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Antioxidant and Antimicrobial Properties of Selected Red Seaweeds from Central Portugal

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Featured Application: Aqueous extracts from selected red seaweeds collected from central Portugal present antioxidant and antimicrobial activity, and therefore may be considered potential natural resources in a biotechnological context.

Abstract: Throughout the ages, macroalgae have provided humankind with elements beneficial to human health, and often with bioactive abilities. Yet, while today we fully acknowledge such potential, especially that of the most widely known species, an even greater number of species remain unaknowledged. This holds particularly true for the highly diverse phylum Rhodophyta (red seaweeds) and, therefore, the present study aims to unveil the antioxidant and the antimicrobial potential of twelve red seaweed species collected in central Portugal. Results obtained from the antioxidant assays ABTS and TPC highlighted the high scavenging capacity of the coralline algae *Corallina officinalis*, *Ellisolandia elongata* and *Amphiroa rigida*, and the high phenolic content of *Porphyra umbilicalis*, whereas the antimicrobial analyses through MIC determination emphasized the activities of *Sphaerococcus coronopifolius* and *Mesophyllum lichenoides* against, respectively, *Bacillus subtilis* and *Saccharomyces cerevisiae*. This study raised awareness of the bioactive potential waiting to be discovered regarding less known Rhodophyta species, such as *Amphiroa rigida* and *Mesophyllum lichenoides*. Therefore, we believe this study provides extra steps in pinpointing Rhodophyta species with bioactive potential, encouraging further studies tailored toward a biotechnological perspective, and, ultimately, influencing current perspectives regarding the exploration of seaweeds.

Keywords: Rhodophyta; red macroalgae; antioxidant activity; ABTS; total phenolic content; antimicrobial activity; minimum inhibitory concentration

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1. Introduction

Macroalgae are a widely diverse group of photosynthetic organisms, with approximately 15,000 described species [1]. Known as "seaweeds" and "sea vegetables", marine macroalgae have been exploited throughout the ages, with seaweed harvesting and usage being activities that are deeply rooted into the tradition and history of many cultures, scattered around the world. Eastern cultures have established the use of seaweeds for food and medicine, and consider them to be of great value, and, on a worldwide scale, they have been relied upon in times of warfare, famine and crisis [2–6]. Among their myriad of uses, around 10,000 are considered edible [7] and can be directly consumed or used in food preparation [7,8], as their nutritional and nutraceutical value has been widely acknowledged [3,8–12].

Today, we are fully aware that valuable natural compounds can be obtained from seaweeds, namely phycocolloids, peptides, polysaccharides, polyunsaturated fatty acids, fibers, calcium carbonate, iodine, pigments, diterpenes, phlorotannin's, vitamins and phenols [7,13–19]. The abovementioned list includes novel bioactive compounds, which are defined as having a biological interaction or effect when applied to living cells, which depends on the applied [20]. Such seaweed-bound compounds can be extracted and incorporated into the food matrix to create new functional foods [8,21] or to develop cosmeceutical, nutraceutical and pharmaceutical products [15,22–28]. The bioactive compounds held by seaweeds are responsible for the different bioactivities already acknowledged by many authors. Numerous studies and reviews have referred to the antioxidant [29–36], antimicrobial [33,37–43], anti-fungal [44], anti-inflammatory [29], anti-cholesterol [45,46], anti-neurodegenerative [47], anti-tumor [32,41,48–52] and prebiotic [14,53,54] properties of these bioactive compounds extracted from seaweeds. On industrial and commercial levels, while seaweed bioactives remain relatively unexploited, efforts are being undertaken to promote the use of seaweeds in food ingredient applications [55].

Among the three main groups of macroalgae (the green ones, belonging to the phylum Chlorophyta; the brown ones, from the Phaeophyceae class; and the red ones, from the phylum Rhodophyta), Rhodophyta include some of the most well-known seaweed species, such as *Porphyra* sp. and *Chondrus crispus*, which are exceptionally popular in Asia [29] and Ireland [56], respectively, especially as an ingredient in their culinary traditions. A number of species have been targeted in several studies, such as *Osmundea pinnatifida* (e.g., [56,57]), *Sphaerococcus coronopifolius* (e.g., [38,51,56]) and *Plocamium cartilagineum* (e.g., [35,50,58,59]), and have become quite popular in an academic context. The coralline algae (Corallinales, Rhodophyta) have become fairly popular for bone health, due to their content of calcium (e.g., [60–62]). However, only the Rhodophyta phylum holds a significantly higher diversity of species, when compared to both Chlorophyta and Phaeophyceae, and many other red seaweeds still require further study. This holds true especially in regions and countries where seaweeds remain highly undervalued, such as Portugal. Portugal holds an incredibly large exclusive maritime zone, with great importance for Portuguese economic development [63], but the remarkable algal abundance and diversity that adorn its shores is still largely unexplored, and the seaweed species remain factually unacknowledged to this day.

