsequent days, the content decreases to 50-60%. The shade-drying method is performed in a solar dryer (Figure 3.3) with two ventilators that promote a horizontal ventilation, using only the heat generated from solar panels. A resistance is connected if there is little sun or to speed up the drying process. The macroalgae are arranged in a horizontal position. Concerning the moisture content, the initial content is of about 90%, decreasing progressively until 15%. The solar dryer temperature with the solar panels and the connected resistance usually ranges from 35ºC to 40ºC. Regarding the drying time, sun-

drying had the duration of approximately of three days, while shade-drying had the duration of approximately two days.

Figure 3.2. Greenhouse used for the sun-drying method, where seaweeds are arranged in a horizontal position. SeaExpert, 2020.

Figure 3.3. Solar dryer used in the shade-drying method. SeaExpert 2020.

The seaweeds were ordered by the Portuguese Institute for the Sea and Atmosphere (IPMA) from the company SeaExpert based in Faial island, Azores, Portugal. Appropriate amounts of each seaweed were freeze-dried, homogenised, and stored at -80ºC until further analysis.

3.2. Proximate composition

3.2.1. Moisture and Ash content

The determination of the content of moisture and ash was based on AOAC methods[153]. Ash is a reduced inorganic residue obtained from the incineration of biological material – it is composed by the minerals obtained from the destruction of organic matter. The parameters moisture and ash indicate the amount of water and inorganic material in the samples studied.

After heating the crucibles for 30 minutes and cooling them on a desiccator for another 30 minutes, they were weighed, and the weight registered. The macroalgae were weighed from 2g to 5g, depending on their morphology and volume. Each macroalgae sample was divided in four crucibles, two duplicates for the macroalgae dried in the sun and two crucibles for the macroalgae dried in the shade. Samples were then left in the oven (Memmert, Model ULE 500, Schwabach, Germany) overnight and weighted after cooling in a desiccator. The moisture content of each sample was calculated according to the formula 3.1:

$$\%\, Moisture\ \left(\%\ \frac{m}{m}\right) = \frac{m1-m2}{m1-m3} \times 100\ (3.1)$$

Where:

m1 = Crucible mass with moist sample (g)

m2 = Crucible mass with dry sample (g)

m3 = Crucible mass (g)

The crucibles with the dried samples were placed in the muffle furnace (Heraeus, Model MR170-E, Hanau, Germany) at 550ºC overnight. The crucibles were then removed from the muffle and placed in the desiccator to cool for 40 minutes, weighed, and then placed again in the muffle furnace for 30 minutes at the same temperature. If the weight doesn't change or increases, less than 0.001g, the ash determination is completed (Figure 3.4). On the other hand, if the weight decreases, the crucibles with the samples return to the muffle furnace for an additional weighing. The ash content in dry matter basis of each sample was calculated according to the formula 3.2.

$$\%\, Ash\left(\%\ \frac{m}{m}\right) = \frac{m1-m2}{m3-m2} \times 100\ (3.2)$$

Where:

m1 = Crucible mass with ash (g)

m2 = Crucible mass (g)

m3 = Crucible mass with dry sample (g)

Figure 3.4. Crucibles with ash at the end of the procedure. Diana Julião, October 2019.

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3.2.2. Protein content

The protein level was quantified by the Dumas method[154], using a conversion factor of nitrogen into protein of 5.0 in the macroalgae samples[155]. This method is based on the combustion

of a sample of known mass in the presence of oxygen, inside a high temperature chamber (900ºC). The nitrogen percentage of the samples was calculated based on thermal conductivity.

The procedure consisted on weighting approximately 100mg of each seaweed in duplicate, and placing them in a LECO FP-528 analyser (LECO Corporation, St, Joseph, MI, USA), which performed the entire combustion process and calculated the nitrogen percentage and the corresponding protein percentage for each sample. Ethylenediaminetetraacetic acid (EDTA) was used to calibrate the standards.

3.2.3. Dietary Fibre content

The determination of total, soluble and insoluble dietary fibre was done with the application of an enzymatic procedure, using the K-TDFR-100A/K-TDFR-200A 04/17 assay kit by Megazime (Megazyme, Bray, Ireland).

Firstly, 1,000 ± 0,005g of duplicated samples of each macroalgae (sun-dried and shade-dried) were weighted (Figure 3.5), 40mL of MES-TRIS blend buffer (pH 8.2) was added to each sample, and the resulting mixtures were stirred.

Figure 3.5. Crucibles with 1,000 ± 0,005g of duplicated samples of each macroalgae (sun-dried and shade-dried). Diana Julião, October 2019.

Each solution was then subjected to a sequential heat-stable enzymatic digestion, with the application of the Megazyme kit. In the first place, each solution was incubated with 50µL of thermostable α-amylase in a water bath (Büchi Laboratoriums-Technik AG, Model 461, Flawil, Switzerland), at 98-100°C, for 30 minutes, with continuous agitation. The solutions were then removed from the hot water bath and cooled to 60ºC. Following, for second incubation, 100µL of protease was added and attained mixture was left in a water bath at 60 ± 1°C, for 30 minutes, with continuous agitation. The solutions were again removed from the hot water bath and 5mL of 0,561 N HCl was added to the mixture with constant agitation to obtain a final pH of 4.1-4.8, measured using a pH meter (Mettler Toledo, Model SevenCompact, Greifensee, Switzerland). Finally, in the third incubation, 200µL of amyloglucosidase was added to each solution, and those solutions were then left in a water bath at 60 ± 1°C, for 30 minutes, with continuous agitation.

3.2.3.1. Insoluble Dietary Fibre

The filtration setup was prepared by weighing Gooch fritted crucibles containing a thin layer of Celite®, and afterwards, wet and redistribute the bed of Celite® in the crucibles using distilled water. Suction was then applied to the crucibles to draw the Celite® onto the fritted glass.

The enzyme mixtures previous prepared were filtered through the crucibles into a kitasato. The residues that remained in the crucibles were washed twice with 10mL of distilled water pre-heated to 70ºC, and the filtrates were transferred to a pre-tared beaker and saved for soluble dietary fibre determination (3.2.3.2). The residues in the crucibles were then washed twice with 10mL 96%, v/v, ethanol, and acetone.

The crucibles containing the residues were dried overnight in an oven (Memmert, Model ULE 500, Schwabach, Germany) at 103ºC and cooled in a desiccator for approximately 1 hour. The crucibles containing Celite® and the dietary fibre residue were weighted.

For the determination of the protein content, approximately 70mg of sample from each crucible were weighed. The remaining content was weighed and used for the ash determination. The methodologies used for the determination of the protein and ash content were the same as used for the total macroalgae biomass determinations.

3.2.3.2. Soluble Dietary Fibre

The filtrates obtained in the insoluble dietary fibre procedure, that were transferred and saved in a pre-tared beaker, were weighted. Distilled water was added to adjust the weight of the combined solution of filtrate and water washings to 80g, and 4 volumes (320mL) of 96%, v/v, ethanol, pre-heated to 60°C, was added. The precipitates were allowed to form at room temperature for 1 hour (Figure 3.6 and Figure 3.7).

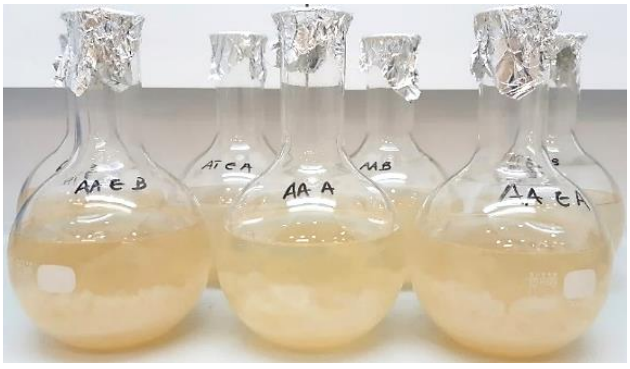

Figure 3.6. Precipitates formed after 1 hour at room temperature, for *Asparagopsis taxiformis* and *Asparagopsis armata*, sun-dried and shade-dried. Diana Julião, November 2019.

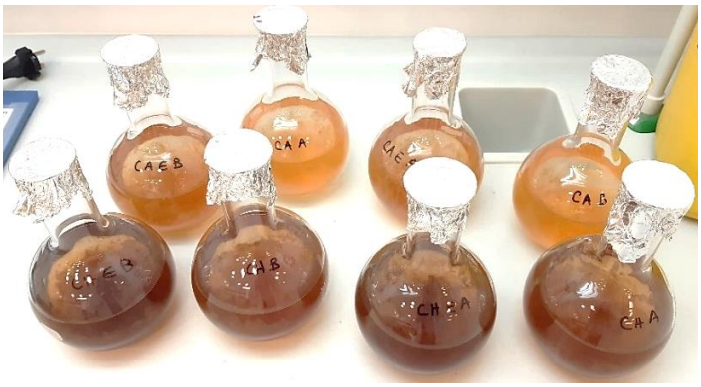

Figure 3.7. Precipitates formed after 1 hour at room temperature, for *Cystoseira abies-marina* and *Cystoseira humilis*, sun-dried and shade-dried. Diana Julião, November 2019.

The filtration setup was prepared by weighing Gooch fritted crucibles, containing a thin layer of Celite®, and afterwards, wet and reorganise the Celite® in the crucibles using 78%, v/v, ethanol. Suction was then applied to the crucibles to attract the Celite® onto the fritted glass.

The precipitated enzyme digest formed for 1 hour (room temperature) was filtered and the residues that remained in the crucibles were washed successively twice with 15mL of 78%, v/v, ethanol, 15mL of 96%, v/v, ethanol, and 15mL of acetone.

The crucibles containing the residues were dried overnight in an oven (Memmert, Model ULE 500, Schwabach, Germany) at 103ºC and cooled in a desiccator for approximately 1 hour. The crucibles containing Celite® and the dietary fibre residue were weighed.

3.2.3.3. Total Dietary Fibre

The soluble and insoluble dietary fibre contents were corrected by the subtraction of protein and ash contents in the residues and the values of the total dietary fibre were obtained from the sum of the values obtained for the insoluble dietary fibre and soluble dietary fibre[152].

3.3. Total Polyphenol content

3.3.1. Extract preparation

The phenolic compounds extraction from the seaweed biomass was executed with two selected solvents - 96% ethanol, v/v, and water Milli-Q – as they belong to the group of safe and environmentally friendly solvents and are the most effective among them. Other solvents included in this group are: 2-propanol, 1-butanol, ethyl acetate and 2-propyl acetate[156].

For the preparation of the extracts, 1.25g of dried seaweed biomass was weighted in duplicates to centrifuge tubes, and 25mL of water Milli-Q was added to one of the duplicates and 25mL of 96% ethanol, v/v, was added to the other. The solutions were homogenised in a Unidrive X1000 Homogenizer Drive (CAT Scientific Inc., California, USA), at a velocity of 30,000 rpm for 1 minute, and agitated overnight in an IKA-VIBRAX-VXR (IKA-Labortechnik, Staufen, Germany) orbital shaker, at a velocity of 200 rpm. After centrifugation at 4ºC, for 10 minutes, at a velocity of 5000x*g*, in a KUBOTA 6800 centrifuge (KUBOTA, Tokyo, Japan), the supernatant was collected with a Pasteur pipette and completed to a final volume of 25mL (Figure 3.8 and 3.9).

Figure 3.8. Supernatant collected from the aqueous extracts, to a final volume of 25mL. From left to right: Sun-dried *Asparagopsis taxiformis*; Shade-dried *Asparagopsis taxiformis*; Sun-dried *Asparagopsis armata*; Shade-dried *Asparagopsis armata*; Sun-dried *Cystoseira humilis*; Shade-dried *Cystoseira humilis;* Sun-dried *Cystoseira abies-marina*; Shade-dried *Cystoseira abies-marina*. Diana Julião, December 2019.

Figure 3.9. Supernatant collected from the ethanolic extracts, to a final volume of 25mL. From left to right: Sun-dried *Asparagopsis taxiformis*; Shade-dried *Asparagopsis taxiformis*; Sun-dried *Asparagopsis armata*; Shade-dried *Asparagopsis armata*; Sun-dried *Cystoseira humilis*; Shade-dried *Cystoseira humilis;* Sun-dried *Cystoseira abies-marina*; Shade-dried *Cystoseira abies-marina*. Diana Julião, December 2019.

3.3.2. Singleton and Rossi assay

The polyphenol content was determined with an adapted version of the Singleton and Rossi method. The Folin-Ciocalteu reagent was used for this method[157].

The Folin-Ciocalteu reagent has a yellow coloration when in its non-reduced form and is formed by a combination of phosphomolybdic acid ($H_3PMo_{12}O_{40}$) and phosphotungstic acid ($H_3PW_{12}O_{40}$) that forms chromogens by reacting with phenols and non-phenolic reducing compounds. The chromogens oxotungstate and oxomolybdate can be detected spectrophotometrically because in alkaline conditions they exhibit a blue colouring proportional to the concentration of polyphenols[158].

A volume of 100µL of each macroalgae extract (aqueous and ethanolic) was pipetted into a vial, in triplicate. To each vial was added 600µL of water Milli-Q and 150µL of twice-diluted Folin-Ciocalteu reagent, and the samples were left in the dark for 5 minutes at room temperature. Subsequently, 750µL of a 2% w/v sodium carbonate solution were added. After being left to react for 1 hour and 30 minutes in the dark at room temperature, absorbance of the samples at 750nm was measured in a Helios Alpha model (Unicam, Leeds, UK) UV-Vis spectrophotometer. The standard used was Gallic acid (GA) (Sigma, Steinheim, Germany) and the phenolic content was expressed as gallic acid equivalents (mg GAE/100g dw) by using a calibration curve of gallic acid (Annex III and IV).

3.4. Antioxidant Activity

3.4.1. DPPH method

The DPPH method was developed by Blois (1958)[159] to measure antioxidant activities, using a stable free radical α,α-diphenyl-β-picrylhydrazyl (DPPH•; $C_{18}H_{12}N_5O_6$, M=394.3 g/mol) (Figure 3.10). It is based on the measurement of antioxidant scavenging capacity against this stable free radical. The odd electron of the nitrogen atom in DPPH is reduced through the reception of a hydrogen atom from antioxidants by the hydrazine. DPPH is characterized as a stable free radical because of the delocalisation of the spare electron, which means that the molecules do not dimerise.

The delocalisation is also responsible for the deep violet colour, with an absorption in ethanol solution at approximately 520nm. Mixing DPPH with a solution that contains a substance that can donate a hydrogen atom, leads to the formation of the reduced form of DPPH – α,α-diphenyl-β-picrylhydrazine (DPPH; C18H13N5O6, M = 395.3 g/mol) - with a yellow coloration[160-161].

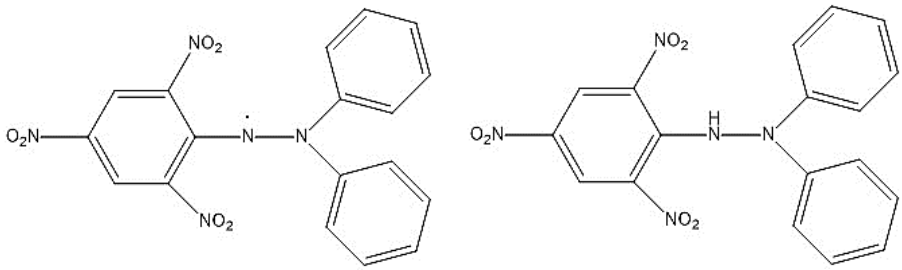

Figure 3.10. DPPH and its stable form. In the left, 2,2-diphenyl-1-picrylhydrazyl (free radical) and in the right, 2,2-diphenyl-1-picrylhydrazine (nonradical)[160].

To start the analysis, 1mL of each extract, aqueous and ethanolic, – as prepared in 3.3.1. - was pipetted in triplicate to a tube, and 2mL of DPPH (Sigma, Steinheim, Germany) 0.15mM methanolic solution was added and the sample was stirred in a vortex. After being left to react for 30 minutes in the dark at room temperature (Figure 3.11), absorbance of the samples at 517nm was measured in a Helios Alpha model (Unicam, Leeds, UK) UV-Vis spectrophotometer. For the blank, water and ethanol 96%, v/v, were used.

Radical scavenging activity was determined by the following formula 3.3[161]:

$$\%\ \text{Inhibition} = \frac{\text{A0} - \text{Asample}}{\text{A0}} \times 100 \quad (3.3)$$

Where:

A0 = Absorption of the blank

Asample = Absorption of the sample

Results were expressed in mg of ascorbic acid equivalents (AA Eq) per g of seaweed (dw) using a calibration curve of ascorbic acid (Annex V and VI).