Therefore, the objective of the present study is to shed light on the antioxidant and antimicrobial activity of twelve red seaweed species commonly found on Portuguese shores. The chosen target species compose a combination of both worldwide renowned species and unexplored species: *Porphyra umbilicalis*, *Ceramium ciliatum*, *Osmundea pinnatifida*, *Chondrus crispus*, *Sphaerococcus coronopifolius*, *Plocamium cartilagineum*, *Corallina officinalis*, *Ellisolandia elongata*, *Amphiroa rigida*, *Jania rubens*, *Mesophyllum lichenoides* and *Liagora viscida*. We expect that the results found will be useful to understand and recognize the significance these species may hold in biotechnological, commercial, nutritional and health contexts.

2. Materials and Methods

2.1. Biomass Harvesting and Processing

Healthy fronds of twelve red seaweed species were harvested in central Portugal, from several locations on the seashore around the region of Peniche (São Marcos: 39°19′10″ N, 9°21′24″ W; Quebrado: 39°22′3″ N, 9°22′26″ W; Consolação: 39°19′27″ N, 9°21′39″ W; Portinho da Areia Norte: 39°22′07″ N, 9°22′41″ W) and Buarcos (Buarcos: 40°09′57″ N, 8°53′05″ W) during low tide, and were kept inside dark cooled boxes until arrival to the laboratory. The selected seaweed species were *Porphyra umbilicalis*, *Ceramium ciliatum*, *Osmundea pinnatifida*, *Chondrus crispus*, *Sphaerococcus coronopifolius*, *Plocamium cartilagineum*, *Corallina officinalis*, *Ellisolandia elongata*, *Amphiroa rigida*, *Jania rubens*, *Mesophyllum lichenoides* and *Liagora viscida*. Apart from the Bangiophyceae *P. umbilicalis*, all species belong to the class Florideophyceae. The corresponding harvest season and coordinates for each species are shown in Table 1.

Table 1. List of the species studied, corresponding code names and harvesting season.

Species	Order	Code	Harvest Season	Coordinates
Porphyra umbilicalis (Linnaeus) J. Agadh	Bangiales	PoUm	Winter	39°19′10″ N, 9°21′24″ W
Ceramium ciliatum (J. Ellis) Ducluzeau	Ceramiales	CeCi	Summer	39°19′27″ N, 9°21′39″ W
Osmundea pinnatifida (Hudson) Stackhouse	Ceramiales	OsPi	Winter	39°22′07″ N, 9°22′41″ W
Chondrus crispus Stackhouse	Gigartinales	ChCr	Spring	40°09′57″ N, 8°53′05″ W
Sphaerococcus coronopifolius Stackhouse	Gigartinales	SpCo	Summer	39°22′3″ N, 9°22′26″ W
Plocamium cartilagineum (Linnaeus) P.S. Dixon	Plocamiales	PlCa	Winter	39°19′10″ N, 9°21′24″ W
Corallina officinalis J. Ellis and Solander 1786	Corallinales	CoOf	Winter	39°19′10″ N, 9°21′24″ W
Ellisolandia elongata (J. Ellis and Solander) K.R. Hind and G.W. Saunders	Corallinales	ElEl	Winter	39°19′10″ N, 9°21′24″ W
Amphiroa rigida J.V. Lamouroux	Corallinales	AmRi	Winter	39°19′10″ N, 9°21′24″ W
Jania rubens (Linnaeus) J.V. Lamouroux	Corallinales	JaRu	Winter	39°19′10″ N, 9°21′24″ W
Mesophyllum lichenoides (J. Ellis) Me. Lemoine	Hapalidiales	MeLi	Winter	39°19′10″ N, 9°21′24″ W
Liagora viscida (Forsskål) C. Agardh	Nemaliales	LiVi	Summer	39°22′3″ N, 9°22′26″ W

In the laboratory, filtered seawater was used to thoroughly rinse the collected biomass, which was subsequently cleaned by removing epiphytes, unhealthy tissue and lingering debris. The healthy, clean biomass was dried in a ventilated oven (25 °C, 48 h) (Binder, FD115), reduced into powder in a blender and finally sieved (<200 µm). The resulting seaweed powder was stored at −20 °C to for further use in all the procedures described below.

2.2. Aqueous Extractions

The aqueous extraction was performed by following a dry biomass:solvent ratio of 1:10 (g.mL^{-1}) and extraction times of 1 h. Briefly, 3 g of powdered dried biomass were mixed with 30 mL of ultra-pure water and left stirring at room temperature (RT), protected from light, for 1 h. The extract was then centrifuged (8000× *g*, 10 min, RT) and the supernatant was collected. The pellet was then subjected to a second extraction, using 10 mL of ultra-pure water. After centrifugation of the second extraction, both supernatants were pooled, filtered (GF/C, Whatman) to remove any lingering powder, freeze-dried and stored at −20 °C. Prior to its use in the assays described below, the dried extracts were dissolved in ultra-pure water at a concentration of 100 mg·mL^{-1}.

2.3. ABTS Radical Scavenging Activity Assay

The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity was measured according to Meng et al. [64] with a few modifications. Briefly, an ABTS stock solution was prepared by mixing equal volumes of 7 mM of ABTS and 2.45 mM of potassium persulfate solutions (both previously prepared in ultra-pure water), and was allowed to stand at room temperature for 12 to 16 h. Afterwards, this stock solution was further diluted with ultra-pure water until reaching an absorbance value of 0.72 ± 0.02 at 734 nm; the new dilution was considered our ABTS working solution. The aqueous extracts were dissolved into different concentrations (20, 40, 60, 80 mg·mL^{-1}).

The reaction was adapted to a microscale, being carried out in a 96-well plate containing 2 µL of the aqueous extract (either at 20, 40, 60, 80 and 100 mg·mL^{-1}) and 198 µL of ABTS working solution. Blank reactions containing 2 µL of ultra-pure water instead of extract, as well as a control made with acid ascorbic (10 mg·mL^{-1}), were also performed. The plate was incubated in the dark for 6 min at room temperature, and the absorbance was measured afterwards at 734 nm (Biotek, Epoch2). The ABTS radical scavenging activity was calculated according to Equation (1).

$$ABTS\ Radical\ Scavenging\ Activity\ (\%) = \frac{AbsC - (AbsS - AbsB)}{AbsC} \times 100 \quad (1)$$

where *AbsC* is the absorbance of the control at 734 nm (ultra-pure water and ABTS), *AbsS* is the absorbance of the sample at 734 nm (extract and ABTS) and *AbsB* is the absorbance of the blank at 734 nm (sample and ultra-pure water). From the results obtained from Equation (1), the Half Maximal Effective Concentration (EC_{50}), defined as "the concentration of a drug that gives half-maximal response" [65], was calculated. The results were interpreted considering: the lower the EC_{50} value, the higher antioxidant activity the respective extract holds.

2.4. Total Phenolic Compound Assay (TPC)

The TPC assay was performed by adapting the Folin-Ciocalteu method developed by Singleton and Rossi [66]. The reaction was adapted to a microscale and carried out in a 96-well plate containing 2 µL of the aqueous extract, 158 µL of ultra-pure water, 10 µL of Folin-Ciocalteu reagent and 30 µL of 1.89 M sodium carbonate solution (Na_2CO_3). Blank reactions containing 2 µL of ultra-pure water instead of the extract were also performed. The plate was then incubated in the dark for 1 h at room temperature, and the absorbance was measured at 755 nm (Biotek, Epoch2). Gallic acid was used as a standard to perform the calibration curve, and the results were expressed as gallic acid equivalent (mg GAE.g^{-1} crude extract).

2.5. Antimicrobial Activity

2.5.1. Microorganism Cultures

The microorganisms *Escherichia coli* (ATCC 10536), *Bacillus subtilis* (ATCC 6633) and *Saccharomyces cerevisiae* (DSM 70449) were separately incubated in Muller Hinton (MH) broth, with shaking, under aerobic conditions. The turbidity of the two bacterial suspensions was adjusted to 0.5 McFarland turbidity standard with 0.85% saline solution, to standardize the assay for the bacterial suspensions. All assays described below were immediately performed after adjusting cell suspension [67].

2.5.2. Minimum Inhibitory Concentration (MIC) Assay

The Minimum Inhibitory Concentration (MIC) assay is a widely common technique in pharmacological studies, which aims to test antimicrobial activity [68], and it is defined as the lowest concentration of an antimicrobial agent that prevents the visible growth of a microorganism within a defined period of time [67]. In the present work, we adapted the method of Lambert and Pearson [69] by employing the broth dilution method in a 96-well plate to determine and compare the MIC of the aqueous seaweed extracts.

Briefly, the stock concentration of seaweed extracts was 100 mg·mL^{-1}. A preliminary screening was performed to access whether the seaweed extracts had any inhibitory activity upon *E. coli*, *B. subtilis* and *S. cerevisiae*, by evaluating their growth in wells containing 145 µL of MH Broth, 50 µL of seaweed extract and 5 µL of bacterial suspension (therefore corresponding to an extract concentration of 25 mg·mL^{-1}). Controls were prepared containing 195 µL MH broth and 5 µL bacterial suspension (positive control), or 200 µL MH broth only (negative control).

The plate was then read at 600 nm twice (Biotek, SynergyH1), at time 0 and 24 h after incubation at 37 °C (*E. coli* and *B. subtilis*) or 28 °C (*S. cerevisiae*). A visual assessment was performed to detect microbial growth after 24 h: the seaweed species whose extracts inhibit

growth were further diluted to find the MIC. In these extracts, serial microdilutions were then performed, starting with a well containing 145 µL of MH Broth and 50 µL of seaweed extract to obtain a sequence of decreasing (by half) extract concentrations, ranging between 25 mg·mL^{-1} and 0.02 mg·mL^{-1} across the plate. In wells containing extract and MH Broth, 5 µL of bacterial suspension was added. Again, the plate was read at 600 nm twice, at 0 h and 24 h after incubation at 37 °C (*E. coli* and *B. subtilis*) or 28 °C (*S. cerevisiae*), and a visual assessment was also performed to determine the MIC. The MIC was considered found in the lowest concentration where the test well replicas were visually less turbid, or with a visibly smaller bacterial deposit, than those of the controls. Microbial growth was calculated according to Equation (2).

$$Microbial\ Growth\ (\%) = \frac{AbsS(t1) - AbsS(t0)}{AbsC(t1) - AbsC(t0)} \times 100 \quad (2)$$

from where *AbsS* is the absorbance of the sample at 600 nm following plate inoculation (t0) and after 24 h of incubation (t1), *AbsC* is the absorbance of the positive control at 600 nm following plate inoculation (t0) and after 24 h of incubation (t1).