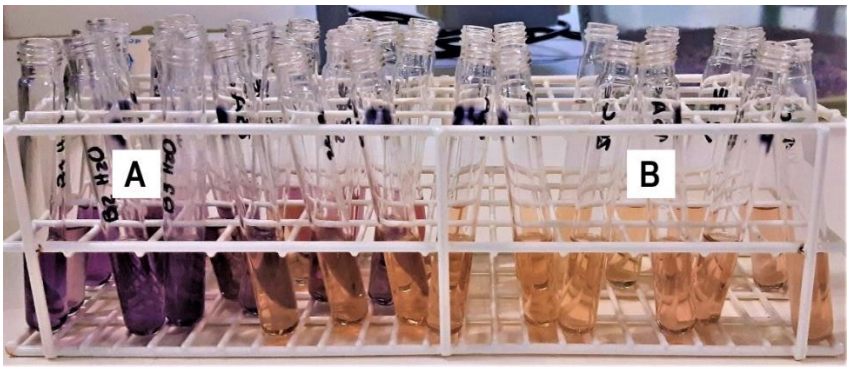

Figure 3.11. Colour variation during the DPPH reaction. DPPH has a characterized violet colour **(A)** but when mixed with an antioxidant substance (capable of donating a hydrogen atom) gradually becomes yellow **(B)**. Diana Julião, December 2019.

3.4.2. FRAP method

The Ferric Ion Reducing Antioxidant Power (FRAP, also Ferric Reducing Ability of Plasma) is a method developed by Benzie & Strain (1996)[162]. As described by the authors, this method is based on a redox-linked colorimetric reaction where the oxidizing species reacts with the antioxidant instead of the "substrate", that is, the antioxidant (reductant) reduces the oxidant, inactivating it. In this case, one reactive species is reduced at the expense of the oxidation of another.

This method is based on antioxidants reduction, at low pH, of the ferric-tripyridyltriazine (Fe^{III}-TPTZ) complex to the ferrous (Fe^{II}) form (Figure 3.12), creating a very intense navy-blue colour with an absorption maximum at 593nm, that confirms the presence of a reductant (antioxidant). The absorbance measured tests the amount of iron reduced and can be correlated with the reducing ability of the antioxidants present in the sample.

To perform this method, the solutions prepared were a 300 mM trihydrate sodium acetate buffer (pH 3.6), a 10 mM 2,4,6-Tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and a 20 mM $FeCl_3.6H_2O$ solution. The FRAP regent was then prepared by adding 300 mM trihydrate sodium acetate buffer (pH 3.6), 10 mM 2,4,6-Tripyridyl-s-triazine (TPTZ) solution, and 20 mM $FeCl_3.6H_2O$ solution in a 10:1:1 (v/v/v) proportion, respectively. Firstly, the FRAP reagent was prepared in a 230:23:23 proportion, and in the second time, with a 150:15:15 proportion.

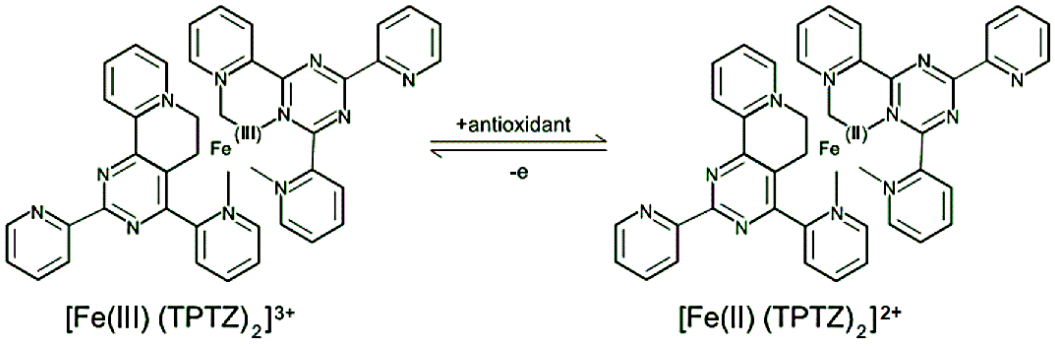

Figure 3.12. Reduction of the ferric-tripyridyltriazine (FeIII-TPTZ) complex to the ferrous (FeII) form by an antioxidant[163].

To start the analysis, 100µL of each extract, aqueous and ethanolic, – as prepared in 3.3.1. - was pipetted in triplicate to a tube, and 3mL of FRAP reagent was added. The mixture was vortexed and incubated for 30 minutes in the dark on a 37ºC bath (Büchi Laboratoriums-Technik AG, Model 461, Flawil, Switzerland). Absorbance of the coloured product (ferrous-tripyridyltriazine complex) (Figure 3.13) was then measured at 595nm in a Helios Alpha model (Unicam, Leeds, UK) UV-Vis spectrophotometer. For the blank, water and ethanol 96%, v/v, were used. Iron Sulphate ($FeSO_4.7H_2O$) was used as standard and the antioxidant content was expressed in µmol of iron(II) sulphate equivalents per g of seaweed (dw) (µmol iron sulphate/g dw) through the calibration curve of iron(II) sulphate (Annex VII).

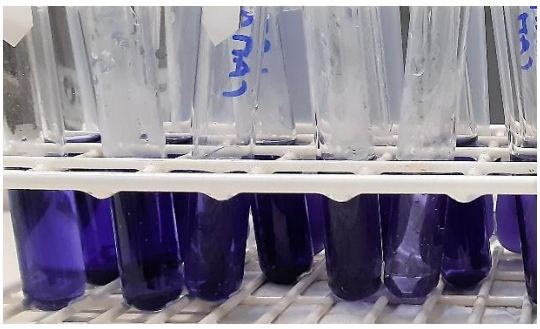

Figure 3.13. Colour variation in the FRAP reaction. The intense blue colour means that the Fe^{III}-TPTZ complex was reduced, indicating the presence of antioxidant activity. Diana Julião, January 2020.

3.4.3. ABTS method

The ABTS method is applied for the measurement of antioxidant activity and is based on the generation of the ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation. The method used was described by Re *et al.* (1999)[164], where the generation of $ABTS^{\bullet+}$ by oxidation of ABTS (Figure 3.14) with potassium persulfate, involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore, that as an absorption maximum at wavelengths 645nm, 734nm and 815nm.

Depending on the reaction time, antioxidant activity and antioxidant concentration, the pre-formed radical cation $ABTS^{\bullet+}$ is reduced by the presence of hydrogen-donating antioxidants to a certain extent. The extent of decolorization observed, that can be translated as the inhibition percentage of the $ABTS^{\bullet+}$ radical cation (Figure 3.15), is determined as a function of concentration *versus* time and calculated according to the Trolox reactivity (standard), under identical conditions[164,165].

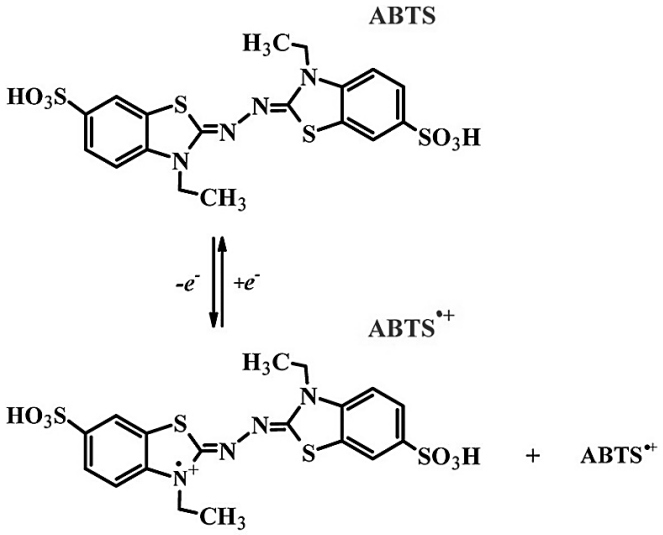

Figure 3.14. One-electron oxidation of ABTS and the formation of cation radicals ($ABTS^{\bullet+}$)[165].

To perform this method was prepared a 2.45 mM potassium persulfate solution, a 5 mM sodium phosphate buffer (pH 7.4) and a 7 mM $ABTS^+$ solution - a specific solution required for this procedure - that was put together by weighing 10mg of ABTS and dissolving it in 2,6mL of the 2.45 mM potassium persulfate solution, then incubated in the dark at room temperature overnight and finally diluting it with 5 mM sodium phosphate buffer (pH 7.4) to obtain a final absorbance value of 0.7 ± 0.02 at 734nm.

To start the analysis, 20µL of each extract, aqueous and ethanolic, – as prepared in 3.3.1. - was pipetted in triplicate to a tube, and 2mL of 7 mM $ABTS^+$ solution was added. The mixture was vortexed and incubated for 6 minutes in a 30ºC bath (Büchi Laboratoriums-Technik AG, Model 461, Flawil, Switzerland) in the dark. Absorbance of the coloured product was then measured at 734nm in a Helios Alpha model (Unicam, Leeds, UK) UV-Vis spectrophotometer. For the blank, water and ethanol 96% w/w were used. Trolox was used as standard and the antioxidant content was expressed in µmol of Trolox equivalents (Trolox Eq) per 100g of seaweed (dw) (µmol eq Trolox/100g dw) using a calibration curve of Trolox (Annex VIII).

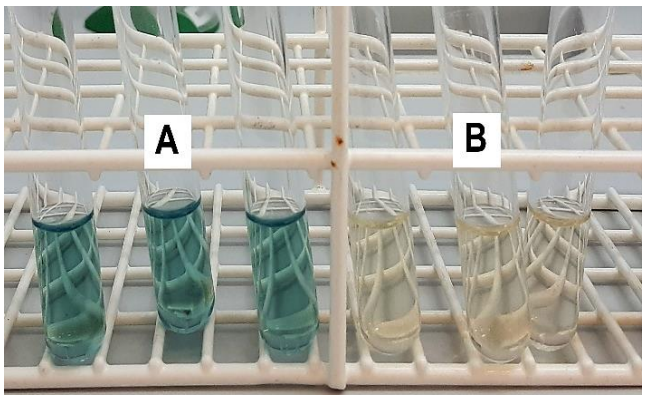

Figure 3.15. Colour variation in the ABTS reaction. The $ABTS^+$ solution exhibits a blue colour **(A)** and the loss of this colour indicates the presence of antioxidant agents **(B)**. Diana Julião, January 2019.

3.5. Beta-glucans (laminarin) content

The quantification of laminarin in seaweed samples was carried out through the measurement of glucose concentration released after enzymatic hydrolysis of laminarin. The method was achieved by using a mixed-linkage beta-glucan assay kit and procedure K-BGLU 08/18 supplied by Megazyme (Bray, Ireland).

To perform the procedure, 80-120mg of each sample was weighed in triplicate and mixed with 0.2mL of ethanol 50%, v/v, and 4mL of 20 mM sodium phosphate buffer (pH 6.5). The mixture was vortex and, on mixing, immediately incubated in a boiling water bath (Büchi Laboratoriums-Technik AG, Model 461, Flawil, Switzerland) for 60 seconds. The mixture was vortexed, incubated for 2 minutes at a 100ºC bath, vortexed again and incubated in a 50ºC bath for 5 minutes. A volume of 0.2mL of lichenase enzyme was added to each tube and the mixture was vortexed and incubated in a stirring water bath for 1 hour at 50ºC with regular vigorous stirring on a vortex. A volume of 0.1mL of 200 mM sodium acetate buffer (pH 4) was added and the mixture was vortexed. The tubes were allowed to equilibrate at room temperature for 5 minutes and centrifuged at 1000×*g* for 10 minutes on a KUBOTA 6800 centrifuge (KUBOTA, Tokyo, Japan). From each sample tube, 0.1mL aliquots were dispensed into the bottom of 3 test tubes, and 0.1mL of β-glucosidase enzyme in 50 mM sodium acetate buffer (pH 4) was added to two of the three test tubes. To the third test tube was added 0.1mL of 50 mM acetate buffer (pH 4) – this tube act as a blank as β-glucosidase is not present to catalyse the hydrolysis of the glycosidic blonds. All test tubes were incubated in a 50ºC water bath (Büchi Laboratoriums-Technik AG, Model 461, Flawil, Switzerland) for 10 minutes. A volume of 3mL of GOPOD (glucose oxidase/peroxidase) reagent was added, and the test tubes were again incubated in a 50ºC water bath for 20 minutes (Figure 3.16). Finally, the absorbance was measured at 510nm in a Helios Alpha model (Unicam, Leeds, UK) UV-Vis spectrophotometer. β-glucan from oats, supplied in the assay kit was used as a standard. Results were expressed in %, w/w, according to the formula 3.4 supplied by the kit:

$$\beta - \text{glucans}\left(\%\frac{\text{w}}{\text{w}}\right) = \Delta\text{Abs} \times \frac{\text{F}}{\text{W}} \times \text{FV} \times 0.9 \quad (3.4)$$

Where:

ΔAbs = Absorbance after β-glucosidase treatment (reaction) minus reaction blank absorbance;

F = Factor for the conversion of absorbance values to µg of glucose = $\frac{100\ (\mu\text{g of D}-\text{glucose})}{\text{absorbance of } 100\ \mu\text{g od D}-\text{glucose}}$.

FV = Final volume (i.e., 9.4mL);

W = Weight in mg of the analysed sample;

0.9 = Factor to convert from free D-glucose, as determined, to anhydro-D-glucose, as occurs in β-glucan.

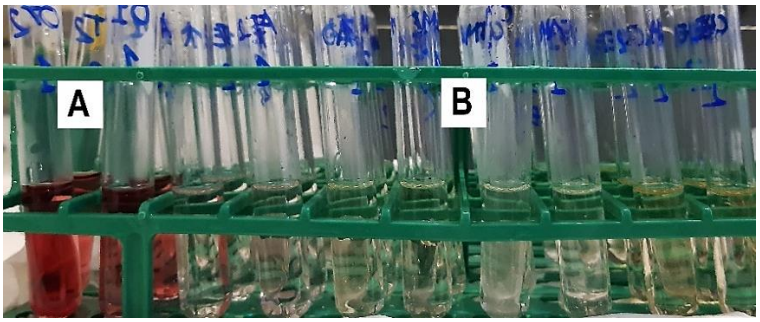

Figure 3.16. (A) Coloration of the D-glucose standard after GOPOD treatment - the pink colour in the mixture indicated the presence of D-glucose, and therefore the presence of β-glucans. **(B)** Coloration of the samples after GOPOD treatment. Diana Julião, January 2020.

3.6. *In vitro* Digestion Model

An *in vitro* digestion model that includes three steps, which simulate the digestive human processes in the mouth, stomach, and small intestine, was applied to determine the bioaccessible antioxidant and anti-inflammatory activity, and essential elements/mineral content. This model was developed by Versantvoort *et al.* (2004)[166] and consequently modified by Afonso *et al.* (2015)[124]. The composition of digestive juices – saliva, gastric, duodenal and bile – was prepared according to Afonso *et al.* (2015)[124].

The chemicals Na_2SO_4, KCl, NaCl, NaH_2PO_4, $CaCl_2$, $NaHCO_3$, HCl, KH_2PO_4 and $MgCl_2{\bullet}6H_2O$, utilized in the preparation of the digestive fluids, were purchased from Merck (Darmstadt, Germany), while NH_4Cl was purchased from Fluka (Buchs, Switzerland). The remaining reagents were purchased from Sigma (St. Louis, MO, USA). Trypsin, and α-chymotrypsin from Sigma (St. Louis, MO, USA) were also added to the duodenal juice. The quantities of these two enzymes were estimated according to the work reported by Gatellier and Santé-Lhoutellier (2009)[167].

Initially, it was weighed 0.5g of each macroalgae (sun-dried and shade-dried) in duplicate and added 4mL of the artificial saliva at a pH 6.8 ± 0.2 for 5 minutes. Afterwards, 8mL of artificial gastric juice (pH 1.3 ± 0.02 at 37 ± 2ºC) was added, and subsequently the pH was adjusted to a final pH of 2.0 ± 0.1 with a pH meter (Mettler Toledo, Model SevenCompact, Greifensee, Switzerland). Then, the samples were placed in a head-over-heels movement in a model Roto-Shake Genie (Scientific Industries Inc., Bohemia, NY, USA) (37 rpm at 37 ± 2ºC) for 2 hours to simulate digestion conditions. After cooling down, a mixture of 8mL of duodenal juice (pH 8.1 ± 0.2 at 37 ± 2ºC), 4mL of bile (pH 8.2 ± 0.2 at 37 ± 2ºC), and 1.3mL of HCO_3^- solution (1 M) was added simultaneously. The final pH of the mixture was set at pH 6.5 ± 0.5 and then agitated for a further 2 hours period in a head-over-heels movement (37 rpm at 37 ± 2ºC). The obtained solution was centrifuged in a Sigma 3K30 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at about 2750×*g* during 5 minutes in order to separate the non-digested from the bioaccessible fraction. The various bioactives and elemental contents were then analysed in the bioaccessible fraction.

To estimate the constituent (C) percentage in the bioaccessible fraction the following formula 3.5 was used:

$$\% \text{ C bioacessible} = \frac{\text{mC bioacessible}}{\text{mC initial}} \times 100 \quad (3.5)$$

Where:

mC = mass of the constituent in the initial or bioaccessible fraction of the macroalgae.

3.7. Anti-inflammatory activity

3.7.1. Extract preparation for *in vitro* anti-inflammatory activity

The anti-inflammatory activity of sun-dried and shade-dried *Asparagopsis taxiformis*, *Asparagopsis armata*, *Cystoseira humilis* and *Cystoseira abies-marina* was determined in aqueous extracts and in the bioaccessible fractions obtained from the *in vitro* digestion model described in 3.6.

Approximately 200mg of each seaweed was weighed and homogenized with 2mL of Milli-Q water, using a Unidrive X1000 Homogenizer Drive (CAT Scientific Inc., California, USA) at a velocity of 30,000 rpm for 1 minute. The macroalgae *Asparagopsis armata* sun-dried and shade-dried, *Asparagopsis taxiformis* shade-dried, *Cystoseira humilis* sun-dried and shade-dried and *Cystoseira abies-marina* shade-dried formed a gelatinised solution with 2mL of Milli-Q water, therefore, another 2mL was added and they were then subjected to another homogenisation at a velocity of 30,000 rpm for 1 minute. The mixtures were exposed to a heat treatment (80ºC for 1 hour) and centrifuged in a KUBOTA 6800 centrifuge (KUBOTA, Tokyo, Japan) for 10 minutes (4ºC, 3000x*g*). The supernatant was collected, and the solvent was evaporated using a nitrogen stream at room temperature using a model Reacti-Therm III nº18940 nitrogen evaporation unit (Pierce, East Lyme, CT, USA). Besides the supernatant, 5mL of each bioaccessible extract was also evaporated using a nitrogen evaporator. The residue was directly dissolved in 100% dimethyl sulfoxide (DMSO) to prepare a stock solution with a concentration of 10 mg/mL. To totally dissolve the residues, the solutions were homogenised using a Unidrive X1000 Homogenizer Drive (CAT Scientific Inc., Paso Robles, CA, USA) at a velocity of 30,000 rpm for 1 minute. From the stock solutions, final solutions of 1 mg/mL were prepared (Figure 3.17 and 3.18).

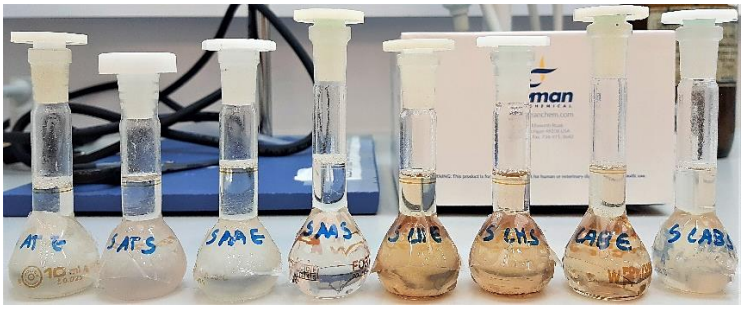

Figure 3.17. Final solutions of 1 mg/mL prepared from the supernatant collected from the aqueous extracts. From left to right: Shade-dried *Asparagopsis taxiformis*; Sun-dried *Asparagopsis taxiformis*; Shade-dried *Asparagopsis armata*; Sun-dried *Asparagopsis armata*; Shade-dried *Cystoseira humilis*; Sun-dried *Cystoseira humilis;* Shade-dried *Cystoseira abies-marina*; Sun-dried *Cystoseira abies-marina*. Diana Julião, June 2020.

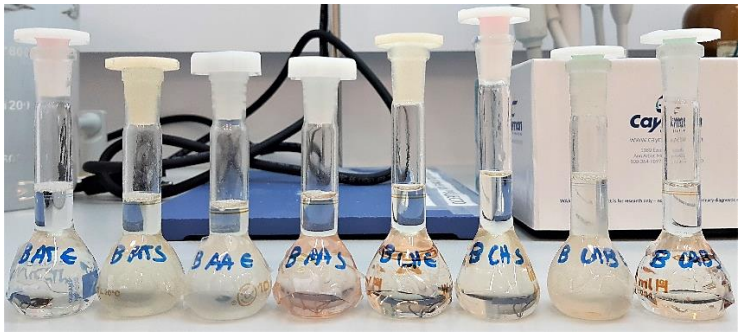

Figure 3.18. Final solutions of 1 mg/mL prepared from the bioaccessible extract. From left to right: Shade-dried *Asparagopsis taxiformis*; Sun-dried *Asparagopsis taxiformis*; Shade-dried *Asparagopsis armata*; Sun-dried *Asparagopsis armata*; Shade-dried *Cystoseira humilis*; Sun-dried *Cystoseira humilis;* Shade-dried *Cystoseira abies-marina*; Sun-dried *Cystoseira abies-marina*. Diana Julião, June 2020.

3.7.2. Cyclooxygenase (COX-2) inhibition method

Cyclooxygenase (COX, also called Prostaglandin H Synthase or PGHS) is a bifunctional enzyme that has COX and peroxidase activities. The COX part of the enzyme converts arachidonic acid to a hydroperoxyl endoperoxide (PGG_2), while peroxidase activity part reduces the endoperoxide to the corresponding alcohol (PGH_2), the precursor of prostaglandins (PGs), thromboxanes, and prostacyclins.

COX-2 is an isoform of COX, responsible for, under acute inflammatory conditions, the biosynthesis of PGs.

The COX inhibitor screening assay directly measures the amount of Prostaglandin F2α ($PGF_{2\alpha}$) generated from arachidonic acid (ARA, 20:4 ω6) in the cyclooxygenase reaction.

This assay is based in a competitive ELISA, where PG-acetylcholinesterase (AChE) conjugate (PG tracer) and PGs compete for a restricted quantity of PG antiserum. The amount of PG tracer that is capable of binding to the PG antiserum is inversely proportional to the concentration of PG, as the concentration of the PG tracer is constant while the concentration of PG varies. The rabbit antiserum-PG (either free or tracer) complex formed binds to a mouse monoclonal anti-rabbit antibody that is attached to the well. The addition of the Ellman's reagent – that consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid) – causes the enzymatic reaction between AChE and acetylthiocholine, that produces thiocholine, while the following reaction between thiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412nm (Figure 3.19).

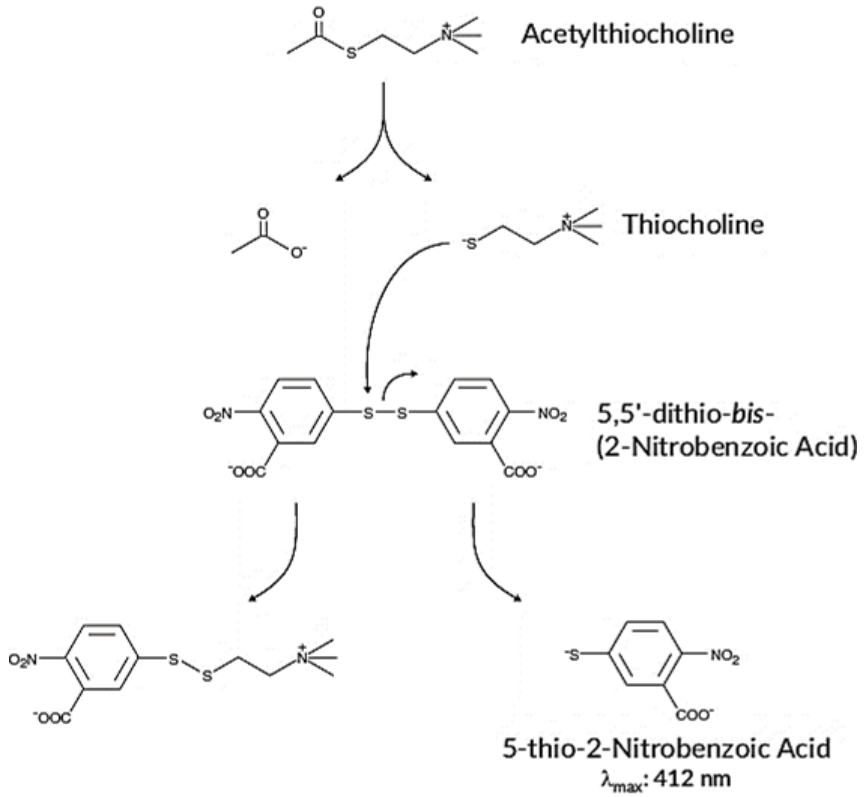

Figure 3.19. Reaction catalysed by acetylcholinesterase. Cayman test kit-560131 (Cayman Chemical Company, Ann Arbor, MI, USA).

The seaweed extracts were tested at 1 mg/mL using a commercial cyclooxygenase (COX) inhibitory screening assay kit, Cayman test kit-560131 (Cayman Chemical Company, Ann Arbor, MI, USA). A volume of 10µl of each extract or DMSO was used. The reaction tubes were then incubated for 10 minutes in a 37ºC bath (Büchi Laboratoriums-Technik AG, Model 461, Flawil, Switzerland). The reaction was initiated by addition of 10µl of arachidonic acid 10 mM and each reaction tube was incubated at 37ºC for 2 minutes. It was terminated by the addition of 30µl of saturated stannous chloride.

The microplate was completed by pipetting the wells correspondent to the blank (Ellman's reagent), non-specific binding (non-immunological binding of the tracer to the well), maximum binding (maximum amount of tracer that the antibody on the absence of the free analyte), total activity (total enzymatic activity of the AChE-linked tracer), prostaglandin screening standards, background COX-2, COX-2 100% initial activity and COX-2 inhibitor macroalgae samples. The prostanoid produced was quantified spectrophotometrically (415nm) in a Model 680 Microplate Reader (BIO-RAD Laboratories, Hercules, CA, USA) via enzyme immunoassay (ELISA) after an 18-hour incubation, washing, addition of Ellman's reagent, and further 90-minute incubation (Figure 3.20). Results were expressed as a percentage of inhibition of COX-2.

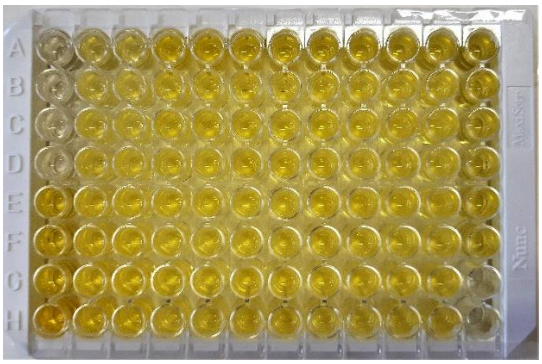

Figure 3.20. Anti-inflammatory activity test plate ready for prostaglandin reading. Diana Julião, June 2020.

3.8. Mineral Composition

The mineral composition of the initial and bioaccessible samples was analysed at the Faculty of Pharmacy of the University of Porto.

3.8.1. Microwave-assisted acid digestion

Samples mineralization was performed using an MLS 1200 Mega Milestone (Sorisole, Italy) microwave digestion system equipped with an HPR-1000/10 S rotor. Approximately 300mg of homogenized dried sample were directly weighed into a microwave oven PTFE vessel and 4mL of nitric acid (HNO_3 69% w/v, Trace*SELECT*®, Fluka, France) and 1mL of hydrogen peroxide (H_2O_2, 30–32% w/w, Primar™, for Trace Metal Analysis, Fisher Chemical, Loughborough, UK) were added. The vessels were closed, and a microwave oven program was performed as follows: 1 minute at 250W, 2 minutes at 0W, 5 minutes at 250W, 5 minutes at 400W and 5 minutes at 650W. After cooling, the sample solutions were diluted to 50mL with ultrapure water (>18.2 MΩ.cm at 25°C), obtained with an arium® pro system (Sartorius, Goettingen, Germany), in decontaminated plastic volumetric flasks. Samples blanks were prepared in the same way. Each sample was digested in triplicate.

For analytical quality control purposes, the same procedure of acid digestion was applied to the certified reference material (CRM) ERM-CD200 seaweed to check the accuracy of the procedure. The results obtained were in good agreement with the certified values (ranged from 96.0% to 107.8%).

3.8.2. Elemental analysis

Elemental analysis was carried out by flame atomic absorption spectroscopy (FAAS) using an AAnalyst 200 instrument (Perkin-Elmer, Uberlingen, Germany) and by inductively coupled plasma mass spectrometry (ICP-MS) using an iCAP™ Q instrument (Thermo Fisher Scientific, Bremen, Germany). The elements Na and K were determined by FAAS. The remaining elements were analyzed by ICP-MS using the following elemental isotopes (m/z ratios): ^{26}Mg, ^{31}P, ^{34}S, ^{43}Ca, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{65}Cu, ^{66}Zn, ^{75}As, ^{111}Cd and ^{208}Pb. The elemental isotopes ^{45}Sc, ^{89}Y, ^{141}Pr, ^{159}Tb and ^{175}Lu were used as internal standards (IS).

Calibration standards for FAAS analysis were prepared from QC Std 3 (100 mg/L) multi-element standard solution (SCP Science, Baie-d'Urfé, Quebec, Canada). Calibration standards for ICP-MS analysis were prepared from a 10 mg/L multi-element standard solution (Periodic table mix 1, Sigma-Aldrich, Buchs, Switzerland). The IS solution was prepared by diluting the multi-element standard solution Periodic table mix 3 (10 mg/L, Sigma-Aldrich, Buchs, Switzerland) to obtain a final concentration of 100 µg/L in 2% HNO_3. This solution was added to both samples and calibration standard solutions to obtain a final IS concentration of 10 µg/L.

3.8.3. Bromine and iodine analysis

Dried samples (approximately 100mg) were weighed directly in borosilicate glass tubes (16×125 mm) previously washed with 0.5% (v/v) TMAH and rinsed with ultrapure water. Bromine and iodine extraction were performed according to the European Standard EN 15111:2007[168].

Bromine and iodine were analyzed by ICP-MS[169] using an iCAP™ Q instrument (Thermo Fisher Scientific, Bremen, Germany) and the elemental isotopes ^{79}Br and ^{127}I were monitored. The ICP-MS instrument was equipped with a Meinhard™ TQ + quartz nebulizer (Golden, CO), a Peltier-cooled baffled cyclonic spray chamber, a standard quartz torch and standard twocone interface design (nickel sample and skimmer cones). High-purity argon (99.9997 %; Gasin, Portugal) was used as the nebulizer and plasma gas. The ICP-MS instrument operational parameters were as follow: RF power 1550 W; plasma gas flow (14 L/min); auxiliary gas flow (0.8 L/min); nebulizer flow rate (1.02 L/min). The internal standard (IS) used was the elemental isotope ^{125}Te and the standard solutions tellurium (TraceCERT®, 1000 mg/L, Sigma-Aldrich, St. Louis, MA, USA), iodide and bromide (both TraceCERT®, 1000 mg/L, Supelco, Bellefonte, PA) were used for the preparation of the internal standard and calibration standard solutions, respectively.

3.9. Statistical analysis

The statistical analysis was achieved using STATISTICA 7 (Stat-sof, Inc., USA, 2004) and the results were expressed as average ± standard deviation.

To test the normality and homogeneity of variance of data, the Kolmogorov-Smirnov's test, and Cochran's C-test (Cochran C., Hartley, Bartlett) were used, respectively. Data that validated these assumptions were analysed by factorial ANOVA distribution using the Tukey HSD to determine the difference in the constituent's contents between species, drying procedure, and extracts (ethanolic and aqueous). For all statistical tests the significance level (α) was 0.05.

4. Results

4.1. Proximate composition

The results obtained regarding the proximate crude composition of the seaweed species *A. armata*, *A. taxiformis*, *C. abies-marina*, and *C. humilis*, either sun-dried or shade-dried, are exhibited in Table 4.1.

Table 4.1. Proximate crude composition (g/100g dry weight for ash, protein, moisture before and after homogenization and lyophilization, and insoluble, soluble and total dietary fibre) of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in the shade.