2.6. Statistical Analysis

All assays and analyses were performed in quadruplicate (n = 4). The EC_{50} of the ABTS assay was calculated in GraphPad Prism 9.0.0. The one-way analysis of variance (ANOVA) was performed upon all treatments, succeeding validation of normality and homogeneity of variances. Whenever this validation was not accomplished, the non-parametric Kruskal–Wallis test was executed instead. All differences were considered significant at p-value < 0.05. Data were expressed as mean ± standard deviation for all data except EC_{50} in ABTS assay, where results were expressed as mean and 95% confidence intervals. All statistical assessments were performed in SPSS Statistics 28 (IBM Corporation, New York, NY, USA).

3. Results

3.1. Antioxidant Activity

3.1.1. ABTS Radical Scavenging Activity Assay

The results obtained from the ABTS assay reveal differences according to the species under study (Figure 1). The values ranged from 18.40 mg·mL^{-1}, with a 95% Confidence Interval (CI) [2.00, 1.85] (*Corallina officinalis*), to 69.41 mg·mL^{-1} with a 95% CI [6.42, 5.32] (*Liagora viscida*), meaning that *C. officinalis* required the lowest concentration of all the species studied to scavenge 50% of the ABTS radical, whereas *L. viscida* required the highest concentration. The seaweeds *Porphyra umbilicalis*, *Chondrus crispus* and all the coralline algae studied (*C. officinalis*, *Ellisolandia elongata*, *Amphiroa rigida*, *Jania rubens* and *Mesophyllum lichenoides*) required less than 30 mg·mL^{-1} of extract to scavenge 50% of the ABTS radical, and the seaweeds *Ceramium ciliatum*, *Sphaerococcus coronopifolius* and *Plocamium cartilagineum* required similar amounts of extract to achieve activity, with no significant differences between them.

3.1.2. Total Phenolic Compound Assay (TPC)

Regarding the results of the TPC assay, we also pinpointed differences between species (Figure 2). The values ranged from 0.323 ± 0.049 mg GAE.g^{-1} (*L. viscida*) to 1.688 ± 0.145 mg GAE.g^{-1} (*P. umbilicalis*), the latter evidently standing out from all other species analyzed, since the values obtained from these do not exceed 0.943 ± 0.060 mg GAE.g^{-1} (*O. pinnatifida*). In addition to *L. viscida*, the seaweeds *C. ciliatum*, *J. rubens* and *M. lichenoides* also presented comparatively lower TPC, between 0.531 ± 0.042 mg GAE.g^{-1} (*C. ciliatum*) and 0.574 ± 0.024 mg GAE.g^{-1} (*J. rubens*). However, all of the aforementioned species were exceptions, as most of the species analyzed presented similar values, ranging from 0.943 ± 0.060 mg GAE.g^{-1} (*Osmundea pinnatifida*) to 0.721 ± 0.058 mg GAE.g^{-1} (*S. coronopifolius*).

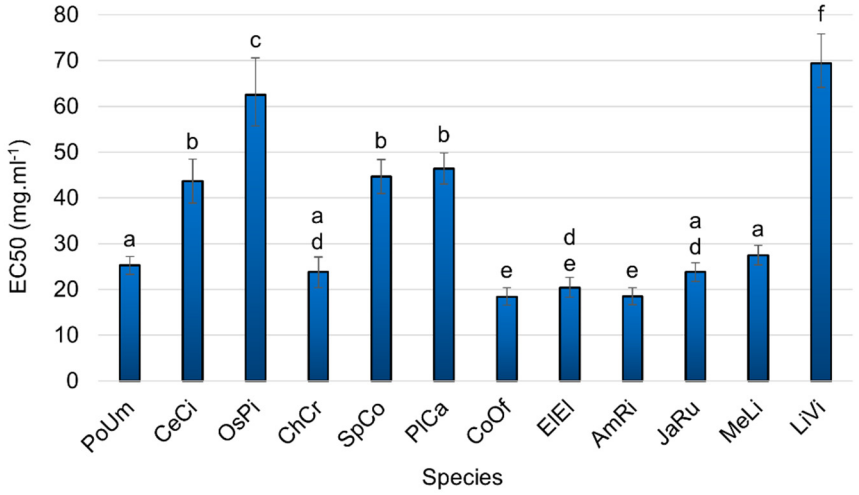

Figure 1. EC_{50} of the ABTS results of the twelve studied red seaweed species expressed in mg·mL^{-1}. PoUm: *Porphyra umbilicalis*, CeCi: *Ceramium ciliatum*, OsPi: *Osmundea pinnatifida*, ChCr: *Chondrus crispus*, SpCo: *Sphaerococcus coronopifolius*, PlCa: *Plocamium cartilagineum*, CoOf: *Corallina officinalis*, ElEl: *Elisolandia elongata*, AmRi: *Amphiroa rigida*, JaRu: *Jania rubens*, MeLi: *Mesophyllum lichenoides*, LiVi: *Liagora viscida*. Values are presented as mean ± 95% CI (n = 4), and lower-case letters (a to f) indicate statistically significant differences in the Tukey HSD test (F(11,35) = 584.734; p = 0.00).