Crude composition	Drying Procedure	***Asparagopsis armata***	***Asparagopsis taxiformis***	***Cystoseira abies-marina***	***Cystoseira humilis***
		(g/100g dw)	(g/100g dw)	(g/100g dw)	(g/100g dw)
Ash	Sun-dried	49.8 ± 1.0^{aA}	56.2 ± 1.0^{bA}	28.1 ± 2.5^{cA}	26.3 ± 1.2^{cA}
	Shade-dried	42.1 ± 0.3^{aB}	52.6 ± 1.3^{bA}	27.7 ± 1.9^{cA}	25.4 ± 1.5^{cA}
Protein	Sun-dried	10.7 ± 0.1^{aA}	13.1 ± 0.4^{bA}	3.5 ± 0.2^{cA}	$3.\ 7 \pm 0.1^{cA}$
	Shade-dried	12.1 ± 0.5^{aB}	11.1 ± 0.3^{aB}	3.8 ± 0.2^{bA}	3.8 ± 0.5^{bA}
Moisture (before homogenization and lyophilization)	Sun-dried	10.4 ± 0.1^{aA}	8.4 ± 1.0^{aA}	9.7 ± 0.4^{aA}	14.4 ± 0.6^{bA}
	Shade-dried	12.1 ± 0.7^{aA}	7.7 ± 0.4^{bA}	6.4 ± 0.5^{bB}	11.2 ± 0.0^{aB}
Moisture (after homogenization and lyophilization)	Sun-dried	3.3 ± 0.0^{aA}	5.3 ± 0.0^{bA}	6.2 ± 0.0^{cA}	4.5 ± 0.0^{dA}
	Shade-dried	6.2 ± 0.3^{aB}	4.4 ± 0.0^{bB}	5.0 ± 0.0^{cB}	4.8 ± 0.1^{bcA}
Insoluble Dietary Fibre	Sun-dried	23.8 ± 4.3^{aA}	20.4 ± 0.0^{aA}	53.6 ± 3.6^{bA}	47.2 ± 3.0^{bA}
	Shade-dried	18.7 ± 5.2^{aA}	18.7 ± 9.7^{aA}	47.6 ± 3.1^{bA}	43.7 ± 3.0^{bA}
Soluble Dietary Fibre	Sun-dried	10.3 ± 5.9^{aA}	13.1 ± 0.0^{aA}	6.1 ± 3.4^{aA}	5.5 ± 3.4^{aA}
	Shade-dried	13.2 ± 4.6^{aA}	8.0 ± 4.3^{aA}	4.8 ± 3.9^{aA}	5.2 ± 5.5^{aA}
Total Dietary Fibre	Sun-dried	34.1 ± 10.2^{aA}	33.5 ± 0.0^{aA}	59.7 ± 7.0^{aA}	52.7 ± 6.4^{aA}
	Shade-dried	31.9 ± 9.7^{aA}	26.7 ± 14.0^{aA}	52.4 ± 7.0^{aA}	49.0 ± 8.5^{aA}

Data: average ± standard deviation. Different lowercase letters in the same row represent statistical differences ($p<0.05$) between seaweed species. Different uppercase letters in the same column represent statistical differences ($p<0.05$) between drying procedures.

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According to the ash content, the *Asparagopsis* genus has an overall higher concentration, since the results obtained for this genus were 50-120% higher than the results acquired for the *Cystoseira* genus, despite the drying method. Sun-dried *A. taxiformis* presented

the highest ash concentration, of 56.2 ± 1.0 g/100g dw, with the shade-dried version being the second highest, with a concentration of 52.6 ± 1.3 g/100g dw. As to the drying procedure, no significant differences were detected, despite the slightly superior concentrations in the sun-dried process.

As to the protein content, one can continue to observe that the concentration values are, once more, higher in the *Asparagopsis* genus, despite the drying method, and the highest protein concentration is 13.1 ± 0.4 g/100g dw, observed in sun-dried *A. taxiformis.* No effect of the drying procedure was detected either generally or for each seaweed species.

The moisture content decreased in a general way from before to after homogenization and lyophilization, as expected, as lyophilization is a process based on the removal of water through the sublimation process. It can be observed that, before homogenization and lyophilization, both *A. taxiformis* and *C. abies-marina* have a lower moisture content, when compared with *C. humilis* and *A. armata,* as well as the lowest moisture decrease, when observing the moisture content after homogenization and lyophilization. Sun-dried *C. humilis* has the highest moisture decrease, of approximately 69%. The macroalgae sun-dried *A. armata* presented the lowest moisture value, after homogenization and lyophilization.

Concerning the insoluble dietary fibre content, the genus *Cystoseira* as an overall superior concentration of 98-187%, when compared with the *Asparagopsis* genus. When observing the drying procedure variable, it is noticeable that all the four sun-dried macroalgae have a slightly higher insoluble dietary fibre concentration, despite the difference not being significative. Contrarily, in the soluble dietary fibre content, the genus *Asparagopsis* has a concentration 30-175% higher. When comparing the results for insoluble and soluble dietary fibre, it can be observed that the four species, either sun-dried or shade-dried, have a higher concentration of insoluble dietary fibre. As to the total dietary fibre, the *Cystoseira* genus as a general higher concentration. No significant differences were detected as to the drying procedure or regarding the genus and species.

4.2. Total polyphenol content

The results obtained regarding the total phenolic content of the seaweed species *A. armata*, *A. taxiformis*, *C. abies-marina*, and *C. humilis*, either sun-dried or shade-dried is exhibited in Table 4.2.

Table 4.2. Total polyphenol content (in mg GAE/100g dw) measured in aqueous (Aq.) and ethanolic (Eth.) extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade.

Extract	Drying Procedure	***Asparagopsis armata***	***Asparagopsis taxiformis***	***Cystoseira abies-marina***	***Cystoseira humilis***
		(mg GAE/100g dw)	(mg GAE/100g dw)	(mg GAE/100g dw)	(mg GAE/100g dw)
Aqueous	Sun-dried	87 ± 8^{aA}	85 ± 4^{aA}	463 ± 13^{bA}	454 ± 74^{bA}
	Shade-dried	115 ± 16^{aA}	85 ± 7^{aA}	639 ± 113^{cA}	386 ± 123^{bA}
Ethanolic	Sun-dried	134 ± 22^{aA}	123 ± 15^{aA}	176 ± 46^{aA}	471 ± 21^{bA}
	Shade-dried	169 ± 32^{aA}	141 ± 19^{aA}	598 ± 116^{bB}	678 ± 51^{bB}

Data: average ± standard deviation. Different lowercase letters in the same row represent statistical differences ($p<0.05$) between seaweed species. For each extract, different uppercase letters in the same column represent statistical differences ($p<0.05$) between drying procedures.

Concerning the aqueous extracts, seaweeds from the *Cystoseira* genus showed higher polyphenol levels than those from the *Asparagopsis* genus despite the drying method. When observing the results from the shade-dried procedure, *C. abies-marina* showed the highest polyphenol level, exceeding 600mg GAE/100g dw, when shade-dried. Results achieved with *Asparagopsis* did not surpassed 115mg GAE/100g dw. No effect of the drying procedure was detected either generally or for each seaweed species.

As to the ethanolic extracts, phenolic contents were higher than determined in the aqueous extracts in the case of *Asparagopsis* seaweeds. Despite this remark, one can continue to observe that there is still a visible difference between the phenolic contents obtained for the two genera, with exception of sun-dried *C. abies-marina*. Shade-drying enabled higher total polyphenol levels in the *Cystoseira* genus, reaching 598-678mg GAE/100g dw.

4.3. Antioxidant activity

The antioxidant activity of the studied seaweed species (*A. armata*, *A.taxiformis*, *C. abies-marina*, and *C. humilis*) either sun-dried or shade-dried, measured by the DPPH, FRAP, and ABTS methodologies is presented in Tables 4.3, 4.4, and 4.5, respectively.

Concerning the DPPH method, the drying procedure produced differences in the antioxidant activity of the aqueous extracts, concerning *C. abies-marina and A. taxiformis* (lower values in the shade-dried seaweed). In the sun-dried samples from the aqueous extracts, both *Cystoseira* species exhibited a very similar value, with higher DPPH values than *A. armata*, being

A. taxiformis intermediate. Concerning the shade-dried samples, the *C. humilis* corresponding extracts had a higher antioxidant capacity than the others, followed by *A. armata*.

Regarding the ethanolic extracts, it is observed a similar tendency of *Cystoseira* species to display higher antioxidant DPPH values, when compared with the *Asparagopsis* species. The highest values were measured for the sun-dried *C. humilis* and shade-dried *C. abies-marina*, whose DPPH inhibition corresponded to 0.19 ± 0.00mg AA Eq/g dw and 0.20 ± 0.00mg AA Eq/g dw, respectively. On the contrary, the ethanolic extracts of *A. taxiformis* showed the lowest DPPH values.

Table 4.3. Antioxidant activity as measured by DPPH (mg Ascorbic Acid Equivalent/100g dw) method in aqueous (Aq.) and ethanolic (Eth.) extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade.

Extract	Drying Procedure	***Asparagopsis armata***	***Asparagopsis taxiformis***	***Cystoseira abies-marina***	***Cystoseira humilis***
		(mg AA Eq/100g dw)	(mg AA Eq/100g dw)	(mg AA Eq/100g dw)	(mg AA Eq/100g dw)
Aqueous	Sun-dried	0.09 ± 0.08^{aA}	0.21 ± 0.03^{abA}	0.33 ± 0.02^{bA}	0.32 ± 0.02^{bA}
	Shade-dried	0.16 ± 0.06^{abA}	0.06 ± 0.10^{aA}	0.11 ± 0.01^{aB}	0.28 ± 0.06^{bA}
Ethanolic	Sun-dried	0.07 ± 0.01^{bA}	<LOD	0.16 ± 0.00^{cA}	0.19 ± 0.00^{dA}
	Shade-dried	<LOD	<LOD	0.20 ± 0.00^{dB}	0.07 ± 0.01^{cB}

Data: average ± standard deviation. Different lowercase letters in the same row represent statistical differences ($p<0.05$) between seaweed species. For each extract, different uppercase letters in the same column represent statistical differences ($p<0.05$) between drying procedures. LOD stands for Limit Of Detection.

The contrast between the two seaweed genera is largely replicated by the FRAP method results of both types of extract. There is a stronger pre-eminence of *C. humilis* in the case of FRAP, since it yield the highest antioxidant activities in the aqueous extract of shade-dried samples (151.0 ± 18.4µmol Fe^{2+} Eq/g dw) and sun-dried samples (62.8 ± 4.4µmol Fe^{2+} Eq/g dw), as well as in the ethanolic extracts of sun- and shade-dried extracts, 75.3 ± 3.14µmol Fe^{2+} Eq/g dw and 248.9 ± 4.4µmol Fe^{2+} Eq/g dw, respectively. According to the results obtained, *C. abies-marina* extracts generated second best activities, as measured by the FRAP method. The values for the *Asparagopsis* seaweed species were typically lower, with the highest value being 8.1 ± 1.7µmol Fe^{2+} Eq/g dw for *A. armata* in an ethanolic extract, shade-dried. The drying procedure only affected *Cystoseira* samples, but in a very consistent and clear way. Sun drying more than halved FRAP antioxidant activity.

Table 4.4. Antioxidant activity as measured by FRAP (µmol Fe^{2+} eq./g dw) method in aqueous (Aq.) and ethanolic (Eth.) extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade.

Extract	Drying Procedure	***Asparagopsis armata***	***Asparagopsis taxiformis***	***Cystoseira abies-marina***	***Cystoseira humilis***
		(µmol Fe^{2+} Eq/g dw)	(µmol Fe^{2+} Eq/g dw)	(µmol Fe^{2+} Eq/g dw)	(µmol Fe^{2+} Eq/g dw)
Aqueous	Sun-dried	4.6 ± 0.4^{aA}	4.0 ± 0.2^{aA}	50.4 ± 2.8^{bA}	62.8 ± 4.4^{bA}
	Shade-dried	5.4 ± 0.3^{aA}	3.9 ± 0.5^{aA}	114.2 ± 10.6^{bB}	151.0 ± 18.4^{cB}
Ethanolic	Sun-dried	6.1 ± 4.3^{aA}	3.2 ± 3.8^{aA}	12.8 ± 0.2^{aA}	75.3 ± 3.1^{bA}
	Shade-dried	8.1 ± 1.7^{aA}	5.6 ± 0.7^{aA}	47.9 ± 7.8^{bB}	248.9 ± 4.4^{cB}

Data: average ± standard deviation. Different lowercase letters in the same row represent statistical differences ($p<0.05$) between seaweed species. For each extract, different uppercase letters in the same column represent statistical differences ($p<0.05$) between drying procedures.

In the case of the ethanolic extracts, antioxidant activity measured by the ABTS method show trends similar to the DPPH and FRAP ones. In the aqueous extracts, the situation is almost opposite.

Indeed, the results obtained for the *Cystoseira* species in the ethanolic extracts are higher, expressing a greater antioxidant activity (as measured by the ABTS method) than those of the *Asparagopsis* species, regardless of the type of drying procedure. The antioxidant activity of the *Asparagopsis* species varies from 6.9 ± 0.5µmol Trolox Eq/g dw to 14.1 ± 0.9µmol Trolox Eq/g dw, while *Cystoseira* species varies from 28.2 ± 4.3µmol Trolox Eq/g dw to 60.6 ± 2.9µmol Trolox Eq/g dw. Hence, there is a sharp divide between both genera.

The discrepancy between genera is also verified in the aqueous extracts from the ABTS method. However, while no antioxidant activity was determined in the two *Cystoseira* species, ABTS values in the aqueous extracts of *A. armata* and *A. taxiformis* exceeded the corresponding values in the ethanolic extracts. Furthermore, whenever drying affected ABTS levels, sun-dried samples' values were 20-50% lower than the results of the shade-dried seaweed.

Table 4.5. Antioxidant activity as measured by ABTS (µmol Trolox Equivalent/g dw) method in aqueous (Aq.) and ethanolic (Eth.) extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, *Cystoseira humilis*) either sun-dried or dried in shade.

Extract	Drying Procedure	***Asparagopsis armata***	***Asparagopsis taxiformis***	***Cystoseira abies-marina***	***Cystoseira humilis***
		(µmol Trolox Eq/g dw)	(µmol Trolox Eq/g dw)	(µmol Trolox Eq/g dw)	(µmol Trolox Eq/g dw)
Aqueous	Sun-dried	18.8 ± 0.3^{cA}	14.3 ± 0.2^{bA}	<LOD	<LOD
	Shade-dried	18.8 ± 1.3^{bA}	17.6 ± 2.3^{bB}	<LOD	<LOD
Ethanolic	Sun-dried	6.9 ± 0.5^{aA}	8.7 ± 2.9^{aA}	28.2 ± 4.3^{bA}	59.2 ± 0.7^{cA}
	Shade-dried	14.1 ± 0.9^{aB}	9.7 ± 0.5^{aA}	57.3 ± 0.7^{bB}	60.6 ± 2.9^{bA}

Data: average ± standard deviation. Different lowercase letters in the same row represent statistical differences ($p<0.05$) between seaweed species. For each extract, different uppercase letters in the same column represent statistical differences ($p<0.05$) between drying procedures. LOD stands for Limit Of Detection.

4.4. Beta-glucan (laminarin) content

Beta-glucan (or laminarin) is a water-soluble polysaccharide[55]. It was first found in *Laminaria* species and is considered to be the food reserve of all brown algae[11]. Their content can reach up to 32–35 %, w/dw[170].

The results obtained for *Cystoseira*, presented in Table 4.6, are justified by the fact that the genus *Cystoseira* does not belong to the group of brown seaweed genera that typically contain beta-glucan in their constitution. Therefore, the results are within expectations. On the contrary, the significant values in the two *Asparagopsis* species require further analysis. Floridean starch, a storage polysaccharide in red seaweeds, does not seem to be an interferent, since it is an α-1,4-glucosidic linked glucose homopolymer with α-1,6-branches[24], while beta-glucan is composed of β-(1→3)-linked glucose, containing randomly β-(1→6) intra-chain branching, with a ratio of approximately 3:1[170].

Table 4.6. Beta-glucan content (in %, dw) of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade.

Drying Procedure	***Asparagopsis armata***	***Asparagopsis taxiformis***	***Cystoseira abies-marina***	***Cystoseira humilis***
	[Beta-glucan] (%, dw)	[Beta-glucan] (%, dw)	[Beta-glucan] (%, dw)	[Beta-glucan] (%, dw)
Sun-dried	0.72 ± 0.05^{cA}	0.39 ± 0.03^{bA}	<LOD	<LOD
Shade-dried	0.25 ± 0.02^{bB}	0.32 ± 0.05^{cB}	<LOD	<LOD

Data: average ± standard deviation. Different lowercase letters in the same row represent statistical differences ($p<0.05$) between seaweed species. Different uppercase letters in the same column represent statistical differences ($p<0.05$) between drying procedures. LOD stands for Limit Of Detection.

4.5. Anti-inflammatory content

The chosen seaweed species (*A. armata*, *A. taxiformis*, *C. abies-marina*, and *C. humilis*) either sun-dried or shade-dried were also studied with respect to the anti-inflammatory activity of their aqueous extracts, as shown in Table 4.7.

The aqueous extracts of *A. armata,* sun-dried and shade-dried, and sun-dried *C. abies-marina* does not have inhibitory capacity of COX-2. Within sun-dried samples, *Cystoseira humilis* has a 26 ± 12% of inhibition of COX-2, with *Asparagopsis taxiformis* possessing approximately half of *C. humilis* inhibitory capacity. In the case of shade-dried samples, *C. humilis* had a higher anti-inflammatory activity — exceeding 30% of inhibition— than *A. armata*, being the other two species at an intermediate level. An effect of the drying procedure on this bioactivity was only observed in the case of *C. abies-marina*, suggesting a detrimental impact of sun-drying.

Table 4.7. Anti-inflammatory activity (% inhibition of COX-2) in aqueous extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade.