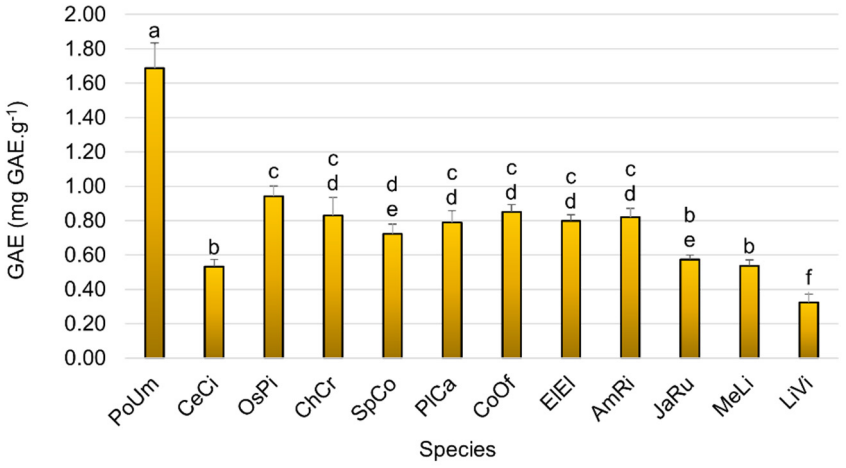

Figure 2. TPC of the twelve studied red seaweed species expressed in mg GAE.g^{-1}. PoUm: *Porphyra umbilicalis*, CeCi: *Ceramium ciliatum*, OsPi: *Osmundea pinnatifida*, ChCr: *Chondrus crispus*, SpCo: *Sphaerococcus coronopifolius*, PlCa: *Plocamium cartilagineum*, CoOf: *Corallina officinalis*, ElEl: *Elisolandia elongata*, AmRi: *Amphiroa rigida*, JaRu: *Jania rubens*, MeLi: *Mesophyllum lichenoides*, LiVi: *Liagora viscida*. Values are presented as mean ± SD (n = 4), and lower-case letters (a to f) indicate statistically significant differences in the Tukey HSD test (F(11,46) = 93.748; p = 0.00).

3.2. Antimicrobial Activity

Minimum Inhibitory Concentration (MIC) Assay

Table 2 shows the bacterial growth calculated within the MIC for each species, which corresponded, for most species, to ≈80% for the gram-positive *Bacillus subtilis* and the yeast *Saccharomyces cerevisiae*. No activity was recorded against the gram-negative *Escherichia coli*, and, thus, data are not shown. *E. elongata* was the only algal species that did not inhibit *B. subtilis* growth (>100%), and all species, except *O. pinnatifida*, *M. lichenoides* and *L. viscida*, failed to inhibit *S. cerevisiae* growth (>100%). All positive controls presented a microbial growth greater than 100%. The statistic Kruskal-Wallis test showed that there

are differences between *B. subtilis* growth in extracts according to algal species ($\chi^2(10) = 30.837$; $p = 0.001$), but no differences between *S. cerevisiae* growth in different algal extracts ($\chi^2(2) = 5.762$; $p = 0.056$).

Table 2. Minimum Inhibitory Concentration (MIC) and respective extract concentrations for twelve red seaweed species, assessed against *Bacillus subtilis* and *Saccharomyces cerevisiae*. PoUm: *Porphyra umbilicalis*, CeCi: *Ceramium ciliatum*, OsPi: *Osmundea pinnatifida*, ChCr: *Chondrus crispus*, SpCo: *Sphaerococcus coronopifolius*, PlCa: *Plocamium cartilagineum*, CoOf: *Corallina officinalis*, ElEl: *Elisolandia elongata*, AmRi: *Amphiroa rigida*, JaRu: *Jania rubens*, MeLi: *Mesophyllum lichenoides*, LiVi: *Liagora viscida*. Values are presented as means ± SD ($n = 4$), or (-) whenever all the concentrations tested yielded more than 100% bacterial growth. Asterisks (**) indicate statistically highly significant differences between the species with the lowest bacterial growth (MeLi) and all other remaining species (*B. subtilis*: Kruskal–Wallis test ($\chi^2(10) = 31.670$; $p = 0.000$)).

Species Code	*B. subtilis*		*S. cerevisiae*	
	MIC (mg·mL^{-1})	Growth (%)	MIC (mg·mL^{-1})	Growth (%)
PoUm	3.13	86.60 ± 2.12 **	-	-
CeCi	6.25	65.29 ± 8.56	-	-
OsPi	1.56	80.80 ± 2.69 **	12.5	81.98 ± 0.23
ChCr	12.5	73.35 ± 2.12	-	-
SpCo	0.02	80.82 ± 3.63 **	-	-
PlCa	3.13	84.03 ± 4.13 **	-	-
CoOf	6.25	83.14 ± 3.49 **	-	-
ElEl	-	-	-	-
AmRi	6.25	83.17 ± 1.48 **	-	-
JaRu	6.25	67.59 ± 1.42	-	-
MeLi	12.5	18.02 ± 2.49	1.56	11.91 ± 2.97
LiVi	6.25	73.31 ± 5.33	12.5	82.79 ± 3.60

On the other hand, complete transparency, comparable to that of the negative control, was found only for *M. lichenoides* extracts when tested against both *B. subtilis* and *S. cerevisiae*, being 12.5 and 1.56 mg·mL^{-1}, respectively; the lowest concentration where full transparency was recorded for this species, corresponding to 18.02 ± 2.97% and 11.91 ± 2.97% of *B. subtilis* and *S. cerevisiae* growth, respectively.