Extract	Drying Procedure	***Asparagopsis armata***	***Asparagopsis taxiformis***	***Cystoseira abies-marina***	***Cystoseira humilis***
		(% inhibition)	(% inhibition)	(% inhibition)	(% inhibition)
Aqueous	Sun-dried	<LOD	15 ± 5^{aA}	<LOD	26 ± 12^{aA}
	Shade-dried	<LOD	16 ± 9^{abA}	13 ± 2^{abB}	37 ± 14^{bA}

Data: average ± standard deviation. Different lowercase letters in the same row represent statistical differences ($p<0.05$) between seaweed species. Different uppercase letters in the same column represent statistical differences ($p<0.05$) between drying procedures. LOD stands for Limit Of Detection.

4.6. Elemental Composition

The chosen seaweed species (*A. armata*, *A. taxiformis*, *C. abies-marina*, and *C. humilis*) either sun-dried or shade-dried were also studied regarding their elemental composition of the aqueous extracts, as shown in Table 4.8.

Concerning essential elements, in the Asparagopsis genus, iodine was the most abundant microelement and sodium the most abundant macro element. In the Cystoseira genus, iron presented the highest concentration regarding microelements, while potassium was the most abundant macro element. Besides that, it is the most abundant mineral found in *Cystoseira humilis* and *Cystoseira abies-marina*. In *Asparagopsis taxiformis* and *Asparagopsis armata*, iodine was the most abundant mineral, with concentrations ranging from 4645.3 ± 119.6 mg/kg dw to 5735 ± 49.2 mg/kg dw. Potassium shows a higher concentration in the *Cystoseira* genus, 37-42% more when compared to the *Asparagopsis* genus. However, the *Asparagopsis* genus are shown to be significantly richer in sodium, manganese, zinc, and bromine. Copper concentrations were similar in the four studied species, being equally found in a low quantity. Regarding contaminant elements, it is noted the high amount of arsenic found in *Cystoseira abies-marina*

(332.2 ± 11.1 mg/kg dw in *C. abies-marina* sun-dried and 367.8 ± 14.0 mg/kg dw in *C. abies-marina* shade-dried. The lead and cadmium concentrations were similar in the four studied species, being equally found in a low quantity. In respect to the drying methods, there is no significant differences that suggest its effect on the results obtained.

Table 4.8. Mineral composition obtained for aqueous extracts of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in shade. Concentration of sodium (Na), magnesium (Mg), phosphorus (P), sulphur (S), potassium (K), calcium (Ca), chromium (Cr), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), arsenic (As), bromine (Br), cadmium (Cd), iodine (I) and lead (Pb) are displayed.

Minerals	Drying Procedure	***Asparagopsis armata***	***Asparagopsis taxiformis***	***Cystoseira abies-marina***	***Cystoseira humilis***
Na (g/kg dw)	Sun-dried	105.0 ± 4.1^{bA}	115.3 ± 3.0^{aA}	28.5 ± 0.6^{cA}	31.4 ± 0.2^{cA}
	Shade-dried	85.6 ± 0.7^{bB}	97.8 ± 0.7^{aB}	30.0 ± 0.7^{cA}	29.5 ± 0.1^{cA}
Mg (g/kg dw)	Sun-dried	9.8 ± 0.6^{bA}	9.2 ± 0.1^{bA}	7.0 ± 0.2^{aA}	12.6 ± 0.3^{cA}
	Shade-dried	9.5 ± 0.3^{bA}	9.2 ± 0.3^{bA}	7.2 ± 0.2^{aA}	11.7 ± 0.5^{cA}
P (g/kg dw)	Sun-dried	2.1 ± 0.1^{aA}	1.7 ± 0.1^{bA}	0.5 ± 0.0^{dA}	0.6 ± 0.0^{cA}
	Shade-dried	2.0 ± 0.0^{aA}	1.7 ± 0.1^{bA}	0.3 ± 0.1^{dA}	0.6 ± 0.0^{cA}
S (g/kg dw)	Sun-dried	36.0 ± 1.5^{aA}	25.3 ± 0.7^{bA}	14.3 ± 0.4^{cA}	16.1 ± 0.6^{cA}
	Shade-dried	31.9 ± 0.8^{aB}	25.9 ± 0.9^{bA}	14.8 ± 0.3^{cA}	15.2 ± 0.4^{cA}
K (g/kg dw)	Sun-dried	14.2 ± 0.4^{aA}	14.7 ± 0.2^{aA}	71.0 ± 1.5^{cA}	64.1 ± 1.0^{bA}
	Shade-dried	13.5 ± 0.4^{aA}	14.0 ± 0.1^{aA}	65.8 ± 1.0^{bB}	65.1 ± 1.5^{bA}
Ca (g/kg dw)	Sun-dried	14.6 ± 0.6^{aA}	14.9 ± 0.9^{aA}	15.2 ± 0.6^{aA}	18.1 ± 0.7^{bA}
	Shade-dried	22.3 ± 0.5^{aB}	17.7 ± 1.2^{bcB}	16.2 ± 0.4^{cA}	18.7 ± 0.8^{bA}
Cr (mg/kg dw)	Sun-dried	2.0 ± 0.3^{bA}	13.4 ± 0.6^{aA}	0.8 ± 0.0^{cA}	0.7 ± 0.0^{cA}
	Shade-dried	5.8 ± 0.4^{aB}	5.0 ± 0.2^{aB}	0.8 ± 0.0^{bA}	0.8 ± 0.0^{bA}
Mn (mg/kg dw)	Sun-dried	21.3 ± 1.5^{bA}	51.3 ± 4.8^{aA}	2.0 ± 0.1^{cA}	5.2 ± 0.6^{cA}
	Shade-dried	36.9 ± 1.5^{aB}	27.4 ± 2.1^{bB}	2.2 ± 0.1^{cA}	4.8 ± 0.3^{cA}
Fe (mg/kg dw)	Sun-dried	557.9 ± 54.1^{bA}	1705.8 ± 74.5^{aA}	168.8 ± 3.0^{dA}	294.8 ± 10.2^{cA}
	Shade-dried	1073.0 ± 81.1^{aB}	917.7 ± 15.2^{bB}	188.0 ± 2.5^{cA}	280.1 ± 7.0^{cA}
Cu (mg/kg dw)	Sun-dried	2.6 ± 0.1^{aA}	2.6 ± 0.1^{aA}	0.6 ± 0.0^{bA}	0.7 ± 0.1^{bA}
	Shade-dried	3.5 ± 0.1^{aB}	2.1 ± 0.1^{bB}	0.7 ± 0.7^{cA}	0.8 ± 0.0^{cA}
Zn (mg/kg dw)	Sun-dried	96.6 ± 2.8^{aA}	17.4 ± 0.3^{bA}	4.4 ± 0.1^{cA}	6.3 ± 0.3^{cA}
	Shade-dried	36.3 ± 0.9^{aB}	11.2 ± 0.4^{bB}	4.7 ± 0.1^{cA}	6.3 ± 0.2^{cA}
As (mg/kg dw)	Sun-dried	16.4 ± 0.8^{aA}	12.9 ± 0.1^{aA}	332.2 ± 11.1^{cA}	48.1 ± 1.2^{bA}
	Shade-dried	18.5 ± 0.8^{aB}	11.3 ± 0.1^{aA}	367.8 ± 14.0^{cB}	58.9 ± 1.2^{bB}
Br (g/kg dw)	Sun-dried	32.6 ± 0.8^{bA}	52.2 ± 0.7^{aA}	0.6 ± 0.0^{cA}	0.8 ± 0.0^{cA}
	Shade-dried	59.2 ± 0.7^{aB}	45.5 ± 0.4^{bB}	0.4 ± 0.0^{cA}	0.9 ± 0.0^{cA}
Cd (mg/kg dw)	Sun-dried	0.6 ± 0.0^{aA}	0.3 ± 0.0^{cA}	0.3 ± 0.0^{cA}	0.4 ± 0.0^{bA}
	Shade-dried	0.5 ± 0.0^{aB}	0.3 ± 0.0^{bB}	0.2 ± 0.0^{bB}	0.4 ± 0.0^{aA}
I (mg/kg dw)	Sun-dried	4645.3 ± 119.6^{bA}	5735.7 ± 49.2^{aA}	88.4 ± 1.6^{cA}	103. 5 ± 3.3^{cA}
	Shade-dried	5734.1 ± 54.4^{aB}	5122.4 ± 25.4^{bB}	47.0 ± 0.7^{cA}	99.6 ± 0.5^{cA}
Pb (mg/kg dw)	Sun-dried	0.7 ± 0.0^{bA}	1.3 ± 0.0^{aA}	<LOD	<LOD
	Shade-dried	1.8 ± 0.1^{aB}	1.7 ± 0.1^{aB}	<LOD	<LOD

Data: average ± standard deviation. Different lowercase letters in the same row represent statistical differences ($p<0.05$) between seaweed species. For each element and species, different uppercase letters in the same column represent statistical differences ($p<0.05$) between drying procedures. LOD stands for Limit Of Detection.

4.7. Bioaccessibility

4.7.1. Anti-inflammatory activity

The bioaccessibility of studied bioactives/bioactivities was determined. However, only anti-inflammatory activity was detected in the bioaccessible fractions of the seaweeds digested *in vitro*. Accordingly, anti-inflammatory activity bioaccessibility was calculated for the four seaweed species (*A. armata*, *A. taxiformis*, *C. abies-marina*, and *C. humilis*) either sun-dried or shade-dried (Figure 4.1). Both sun- and shade-dried *A. armata* as well as sun-dried *C. abies-marina* did not show any anti-inflammatory activity in their biomass (Table 4.7), thus this bioactivity was also not found in the respective bioaccessible fractions. Moreover, bioaccessibility factor was also zero for other samples (shade-dried *C. abies-marina* and both samples of *C. humilis*). Only *A. taxiformis* samples displayed anti-inflammatory activity in the bioaccessible fraction. This activity was similar in sun- and shade-dried samples and only slightly lower than the values prior to digestion, thus rendering bioaccessibility factors in the 90-100% interval.

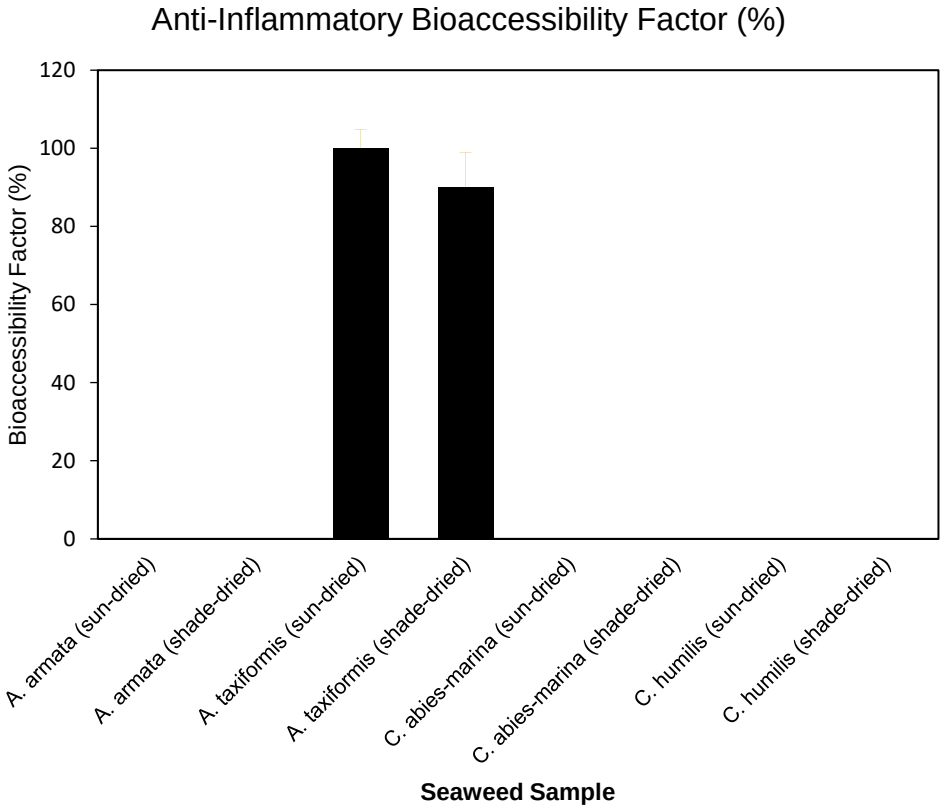

Figure 4.1. Bioaccessibility of the anti-inflammatory activity in the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina* and *Cystoseira humilis*) either sun-dried or dried in the shade.

4.7.2. Elemental Composition

The chosen seaweed species (*A. armata*, *A. taxiformis*, *C. abies-marina*, and *C. humilis*) either sun-dried or shade-dried were also studied with respect to bioaccessible elemental composition of their aqueous extracts, as shown in Table 4.9.

Concerning essential elements, bromine was the element found to have the highest bioaccessibility percentage on all four studied species, sun-dried and shade-dried, ranging from 84.0 ± 2.8% in sun-dried *C. abies-marina* to 101.5 ± 0.7% in C*.* shade-dried *abies-marina*. Besides that, high percentages were also obtained for magnesium and iodine. The *Asparagopsis* genus presented high values concerning calcium (28.5 ± 4.9-40.0 ± 9.9%). In contrast, the *Cystoseira* genus presented low results for calcium. Zinc and iron also presented low bioaccessibility percentages for the four seaweed species. The value obtained for zinc bioaccessibility with respect to sun-dried *A. armata* – 38.5 ± 2.1% – is in disagreement with the remaining values obtained for this element – 0.0 ± 0.0%. The values attained for copper were significant in the *Asparagopsis* genus (37.5 ± 7.8-40.0 ± 5.7%), but low in the *Cystoseira* genus. Regarding contaminant elements, arsenic displays high results in all four seaweed species, ranging from 55.0 ± 2.8% in shade-dried *C. humilis* to 86.5 ± 0.7% in shade-dried *A. taxiformis*. Cadmium also shows high percentages in the four seaweeds, from 33.5 ± 0.7% in shade-dried *C. humilis* to 67.5 ± 2.1% in sun-dried *A. taxiformis*. Lead presented low concentrations in the four seaweeds studied. With respect to the drying methods, there are few significant differences that suggest its influence in the results obtained.

Table 4.9. Elemental bioaccessibility obtained for aqueous extracts after *in vitro* digestion. Of the studied seaweed species (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Cystoseira abies-marina*, and *Cystoseira humilis*) either sun-dried or dried in the shade.

Minerals	Drying Procedure	***Asparagopsis armata***	***Asparagopsis taxiformis***	***Cystoseira abies-marina***	***Cystoseira humilis***
		(%)	(%)	(%)	(%)
Mg	Sun-dried	63.5 ± 3.5^{aA}	67.5 ± 7.8^{aA}	58.5 ± 0.7^{aA}	63.5 ± 0.7^{aA}
	Shade-dried	62.5 ± 2.1^{abA}	70.5 ±0.7^{aA}	55.5 ± 0.7^{bA}	63.5 ± 0.7^{abA}
S	Sun-dried	33.0 ± 1.4^{aA}	48.0 ±8.5^{aA}	34.0 ± 4.2^{aA}	40.0 ± 1.4^{aA}
	Shade-dried	45.0 ± 0.0^{aA}	44.0 ±2.8^{aA}	26.5 ± 2.1^{bA}	35.5 ± 3.5^{abA}
Ca	Sun-dried	28.5 ± 4.9^{abA}	40.0 ±9.9^{aA}	1.0 ± 1.4^{cA}	13.0 ± 0.0^{bcA}
	Shade-dried	30.0 ± 5.7^{abA}	38.0 ±1.4^{aA}	<LOD	13.5 ± 2.1^{bcA}
Cr	Sun-dried	7.0 ± 4.2^{abA}	2.0 ±1.4^{abA}	4.0 ± 5.7^{bA}	6.0 ± 2.8^{bA}
	Shade-dried	21.0 ± 0.0^{aB}	4.5 ±0.7^{bA}	<LOD	1.0 ± 1.4^{bA}
Fe	Sun-dried	4.5 ± 0.7^{abA}	2.0 ±0.0^{bA}	3.5 ± 2.1^{abA}	6.0 ± 0.0^{aA}
	Shade-dried	3.5 ± 0.7^{abA}	4.0 ±0.0^{abA}	1.0 ± 0.0^{aA}	7.0 ± 1.4^{bA}
Cu	Sun-dried	40.0 ± 5.7^{aA}	37.5 ±7.8^{aA}	<LOD	<LOD
	Shade-dried	38.0 ± 5.7^{aA}	38.5 ±12.0^{aA}	<LOD	<LOD
Zn	Sun-dried	38.5 ± 2.1^{aA}	<LOD	<LOD	<LOD
	Shade-dried	<LOD	<LOD	<LOD	<LOD
As	Sun-dried	76.5 ± 3.5^{aA}	82.5 ±10.6^{aA}	66.0 ± 8.5^{aA}	65.5 ± 2.1^{aA}
	Shade-dried	77.5 ± 2.1^{aA}	86.5 ±0.7^{aA}	67.5 ± 0.7^{abA}	55.0 ± 2.8^{bA}
Br	Sun-dried	89.0 ± 2.8^{aA}	94.0 ±5.7^{aA}	84.0 ± 2.8^{aA}	90.0 ± 1.4^{aA}
	Shade-dried	100.0 ± 0.0^{aB}	98.5 ±2.1^{aA}	101.5 ± 0.7^{aB}	91.0 ± 1.4^{aA}
Cd	Sun-dried	44.5 ± 2.1^{aA}	67.5 ±2.1^{bA}	50.0 ± 4.2^{aA}	43.5 ± 2.1^{aA}
	Shade-dried	46.5 ± 2.1^{bA}	65.5 ±2.1^{aA}	42.5 ± 0.7^{bcA}	33.5 ± 0.7^{cB}
I	Sun-dried	73.0 ± 2.8^{aA}	74.5 ±3.5^{aA}	35.5 ± 0.7^{cA}	49.0 ± 0.0^{bA}
	Shade-dried	76.0 ± 4.2^{aA}	64.5 ±0.7^{bB}	57.0 ± 0.0^{bB}	40.5 ± 2.1^{cA}
Pb	Sun-dried	<LOD	5.5 ±0.7^{bA}	<LOD	<LOD
	Shade-dried	3.5 ± 0.7^{bB}	8.5 ±0.7^{aA}	<LOD	<LOD

Data: average ± standard deviation. Different lowercase letters in the same row represent statistical differences ($p<0.05$) between seaweed species. For each element and species, different uppercase letters in the same column represent statistical differences ($p<0.05$) between drying procedures. LOD stands for Limit Of Detection.