4. Discussion

In the present work, we assessed the antioxidant and antimicrobial activity of twelve seaweed species that regularly occur across the central coast of Portugal. Our target species, *Porphyra umbilicalis*, *Ceramium ciliatum*, *Osmundea pinnatifida*, *Chondrus crispus*, *Sphaerococcus coronopifolius*, *Plocamium cartilagineum*, *Corallina elongata*, *Ellisolandia elongata*, *Amphiroa rigida*, *Jania rubens*, *Mesophyllum lichenoides* and *Liagora viscida*, were generally available for gathering, depending on the season. The results regarding the antioxidant and antimicrobian activity of the twelve aqueous seaweed extracts analyzed showed distinct and interesting results across taxa, both for ABTS and TPC analysis, as well as the MIC evaluation. It is, however, of utmost importance to keep in mind that, since each species was collected in one specific season and sample area only (following their availability), and since species can show natural variability shaped by seasonal patterns, environmental gradients [55], and even life-cycle [70], in addition taxonomic classification itself, extrapolation endeavors and comparisons across studies must be sensibly conducted.

4.1. Species-Specific Notes

Algae can occur in complex habitats and sometimes in extreme environmental conditions, which play a role in shaping the development of defense strategies, such as metabolite production [63]. All species considered in the present study were harvested in the intertidal region, which, over the course of a single day, can suffer from a range of environmental shifts in temperature, light, salinity, air desiccation and water motion, depending on tidal

influence at any given time [71]. Especially near the upper limits of the intertidal area and during the low tide, seaweeds are exposed to drought, solar radiation, extreme salinities and temperature shifts [71–73]. For example, species such as *P. umbilicalis*, *C. crispus* and *O. pinnatifida* were collected in the upper intertidal region where they are out of water during low tide, exposed to air, and are, thus, fully exposed to all of the extreme conditions mentioned above. This scenario is, therefore, highly oxidative and both physically and physiologically stressful for most of the other algal species considered, which were collected in the lower portion of the intertidal region and have been mostly, if not at all times, fully submerged. *P. umbilicalis* present, by far, the highest phenol content when compared to the other studied algae, as well as a high scavenging ability. These results likely indicate strong antioxidant activity, as it is known that seaweeds are able to develop antioxidant shielding mechanisms and strategies to withstand highly oxidative environments [36,74].

Indeed, *P. umbilicalis* is a seaweed that stood out from the other red seaweed species in most analyses performed in the present study. In the ABTS assay, *P. umbilicalis* is able to scavenge ABTS+ with a lower extract concentration when compared to the majority of the species analyzed. Regarding antimicrobian activity, *P. umbilicalis* shows activity against *Bacilus subtilis*, but no activity recorded for *Escherichia coli* or *Sacharomyces cerevisiae*. One of the major constituints of the genus *Porphyra* is porphyran, which is a linear sulphated polyssacharide that can be extracted with water [75], beneficial to human health and with a diverse array of bioactivities, namely antioxidant [29]. It was also reported by Vega et al. [76] that *P. umbilicalis* had comparatively higher concentrations of polyphenols and Micosporin-like Aminoacids (MAAs) (from a selected pool of species analyzed), which were not only better extracted with water, but also showed antioxidant activity. Therefore, it remains to be seen which component(s) of our aqueous extracts of *P. umbilicalis* is (are) responsible for the bioactivities observed in the ABTS and the MIC assay, although phenols stand as the leading hypothesis, considering they are present in large amounts in this seaweed when compared to all other species analyzed. In addition to the environmental fluctuations previously discussed that may have possibly played a role in shaping the bioactivity potential of *P. umbilicalis*, we must also consider the relevance of taxonomic position, which perhaps may have influenced the significant difference between *P. umbilicalis* and all other species analyzed regarding the polyphenol content. While all the other red seaweed species presently considered belong to class Florideophyceae, *P. umbilicalis* is a Bangiophyceae, meaning it is an ancient algae shaped by the environment through the ages, and, thus, stands perfectly adapted today. Florideophyceae and Bangiophyceae are, thus, highly divergent [77], a fact that may have implications on their bioactivity potential.

C. ciliatum has shown bioactive potential, although not outstanding when regarding all the other species studied, presenting one of the highest EC_{50} values and being one of the species with the lowest polyphenol content. Regarding antimicrobian activity, the extracts of this seaweed inhibit the growth of *B. subtillis*, similarly to most other seaweed species. A previous study assessed the antimicrobial activity of ethanolic extracts of *C. ciliatum*, which also showed modest potential as an antibacterial and antifungal agent, when compared to a large pool of green, brown and red seaweeds [78]. Methanol, dichloromethane and *n*-hexane extracts of *C. ciliatum* also failed to inhibit *E. coli*, but the dichloromethane extract showed activity against *B. subtilis* [39], which was still of little import when compared to the greater results obtained for other algal species by the authors. *C. ciliatum* stands as an example of one seaweed species that is far from being explored or acknowledged in academic settings regarding the current topic, which may produce an opportunity to study it further.