5. Discussion

5.1. Proximate Composition

According to Kumar *et al.* (2011)[171], the ash content in macroalgae can reach up to 55%, and the values obtained in the present work are in concordance with this observation. A study with *Asparagopsis taxiformis* collected in the Hawaiian islands report an ash content value of 36.0%[172]. Another study presents for *A. taxiformis* an ash value of 44.4% when collected in the Azores islands[152]. The values presented in literature are lower than the values obtained in this study (56.2 ± 1.0 g/100g dw for sun-dried *A. taxiformis* and 52.6 ± 1.3 g/100g dw for shade-dried *A. taxiformis*). Literature for *Asparagopsis armata* is very scarce, however, the values obtained for this macroalgae (49.8 ± 1.0 g/100g dw for sun-dried *A. armata* and 42.1 ± 0.3 g/100g dw for shade-dried *A. armata*) are in agreement with the literature presented for *A. taxiformis*. As for the *Cystoseira* genus, studies report an ash value of 23.9 g/100g dw for *C. tamariscifolia* and 20.4 g/100g dw for *C. humilis,* both collected in Portugal[173], while Fonseca et al. (2020)[174] reported a value of 25.2 ± 0.4 g/100g dw for sun-dried *C. abies-marina*. The results obtained in the present study for *C. humilis* and *C. abies-marina* agree with the literature presented. Other macroalgae from the *order* Fucales also present similar ash content - *Fucus vesiculosus* and *Sargassum obtusifolium*, with reported values of 30.10 ± 0.20 g/100g dw[94] and 28.9 ± 0.4 g/100g dw, respectively[172].

The protein content, one of the major biochemical components of macroalgae, is variable, and the highest contents are generally found in green (Chlorophyta) and red (Rhodophyta) seaweeds (10-30% dw) in comparison to brown (Phaeophyceae) seaweeds (5-15% dw)[175].

Previous studies with *Asparagopsis taxiformis* show slightly higher protein levels - 18.7 ± 0.1 g/100g dw, from macroalgae also collected in the Azores islands[152] and 17.55 ± 0.11 g/100g dw from macroalgae collected in the Madeira Archipelago[176] – when compared with the results obtained in this study (13.1 ± 0.4 g/100g dw for sun-dried *A. taxiformis* and 11.1 ± 0.3 g/100g dw for shade-dried *A. taxiformis*). A study with *Asparagopsis armata* reports a protein content of 18.3 g/100g dw[151], a value also slightly higher than the results obtained (10.7 ± 0.1 g/100g dw for sun-dried *A. armata* and 12.1 ± 0.5 g/100g dw for shade-dried *A. armata*). Vizetto-Duarte *et al.* (2016)[173] presented a value of 10.34 g/100g dw, for *Cystoseira humilis* protein content and Fonseca *et al* (2020)[174] reported a protein content of 7.4 ± 0.2 g/100g dw for sun-dried *Cystoseira abies-marina*. Both values are higher when compared with the values obtained for *C. humilis* and *C. abies-marina*, in the present work (Table 4.1). However, Catarino *et al.* (2018)[79] reported for the brown algae *Fucus vesiculosus* a range of 1-11 g/100g dw for protein content, meaning that, the values obtained were lower than expected but, nevertheless, similar values have been reported for brown algae.

The moisture content of fresh marine algae is very high and can account for up to 94% of the biomass[8]. Fresh *Cystoseira humilis* has a moisture content that reaches up to 57.06% ww[173] and *Asparagopsis taxiformis,* 90.4% ww[172]. When the macroalgae goes through a drying process, its moisture content decreases significantly. Literature presents a value of 4.50 ± 0.20 g/100g dw

for *Asparagopsis taxiformis* moisture content, after lyophilization[176]. This value agrees with the results obtained (5.3 ± 0.0 g/100g dw for sun-dried *A. taxiformis* and 4.4 ± 0.0 g/100g dw for shade-dried *A. taxiformis*). The values attained for *Asparagopsis armata* (3.3 ± 0.0 g/100g dw for sun-dried *A. armata* and 6.2 ± 0.3 g/100g dw for shade-dried *A. armata*) also agree with the value present in literature for *A. taxiformis*. The higher values obtained for *A. armata* and *A. taxiformis* before lyophilization and homogenization, when compared with the results from after lyophilization and homogenization, are expected, as lyophilization is a process in which water is frozen, followed by its removal from the sample, initially by sublimation (primary drying) and then by desorption (secondary drying)[177]. Fonseca *et al.* (2020)[174] presented a value for sun-dried *C. abies-marina* moisture content of 6.2 ± 0.0 g/100g dw and *Sargassum muticum*, from the *order* Fucales, was reported to have a moisture content of 9.6 ± 0.1 g/100g dw[175], values similar to those obtained for *Cystoseira humilis* and *Cystoseira abies-marina*, before lyophilization and homogenization (6.4-11.4 g/100g dw).

The content of total dietary fibre in macroalgae may range from 33–50 g/100g dw[72]. Literature for *Asparagopsis taxiformis* shows a value for: insoluble dietary fibre of 15.7 ± 0.7% dw; soluble dietary fibre of 4.6 ± 0.1% dw; and total dietary fibre of 20.1 ± 0.9% dw[152]. Results obtained for *A. taxiformis* and *A. armata* are similar (Table 4.1), but slightly higher when compared with the values reported in literature. One can remove the possibility that the results are dissimilar due to variations in the determination method since the determination of dietary fibre in both cases was accomplished using the same enzymatic procedure (based on the kit from Megazyme). *Fucus vesiculosus*, from the *order* Fucales, is presented in literature with an insoluble dietary fibre content of 40.3 ± 1.0% dw, a soluble dietary fibre content of 9.8 ± 0.8% dw and a total dietary fibre content of 50.1 ± 1.8% dw[72]. Moreover, Fonseca *et al*. (2020)[174] reported for sun-dried *C. abies-marina* a total dietary fibre of 46.2 g/100g dw, a soluble dietary fibre of 1.8 g/100g dw and an insoluble dietary fibre of 44.4 g/100g dw. These values are similar to the values obtained for both *Cystoseira humilis* and *Cystoseira abies-marina* (Table 4.1), as no significant differences with respect to the drying method or in between *Cystoseira* species were observed.

5.2. Total polyphenol content

The phenolic content in macroalgae is not consistent since factors like habitat, maturity and time of the year are known to affect their bioactive compounds. This is shown by Chkhikvishvili & Ramazanov (2000)[178], that reported a range of 30-3,000mg GAE/100 g dw for phenolic contents in seaweed.

Values reported by Nunes et al. (2018)[179] for *Asparagopsis taxiformis* phenolic content – 470-620mg GAE/100g dw, when in aqueous extracts, and 1430-1710mg GAE/100g dw, in ethanolic extracts – are higher when compared with the results obtained in this study. However, the concentration of phenolic content attained for *A. taxiformis* in the ethanolic extracts is higher when compared with the aqueous extracts, as in the results presented in the current study. Moreover, results reported by Regal *et al.* (2020)[152] are in the range of values obtained in this study, presenting a phenolic concentration of 119 ± 8mg GAE/100g dw for aqueous extracts and

a concentration of 49 ± 1mg GAE/100g dw for ethanolic extracts, despite the results for the aqueous extracts being superior in this case.

In the case of *Asparagopsis armata*, literature presents results, using methanol and dichloromethane as solvents, with a phenolic content of 207 ± 0.6 mg GAE/100g dw and 1421 ± 1.4 mg GAE/100g dw, respectively[180]. The discrepancy observed supports the importance of the solvent choice.

Brown seaweeds are sometimes reported to have low phenolic content in the range of 25-218 mg GAE/100g dw, determined in aqueous extracts and using phosphate buffer (pH 7.0), as disclosed by Demirel *et al.* (2012)[181]. In the case of the *Cystoseira* genus, Fariman *et al.* (2016)[182] reported for *C. indica* a range of 80-130g GAE/100g dw for aqueous extracts. Despite this results, Pinteus *et al.* (2017)[180] show that the *Cystoseira* genus may present very high phenolic contents (>1,000mg GAE/100 g dw in aqueous extracts), even surpassing the current study's results.

Furthermore, while for the red seaweed *Kappaphycus alvarezii v*alues were quite low, not exceeding 60mg GAE/100g dw[183], the comparison of *K. alvarezii* dried by different methods revealed higher phenolic content in shade-dried samples than in sun-dried ones. Such effect was only observed in the ethanolic extracts of the Azorean *Cystoseira* seaweeds.

The divergence of the phenolic content within the same species is presented in literature, through the comparison of different values obtained for the phenolic content of macroalgae. This variability may be related to UV radiation level variability (depending on geographic location and season), since higher UV exposure may lead to higher phenolic contents[184].

Concerning the drying process, it is stated that an intense and/or prolonged exposure to high temperatures may be responsible for a significant loss of natural antioxidants in raw materials from plants, as most of the compounds are relatively unstable[185]. The decrease in phenolic content, after the drying process, can be ascribed to the binding of polyphenols with other compounds (e.g. proteins) or to alterations in their chemical structure. These findings are in concordance with results reported by other researchers, who suggested that polyphenols are thermolabile and irreversible chemical changes can occur in their content due to extended heat treatments[186]. The sun-drying process may have further reduced the level of ethanol soluble phenolic substances in *Cystoseira* seaweeds. Sun-drying is strongly dependent on the weather and the length of the day. The phytochemical content might suffer a stronger reduction because of the long drying time (3 to 4 days under direct sunlight). Its slower drying rate also likely increased the leaching effect and prolonged exposure time to air. In literature, it is claimed that ascorbate, tocopherols and carotenoids are affected by dehydration, whether by sun or artificial heat sources, as a result of exposure to UV light, heat, and air[187]. Nevertheless, Rajauria *et al.* (2010)[188] and Norra *et al.* (2017)[189] reported a substantial increase in the phenolic content and antioxidant activity when exposed to higher temperatures. The difference caused by the drying and its particular process, translated into the different values present in the literature related to the phenolic content, either leading to lower or unchanged levels, as in the current study, or to higher levels. As a result, further research is needed.

5.3. Antioxidant Activity

The combination of three different methods for the assessment of the antioxidant activity allows a broader knowledge of the antioxidant capacity of each macroalgae studied.

Concerning the DPPH method, there was a not inconsiderable antioxidant activity in the macroalgal extracts. Regal *et al.* (2020)[152] disclosed for the aqueous extracts of *Asparagopsis taxiformis* values in the range of 14-46mg AA Eq/100g dw. The results obtained in this study for sun-dried *A. taxiformis* and shade-dried *A. armata* are in agreement with the presented results. Both types of extract of *Cystoseira abies-marina* are also in agreement with literature[174]. Regarding *Cystoseira humilis* and *Asparagopsis armata*, there are no published results and, therefore, comparison is not possible. Literature concerning the FRAP method is more scarce, and if available, the few results are in agreement with the values obtained in the present study, as observed for *C. abies-marina*[174]. Lastly, taking into account the ABTS method, Campos *et al.* (2019)[190] reported results analogous to the ones obtained in the present work. These authors reported higher ABTS values in the aqueous extracts than in the ethanolic extracts. Likewise, Regal *et al.* (2020)[152] presented, for *A. taxiformis*, an aqueous extract with higher antioxidant activity (8.4 ± 1.4µmol Trolox Eq/ g dw), as measured by ABTS, when compared with the ethanolic extract (2.6 ± 0.4µmol Trolox Eq/ g dw). These values were lower for *A. taxiformis*, in comparison with the current study. In relation to *C. abies-marina*, the results presented by Fonseca *et al.* (2020)[174] for aqueous extracts – 43 ± 1µmol Trolox Eq/g dw – show a starker divergence when compared with the current ABTS results. These disagree with the phenolic content, DPPH, and FRAP results, which point to a strong antioxidant activity of the aqueous extracts of *C. abies-marina*. Hence, further research is required.

In the present study, the drying method did not affect most results in these three methods, having a scarce influence. Despite, in most cases, the sun-drying method affected negatively the antioxidant activity (as it is measured by ABTS and FRAP), in the case of the DPPH method, there was an increase in the antioxidant activity when the macroalgae were exposed to sun-drying, with respect to shade-dried samples. As previously mentioned in 5.2., Norra *et al.* (2017)[189] and Rajauria *et al.* (2010)[188] reported an enhancement of the antioxidant activity when the sun drying method was applied, contradicting the proposition that sun-drying lowers the antioxidant potential. According to Ling *et al.* (2015)[183], DPPH also yielded an higher antioxidant activity for *K. alvarezii* when sun-dried, thus opposing the variation measured by FRAP and ABTS. These two last methods largely agree with the phenolic content, just as in the current study[183]. Therefore, it is possible that sun-drying may induce a steep reduction of the levels of some bioactive components in the seaweed biomass that affect antioxidant potential assessed by FRAP and ABTS, but may trigger the formation of other compounds that may enhance antioxidant activity measured by the DPPH methods. The results presented in the current study, on Azorean macroalgae, show substantial agreement in the antioxidant activity determined by different methods, especially if the aqueous extracts measured by ABTS are not considered in the comparison.

While FRAP only reflects antioxidants acting through single electron transfer, ABTS enables the evaluation of antioxidant activity by single electron transfer (direct reduction of $ABTS^{+\bullet}$) or radical quenching by hydrogen atom transfer[191]. In addition, ABTS has been deemed more sensitive than DPPH, being divergent results between these two methods a common finding[192]. Taking into account that the used methods – DDPH, FRAP and ABTS –, are not equivalent and reflect different antioxidant properties, the observed convergence in their results is significant. Furthermore, there was some degree of convergence of the antioxidant activity with the total phenolic content. Namely, higher values for the two *Cystoseira* species. Since phenolic compounds act as antioxidants through single electron transfer, FRAP may correlate with the concentration of phenolic substances in the seaweed biomass, which seems to be empirically supported by the current study's results. Likewise, Alcalde *et al.* (2019)[193] have observed a correlation between phenolic content and FRAP (R=0.92), thus suggesting that the two parameters provide similar information. For other antioxidant assays, poorer correlations were obtained, meaning that there were divergences. The differences in the sensitivities to compounds in the assays seem to underlie these observations[193].

5.4 Beta-glucan (laminarin) content

Beta-glucans are storage polysaccharides present in brown seaweed (e.g., *Laminaria* or *Saccharina* spp.) and their content can represent up to 32–35%, w/dw. They are linear polysaccharides composed of β-(1→3)-linked glucose, containing randomly β-(1→6) intra-chain branching, with a ratio around 3:1[131,170]. The *Cystoseira* results obtained in this study are expected as this brown seaweed genus typically does not contain beta-glucan compounds. However, the significant values in the two *Asparagopsis* species require further study. Floridean starch, a storage polysaccharide in red seaweeds, does not seem to be an interferent, since it is an α-1,4-glucosidic linked glucose homopolymer with α-1,6-branches[24].

5.5. Anti-inflammatory activity

Studies that focus on the anti-inflammatory activity of macroalgae are relatively scarce and the methodologies used vary, including *in vitro* and *in vivo* assessments[190,194-195]. The comparison of results, obtained from different studies, is, thus, difficult. Despite the complications, there are studies reporting anti-inflammatory activity in brown seaweeds [196,197].