O. pinnatifida is an edible species, widely common in central Portugal, and studies have unveiled its biological properties, namely antioxidant, antidiabetic and prebiotic. *O. pinnatifida* did not stand out regarding antioxidant activity reported in the ABTS assay; however, it showed an appreciable amount of polyphenols and was one of the few seaweeds that showed inhibitory ability against *B. subtilis* and *S.cerevisiae*. This is especially relevant in the MIC respective to *B. subtillis*, the growth of which was inhibited by a lower

concentration than what was generally required to produce the same effect, when taking into consideration the concentrations of all the other seaweed extracts currently analyzed. It remains to be assessed which specific compounds are responsible for this activity. Rodrigues et al. [79] shed light on the chemistry and structure of enzymatic extracts obtained from this Rhodophyta, finding the presence of agarans within this algae. *O. pinnatifida* has a reportedly high concentration of MAA, extracted with water, with a positive correlation with antioxidant activity [76]. These compounds, agarans or MAA, perhaps play a role in the activities found in the present study, although it is equally likely that this activity is caused by other groups of compounds, such as pigments.

The seaweed *C. crispus* showed potential in the ABTS assay, presenting a low EC50 value, while, in the TPC assay, it had a polyphenol content comparable to most of the remaining seaweeds analyzed. In the MIC assay, *C. crispus* extracts only inhibited *B. subtillis* growth; furthermore, it is one of the species that required the highest concentration to induce such inhibition, from all the species analyzed. Nevertheless, the aqueous extracts show potential for further study, specifically in discovering which compounds are responsible for the measured bioactivities. *C. crispus* is a well-known edible carrageenophyte species, holding many benefits for human health, which includes having great bioactivity potential [44–46,48]. The phenolic fraction of a number of carrageenophyte species reportedly holds antimicrobian activity against fungi [80]. It is noteworthy, however, that *C. crispus* stands as one example where the composition of a single compound, carraagenan (and consequent bioactivities it may hold), significantly varies between the tetrasporophyte and the gametophyte life stages, which are otherwise isomorphic [70].

Although the antioxidant activity of *S. coronopifolius* stood among the average values unveiled for all seaweed species, both for the ABTS and the TPC assay, this red seaweed stood out as the one with the lowest MIC against *B. subtilis* (although it failed to inhibit both *E. coli* and *S. cerevisiae* growth). *S. coronopifolius* is a fairly well-studied seaweed in the academic context, although it remains to be as acknowledged as seaweeds such as *P. umbilicalis* and *C. crispus*. Regarding its bioactive potential, *S. coronopifolius* has been widely reported as a source of important metabolites with biological activity, namely antioxidant [81], antimicrobial [41], anti-inflammatory [82] and anti-tumoral activities [41,50,51,82]. A previous study reported the noteworthy antimicrobial activity of *S. coronopifolius* compared to a range of selected seaweed species, whose range of extracts with different solvents (*n*-hexane, dichloromethane and methanol) inhibit the growth of *S. cerevisiae*, while only the *n*-hexane extract inhibits the growth of *B. subtilis* and, similarly to our study, showed no inhibition against *E. coli* [39]. Terpenes, commonly found in brown algae, but less so in red algae, have been isolated from *S. coronopifolius* and targeted in several studies evaluating their potential as anti-cancer [51,83–86], antibiotic [87] and anti-fouling [88–90] agents. The variations found in the present study are probably explained by the different extraction method, where only aqueous extracts were tested.

P. cartilagineum is an algae that showed activity in both antioxidant and antimicrobial assays. Although the values achieved were not particularly outstanding, it presented polyphenols in amounts comparable to those found in the majority of the species analyzed. Similarly to the other species, it presented activity neither against *E. coli* nor *S. cerevisiae*, but inhibited *B. subtilis* growth. *P. cartilagineum* lacks dedicated studies regarding its bioactivity potential, thus offering an opportunity to explore it further. Authors have shown the antiproliferative activity of the dichloromethane extracts of *P. cartilagineum* [49] or studying the terpenes *P. cartilagineum* produces, with authors isolating and characterizing these compounds [91–94]. A number of authors also found that the Antarctic *P. cartilagineum*, being completely adapted to polar environments, produces specific metabolites that can potentially be used in skin therapeutics [95].

The coralline algae (*C. officinalis*, *E. elongata*, *A. rigida* and *J. rubens*) showed similar results in most analyses, and thus are discussed together. The whole group shows the lowest antioxidant activity by the ABTS assay when compared to all the other species analyzed. All coralline algae, except *J. rubens*, showed similar polyphenol quantities. Regarding

antimicrobial activity, *C. officinalis*, *A. rigida* and *J. rubens* required the same concentration of the aqueous extract to inhibit growth, while *E. elongata* showed no antimicrobial activity at all. None of these algae presented activity against *E. coli* or *S. cerevisiae*. Coralline seaweeds have shown potential as a dietary calcium supplement to promote bone health [60–62]. Other authors have unveiled the antimicrobial potential of dichloromethane extracts of *C. officinalis* against *E. coli* [96], the antioxidant [97,98], anti-inflammatory and anticancer potential of methanolic extracts of *J. rubens* and *E. elongata* (formerly *C. elongata*) [98], and the insecticidal [99], anti-tumour [100] and anti-bacterial [101] potential of the methanolic, organic and ethanolic extracts, respectively, of *J. rubens*.