For instance, Yang *et al.* (2010)[197] reported anti-inflammatory activity in ethyl acetate extracts of *Petalonia binghamiae.* This macroalgae inhibited lipopolysaccharide-induced nitric oxide (NO) and prostaglandin E2 (PGE_2) production, and reduced lipopolysaccharide-induced expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the protein level in a concentration-dependent manner. In another case, Kang *et al.* (2012) suggested that sargaquinoic acid, a compound isolated from *Sargassum siliquastrum*, a brown algae from the Sargassacea *family*, specifically prevents nitric oxide (NO) production in lipopolysaccharide-stimulated macrophages, via modulation of different signalling pathways.

Moreover, Oh *et al.* (2016)[198], reported that ethanolic extracts of *Sargassum serratifolium*, at low concentrations, efficiently inhibited the secretion of proinflammatory cytokines and inflammatory mediators. This inhibitory effect on the expression of inflammatory mediators suggested its potential as a therapeutic agent for treating neuroinflammatory diseases.

For the seaweed species selected to the current study, almost no literature is available. A study by Oumaskour *et al.* (2013)[199] measured anti-inflammatory activity through the inhibition rate of the phospholipase A2 enzyme. A dichloromethane/ methanol (50:50) *Asparagopsis armata* extract presented a 100% inhibition of phospholipase A2 activity. These results contrast with the results obtained in the present study, where *A. armata* shows an absence of inhibition of COX-2. The difference in the results obtained for the same macroalga species demonstrates that, just as with the antioxidant properties, a compound that as the capacity to hinder a specific enzyme, may be harmless to another enzyme.

As said before, there are a varied group of methodologies used for the determination of anti-inflammatory activity, and the ones presented above are different from the one used in the current study, where the activity of the enzyme COX-2 was reduced by inhibition.

The anti-inflammatory activity of green seaweeds was tested using the same method as in the present work, based on the inhibition of COX-2. The seaweed aqueous extracts (10% w/v) were dissolved in DMSO (1 mg/mL) and the COX-2 activity inhibition ranged between 31 and 45%[195]. A COX-2 inhibition of 40 ± 7% by aqueous extracts of *Petalonia binghamiae* was also reported[190]. These values are similar to those attained with the aqueous extracts of the Azorean *C. humilis*.

Once again, as observed in antioxidant activity, the sun-drying method did not induce much more damage when compared to the shade-drying method. It is possible to assume that the sun-drying method is not a determining factor in the anti-inflammatory bioactivity of the compounds present in the studied seaweeds, that is, they may be less susceptible to heat and UV light. Actually, there are many groups of compounds that display anti-inflammatory activity, such as phenolic compounds, carotenoids and quinones[200]. However, no correlation is observed between phenolic concentration in the aqueous extracts (Table 4.2) and their anti-inflammatory activity, so it is possible that other compounds, such as quinones, are responsible for the anti-inflammatory activity of *C. humilis* and other seaweed species[200,201].

5.6. Elemental Composition

Macroalgae are capable of accumulating minerals essential for human nutrition that are usually absent from freshwater and crops grown in soils deprived from minerals[78]. However, it is important to analyse the mineral composition of seaweeds, since, when in excess, minerals can have toxic/poisoning effects.

Concerning the elemental composition, results found for the *Cystoseira* genus agree with the available literature, especially regarding the high potassium concentrations. More specifically, Vizetto-Duarte *et al*. (2016)[173] reported values between 16 g/kg dw and 60 g/kg dw for five *Cystoseira* species and Rupérez (2002)[94] reported a value of 85.5 g/kg dw for *Cystoseira trinodis*.

Values for calcium (9.43-26.5 g/kg dw), magnesium (5.7-18.5 g/kg dw) and sodium (7.6-16.4 g/kg dw) are also reported by Vizetto-Duarte *et al*. (2016)[173]. The values obtained in the present work for the *Cystoseira* genus are within the ranges presented in literature for calcium and magnesium, but the results attained for sodium are higher, when compared with the literature presented. However, *Fucus vesiculosus*, a macroalga species from the order *Fucales*, is presented in literature with a sodium concentration of 54.6 g/kg dw[94], a value higher than those obtained for the *Cystoseira* genus. As for the *Asparagopsis* genus, values for calcium (6.1 g/kg dw)[202], potassium (14.7 g/kg dw) [202], magnesium (8 g/kg dw)[203] and sodium (66 g/kg dw)[203], were also reported. The results presented for potassium and magnesium are in concordance with the results obtained in this study for *A. taxiformis* and *A. armata*, however, the results obtained for calcium and sodium are higher than the values in literature. The *Asparagopsis* genus shows high levels of sodium in their constitution, varying from 85.6 ± 0.7 g/kg dw to 115.3 ± 3.0 g/kg dw. Even taking into account the issue of seaweed washing with either fresh or salt water, high sodium levels may be assumed as a weakness of seaweed consumption[82,96].

Moreover, values for iron, manganese, and zinc were reported by Vizetto-Duarte *et al*. (2016)[173]. The iron results attained in this study for the *Cystoseira* genus are similar to the values presented in literature (109.6-508.1 mg/kg dw). However, the results obtained for manganese and zinc are lower than the results presented in literature (14.3-398.5 mg/kg dw for manganese and 9.4-720.5 mg/kg dw for zinc). Machado *et al.* (2014)[202] reported a sulphur value of 13.1 g/kg dw for *Cystoseira trinodis* and Rupérez (2002)[94] presented a value for copper concentration in *Fucus vesiculosus* (<5 mg/kg dw). These values agree with the results presented in this work for the *Cystoseira* genus. Roque *et al.* (2019)[203] reported, for *A. taxiformis*, concentration values concerning zinc (23.7 mg/kg dw), iron (6241 mg/kg dw), manganese (112.7 mg/kg dw) and copper (8.7 mg/kg dw). The zinc concentration reported is in agreement with the values obtained in the present work, regarding *A. taxiformis* and *A. armata*; as for iron, manganese, and copper, the results obtained in the present study for *A. taxiformis* and *A. armata* are lower. Regarding phosphorus, this mineral is present in similar ranges in brown and red seaweeds, with different concentrations ranging from 0.5 to 7 g/kg dw[82]. The results obtained in the present work agree with the range presented.

Concerning the *Asparagopsis* genus, the iodine concentration values obtained in the present work are high, ranging from 4645.3 ± 119.6 mg/kg dw to 5735 ± 49.2 mg/kg dw. Nunes *et al.* (2018)[179] also reported an extremely high value for iodine concentration in *Asparagopsis taxiformis* (11600 ± 300 mg/kg dw). The Scientific Committee on Food established an upper limit for iodine intake of 0.6 mg a day for adults[97], which means that with the high levels obtained for the *Asparagopsis* genus in the present work, an adult can only consume 0.11-0.13g a day of these seaweeds.

The high arsenic level found in *C. abies-marina* deserves a special attention. Fonseca *et al.* (2020)[174] also reported high values for sun-dried *C. abies-marina* (340 ± 0 mg/kg dw), also harvested in the Faial island (Azores Archipelago). Nevertheless, the results found in literature for arsenic concentration in seaweeds are generally much lower. For instance, *Cystoseira barbata*

harvested from different regions of the Black Sea, showed arsenic concentrations between 3.85 mg/kg dw and 5.55 mg/kg dw[204]. High arsenic levels were found, however, in *C. barbata* collected from the Venice lagoon (242 ± 104 mg/kg dw, with a maximum of 360 mg/kg dw), but those high levels are justified by the high pollution present in that area[205]. It has been claimed that *C. barbata* is an arsenic hyperaccumulating species (As contents exceeding 100 mg/kg dw). This may also apply to *C. abies-marina*. Considering this species was harvested in the Azores islands, a region that is not heavily polluted, further research, including arsenic speciation, is needed[174].

A higher content of sodium and potassium is usually found in algae, when compared with vegetables, but Na/K ratios found are lower. *A. armata* and *A. taxiformis* show a Na/K ratio between 2.3 and 7.4, while *C. humilis* and *C. abies-marina* show a 0.5 ratio. This is interesting from a nutritional point of view as it can be observed that the Na/K ratio found in the *Cystoseira* genus is below 1.0 and low ratios are shown to promote the decrement of blood pressure. However, the intake of diets with a high Na/K ratio have been related to hypertension[95,206]. The values obtained for the *Cystoseira* genus are in agreement with the literature presented for five *Cystoseira* species (0.15-0.97)[173].

The Ca/Mg ratio is also important concerning calcium absorption as a deficient magnesium intake can lead to an excessive accumulation of calcium in soft tissues, consequently resulting in arthritis and appearance of kidney stones[207-208]. The Ca/Mg ratio in the *Asparagopsis* genus ranged from 1.5 to 2.3, while in the *Cystoseira* genus it ranged from 1.4 to 2.3. The low ratios found in all four seaweeds suggest that calcium and magnesium are present in similar concentrations.

Regarding contaminant elements, the European Commission Regulation set the maximum allowed levels of cadmium to be less than 3 mg/kg dw in food supplements that are exclusively or mainly composed of dried seaweed or seaweed-derived products[97] and established the maximum arsenic levels in complementary feed and/or a complete feed meal at 40 mg/kg and 10 mg/kg, respectively[98]. Regarding cadmium, the four studied seaweeds show levels below the maximum allowed. However, concerning arsenic, only the *Asparagopsis* genus shows values below the maximum allowed for complementary feed. These results stand out because, as already mentioned, *A. taxiformis* shows anti-methanogenic activity and, according to Roque *et al.* (2019)[151], the production of methane in cattle rumen can decrease up to 90% by incorporating 5% *A. taxiformis* organic matter in dairy cattle feed.

5.7. Bioaccessibility

5.7.1. Anti-inflammatory activity

Regarding the bioaccessibility of the anti-inflammatory activity, there are no published results that allow for a comparison with the results obtained in the present work, especially with the methodology used - inhibition of COX-2. A study with green seaweeds, using the same methodology, reported values not significantly different from zero for anti-inflammatory bioaccessibility, as *Chaetomorpha linum* presented the highest bioaccessibility, of approximately

30%[195]. Low values were also observed in the present study regarding *Cystoseira* species. Thus, it is important to further analyse the high bioaccessibility results obtained for *A. taxiformis.*

Regarding *Cystoseira* species, preparation of more refined extracts for nutraceutical and pharmacological applications or seaweed processing through decoction to produce a tisane — especially in the case of *C. humilis*, which exhibited a substantial anti-inflammatory activity before digestion — may represent a solution to the poor bioaccessibility, thereby making the anti-inflammatory compounds available for absorption at the gastrointestinal tract.

5.7.2. Elemental Composition

The determination of elemental bioaccessibility in macroalgae is of great importance, mainly due to the possible presence of toxic elements, but also because of the many advantages of nutritionally important minerals and trace elements. Low bioaccessibility percentages were expected as the human digestive enzymes have an inability in breaking down the polysaccharides in algal cell walls.

Iodine, bromine, magnesium, and cadmium showed the highest bioaccessibility percentages. The results for iodine concerning *A. taxiformis* are of interest since, as before mentioned, this macroalgae has a high concentration regarding this element and the upper limit established for iodine intake is only of 0.6mg a day for adults[97]. In contrast, copper, zinc, and lead, regarding the *Cystoseira* genus, showed to be not bioaccessible. The discrepancy observed in the result presented for zinc regarding sun-dried *A. armata* (38.5 ± 2.1% dw) may be due to a methodological error when analysing the sample. Arsenic showed a significant bioaccessibility percentage.

Desideri *et al.* (2018)[121] also reported high bioaccessibility values for cadmium and arsenic in two brown seaweeds – a cadmium bioaccessibility percentage of 91.0 ± 24.9% dw and an arsenic percentage of 45.9 ± 0.72% dw, were presented for *Aschophyllum nodosum*, while for *Fucus vesiculosus*, was reported a cadmium bioaccessibility percentage of 86.5 ± 16.6% dw and an arsenic percentage of 48.5 ± 2.9 %.

As for the low iron bioaccessibility, Boato *et al.* (2002)[209] observed that fruit juices high in polyphenols content displayed limited bioavailability of iron by forming iron–polyphenol complexes, preventing absorption by the cells. Knowing that *C. abies-marina and C. humilis* have a high polyphenolic content, the low bioaccessibility of iron observed may be due to the formation of such complexes. To explain the low bioaccessibility of other minerals, Taboada *et al.* (2010)[206] suggested that binding of certain minerals to the polysaccharides present in seaweeds may limit its absorption.

6. Conclusions and Future Perspectives

The analysis of bioactive contents and bioactivity levels in the samples of the four studied seaweed species - *Asparagopsis taxiformis*, *Asparagopsis armata*, *Cystoseira abies-marina* and *Cystoseira humilis* - subjected to alternative drying processes offered valuable insights regarding the nutraceutical and biotechnological potential of these undervalued marine resources.

The seaweeds from the *Cystoseira* genus showed high polyphenol levels (176-678mg GAE/100 g dw), which clearly surpassed those measured in the *Asparagopsis* genus regardless of the used drying process. This was partially reflected in the antioxidant activity, as determined by DPPH and FRAP methods, which pointed to a tendency for *Cystoseira* species to generate more antioxidant extracts. The influence of the drying technique upon the antioxidant activity was relatively limited, since in many instances there was no effect. In any case, there was substantial agreement in the antioxidant activity as determined by different methods, especially if the aqueous extracts measured by ABTS are excluded from the comparison. Concerning anti-inflammatory activity, sun-dried and shade-dried *A. armata* as well as sun-dried *C. abies-marina* did not show any inhibitory capacity of COX-2. In the case of shade-dried samples, *C. humilis* had a higher anti-inflammatory activity (>30 % COX-2 inhibition) than *A. armata*, being the other two species at an intermediate level. However, this activity in *C. humilis* was not rendered bioaccessible. Indeed, only *A. taxiformis* displayed anti-inflammatory activity in the bioaccessible fraction, leading to bioaccessibility factors in the 90-100% interval. Therefore, though bioactivities were higher in the studied species of the *Cystoseira* genus, *Asparagopsis* species and, in particular, *A. taxiformis*, also had a valuable bioactive potential. Sun-drying produced more negative effects than shade-drying, but these effects were not very extensive.

Minerals like bromine, magnesium, and iodine, showed high bioaccessibility in all four macroalgae. From the contaminant analyses, it can be stated that *Cystoseira abies-marina* displayed high levels of arsenic. Moreover, it is to note its high bioaccessibility values, which may be an additional concern.

Future work should aim at the preparation of more refined extracts or seaweed processing through decoction to produce a tisane as ways to improve bioaccessibility and ensure nutraceutical applications. Therefore, it would also be of interest to develop potential nutraceutical/functional products, as well as proceed with their bioaccessibility studies. Besides that, it would be relevant to specify arsenic in *C. abies-marina,* for a improved assessment of the risk related to this seaweed consumption, and proceed with a more in-depth analysis regarding *A. taxiformis* brominated compounds, such as bromoform, due to their anti-methanogenic activity. This specific research should include the identification and detailed quantification of these brominated compounds and the assessment of their bioaccessibility.

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8. Annexes

Annex I – Reference to submitted paper (Phycological Research)

The Effect of Drying Process on Undervalued Brown and Red Seaweed Species: Bioactivity Alterations

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For Review Only

Abstract

The effect of drying on two brown seaweed (*Cystoseira abies-marina, Cystoseira humilis*) and two red seaweed species (*Asparagopsis armata* and *Asparagopsis taxiformis*) was evaluated using as touchstone relevant biological activities, such as antioxidant and anti-inflammatory properties. Moreover, some significant bioactives, such as polyphenols and beta-glucans were determined.
Seaweeds from the *Cystoseira* genus showed high polyphenol levels (176-678 mg GAE/100 g dw), which clearly exceeded those determined in the *Asparagopsis* genus regardless of drying process. This was partially reflected in the antioxidant activity as measured by DPPH and FRAP methods, which showed that extracts from the *Cystoseira* species were often more antioxidant than those from *Asparagopsis* species. The influence of the drying technique upon the antioxidant activity was relatively limited, since in many instances there was no effect. Concerning anti-inflammatory activity, in the case of shade-dried samples, *C. humilis* had a higher activity (>30 % COX-2 inhibition) than *A. armata*. However, this activity in *C. humilis* was not rendered bioaccessible. Indeed, only *A. taxiformis* displayed anti-inflammatory activity in the bioaccessible fraction, leading to bioaccessibility factors in the 90-100 % range. Therefore, though bioactivities were higher in the *Cystoseira* species, *Asparagopsis* species also had a positive bioactive potential. Sun-drying produced more negative effects than shade-drying, but these effects were not very extensive.

Keywords: *Asparagopsis armata*; *Asparagopsis taxiformis*; *Cystoseira abies-marina*; *Cystoseira humilis*; biological activities; bioaccessibility;

Figure 8.1. First page of the document submitted to the journal of Phycological Research for publication.

Annex II

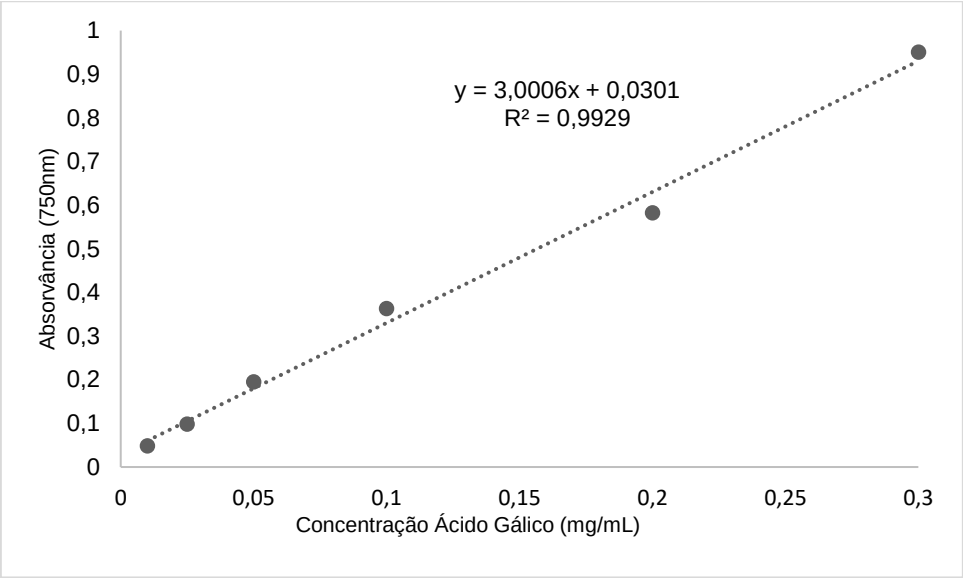


Figure 8.2. Gallic acid calibration curve for polyphenol quantification, for the aqueous extract, obtained through solutions with concentrations of 0.01 mg/mL, 0.025 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL and 0.3 mg/mL.

Annex III

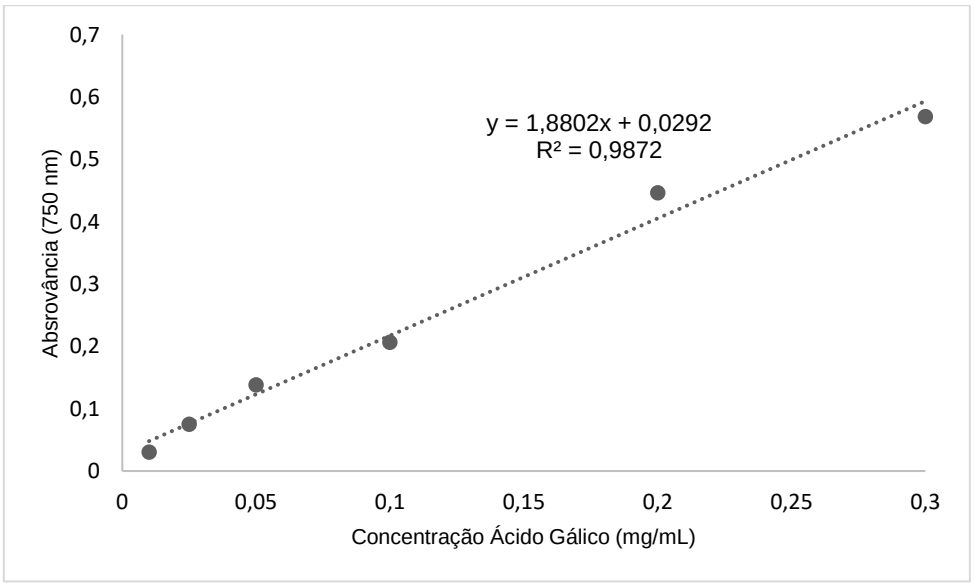


Figure 8.3. Gallic acid calibration curve for polyphenol quantification, for the ethanolic extract, obtained through solutions with concentrations of 0.01 mg/mL, 0.025 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL and 0.3 mg/mL.

Annex IV

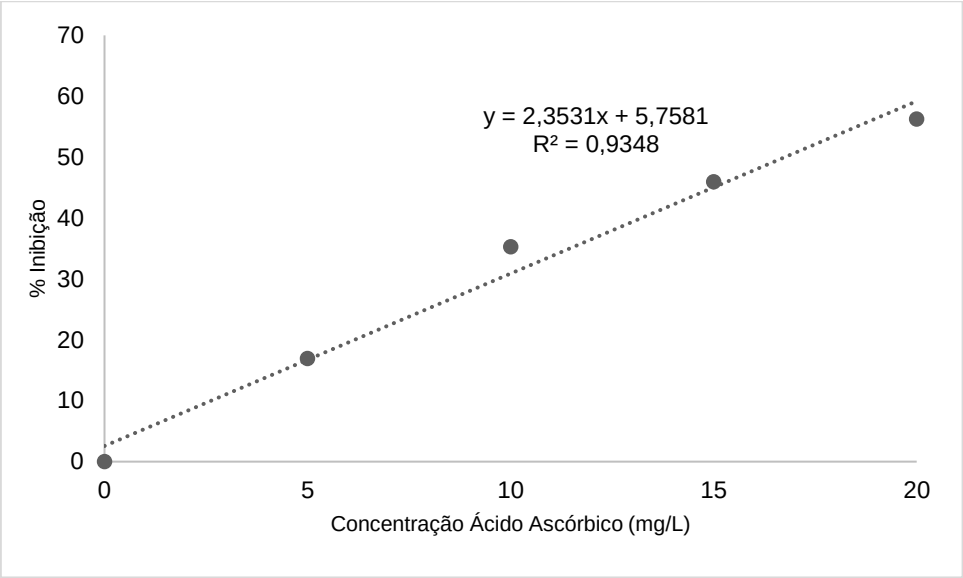


Figure 8.4. Ascorbic Acid calibration curve for the DPPH assay, for the aqueous extracts, obtained through solutions with concentrations of 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L and 25 mg/L.

Annex V

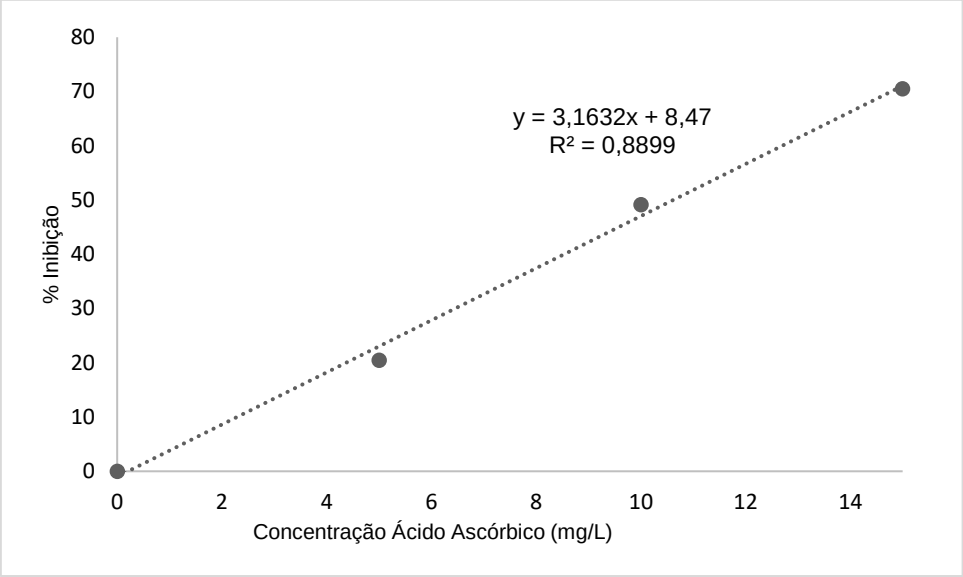


Figure 8.5. Ascorbic Acid calibration curve for the DPPH assay, for the ethanolic extracts, obtained through solutions with concentrations of 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L and 25 mg/L.

Annex VI

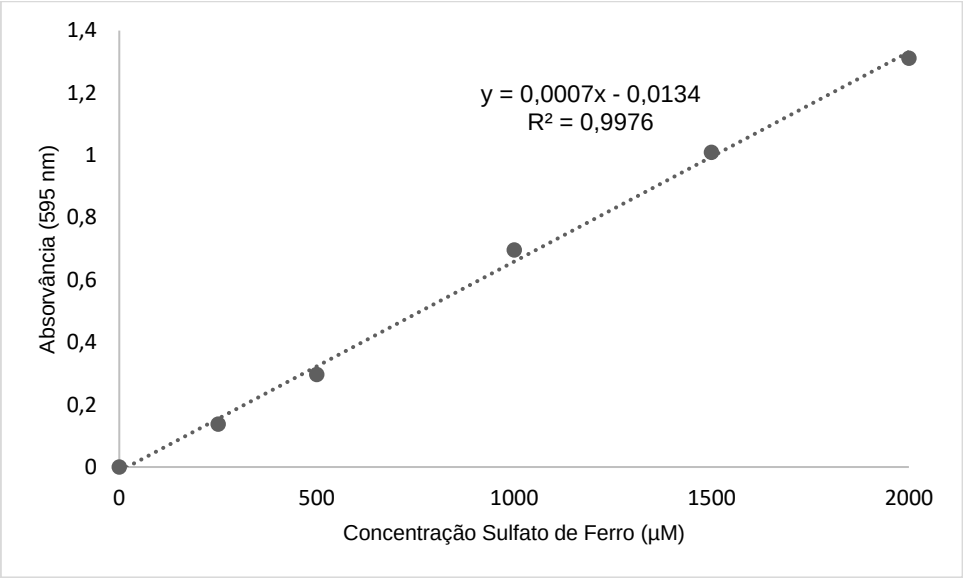


Figure 8.6. Iron(II) sulphate calibration curve for the FRAP assay, for the ethanolic and aqueous extracts, obtained through solutions with concentrations of 250 µM, 500 µM, 1000 µM, 1500 µM and 2000 µM.

Annex VII

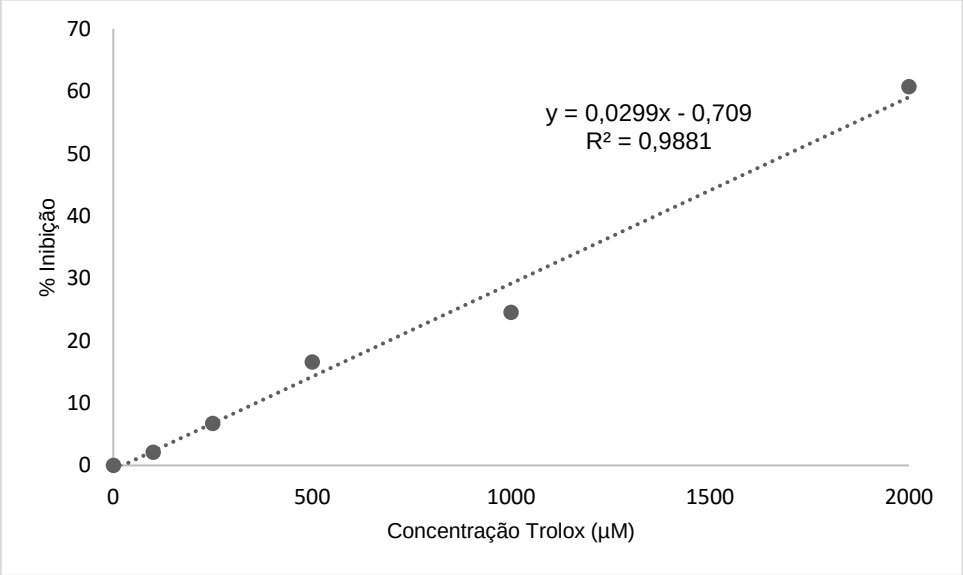
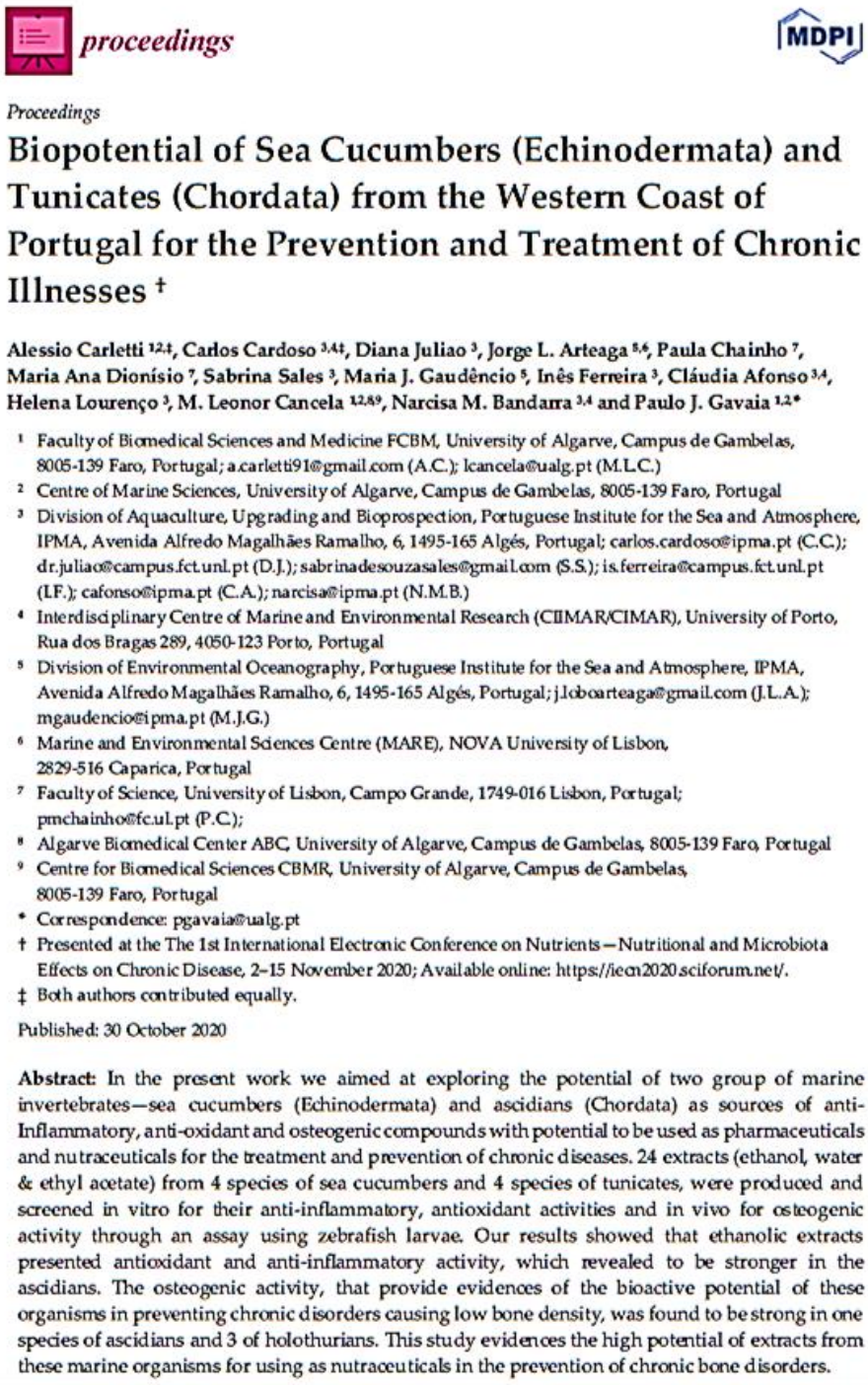


Figure 8.7. Trolox calibration curve for the ABTS assay, for the ethanolic and aqueous extract, obtained through solutions with concentrations of 100 µM, 250 µM, 500 µM, 1000 µM, and 2000 µM.

9. Additional Information

During the elaboration of this project, I was given the possibility of co-authoring an article (Figure 8.2) that, despite having no direct contribution to the present work, helped me with the better understanding and execution of techniques also performed in this work.



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Biopotential of Sea Cucumbers (Echinodermata) and Tunicates (Chordata) from the Western Coast of Portugal for the Prevention and Treatment of Chronic Illnesses †

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Abstract: In the present work we aimed at exploring the potential of two group of marine invertebrates—sea cucumbers (Echinodermata) and ascidians (Chordata) as sources of anti-Inflammatory, anti-oxidant and osteogenic compounds with potential to be used as pharmaceuticals and nutraceuticals for the treatment and prevention of chronic diseases. 24 extracts (ethanol, water & ethyl acetate) from 4 species of sea cucumbers and 4 species of tunicates, were produced and screened in vitro for their anti-inflammatory, antioxidant activities and in vivo for osteogenic activity through an assay using zebrafish larvae. Our results showed that ethanolic extracts presented antioxidant and anti-inflammatory activity, which revealed to be stronger in the ascidians. The osteogenic activity, that provide evidences of the bioactive potential of these organisms in preventing chronic disorders causing low bone density, was found to be strong in one species of ascidians and 3 of holothurians. This study evidences the high potential of extracts from these marine organisms for using as nutraceuticals in the prevention of chronic bone disorders.

Figure 9.1. First page of the article of my co-authoring collaboration, published in Sciforum.