M. lichenoides is a seaweed that, although scarcely studied, showed promise in most analyses performed in the present study. Specifically, the ABTS results show its potential as an antioxidant comparable to that found for coralline algae, which, as previously mentioned, presented the most promising results. However, in the TPC assay it shows a lower phenolic content when compared to the other species analyzed. Hence, compounds other than phenols are responsible for this bioactivity. As for its antibacterial potential, *M. lichenoides* is one of the few three seaweed species whose aqueous extracts can severely inhibit the growth of both *B. subtilis* and *S. cerevisiae*. *M. lichenoides* shows quite interesting results, further worth researching, especially given that this seaweed has not yet been given attention or focus regarding its bioactive potential, as far as we are aware. The few studies available seem to mostly focus on the distribution and systematics of the entire genus *Mesophyllum* [102,103].

L. viscida is the seaweed that presented the lowest antioxidant potential of all the species studied, showing the highest EC_{50} values, and the lowest polyphenol content, when compared to the overall results presently obtained. Similarly to most species studied, *L. viscida* aqueous extracts inhibit *B. subtilis* growth; however, *L. viscida* is one of the three species that inhibit *S. cerevisiae* growth as well. These results may indicate that *L. viscida* is worth further study within the scope of microbiology, as this seaweed has previously shown antimicrobial activity against bacteria and fungi [104]. To this day, and considering the currently studied species, *L. viscida* remains one of the seaweed species severely lacking research focused on its biotechnological potential.

The results obtained in the present work can be summarized by noting the species that stood out in each assay: *Corallina officinalis* and *Porphyra umbilicalis* for the highest antioxidant potential, measured by the ABTS and the TPC assay, respectively, and *Sphaerococcus coronopifolius* and *Mesophyllum lichenoides* for the highest antibacterial potential against, respectively, *Bacillus subtilis* and *Saccharomyces cerevisiae*. All of the species showed quite noticeable antioxidant or antimicrobial activities, but generally performed far better in either the antioxidant or the antimicrobial assessment, but not both at once. There are questions that remain unanswered, namely the nature of the metabolite(s) accountable for the measured activities, and whether changing the extraction method or solvent would yield different results.

4.2. Additional Considerations

The importance of seaweeds as bioactive compound providers is supported by the efficiency of the chosen extraction method [79]. The extracts in the present study were obtained after drying the seaweed biomass at 25 °C in order to preserve its properties. The adoption of higher temperatures or aggressive solvents may compromise these properties, deteriorate sensitive and unstable compounds, and, in turn, compromise the bioactivities they may eventually possess [55]. Novel, efficient and conservative extraction techniques that optimize time, yield, cost and sustainability are mostly desired and are being researched. In the present work we performed a simple, sustainability-focused water extraction at room temperature. Therefore, we question whether applying popular extraction techniques, such as ultrasound, enzymes or supercritical fluids, among other techniques, would both enhance extract yields, still preserve the bioactivities found and still remain sustainable and safe. We are also aware that aqueous extractions probably target a very specific group

of water-soluble metabolites with high polarity, leaving out compounds with medium to low polarity that are only extracted with a different range of solvents. Therefore, it remains to be investigated whether applying a wider selection of solvents would yield different results, not only with respect to bioactivities (since we would be targeting a completely different class of metabolites), but also in terms of extraction yields, and still remain environmentally friendly.

Rhodophyta is one of the largest groups of macroalgae and contains many compounds that are diverse in both structure and bioactivity [105]. One sound example of such compounds, common to all Rhodophyta, are phycobiliproteins (PBP), which constitute a major portion of the red algal cell proteins (which in turn compose a high percentage of the red algal dry biomass). PBP are known to have a wide range of biological activities, easily extracted with water or phosphate buffers, but are highly unstable when exposed to light and high temperatures. Therefore, extraction methods that specifically target this class of compounds must be wisely weighted [106], and we are currently making progress on this specific topic. Additionally, future steps call for the structural clarification of the bioactive components that are responsible for the activities found in the present study. Characterization of bioactive components in seaweeds is a field yet largely unexplored, but one that is mostly needed, given that macroalgae are not only key part of the traditional diet of many eastern countries, but are also becoming a global trend in the human diet.

5. Conclusions and Final Considerations

Humankind has sought to adopt a healthier lifestyle by reining in unruly dietary impulses and instead choosing natural and healthier regimes, to ultimately achieve a better quality of life. We have been quickly finding out it is accessible, beneficial and affordable to adopt a diet consisting of natural food sources, filled with multiple benefits to the human body, to replace the once popular but unhealthy fast foods and overly processed meals. As such, many entrepreneurs have seen this as an opportunity to adapt traditional methods of seaweed exploration into a more industrialized setting to quickly and effectively obtain products from natural sources, which can be then easily accessed by whoever wishes it. Either by implementing large-scale production of seaweed biomass, or by developing effective methods to attain high yields of metabolites from seaweeds, or studying which species and metabolites are worth investing in, steps have been taken worldwide by many contributors in order to achieve this goal.

All of the species studied in the present work occur in relative abundance throughout the Portuguese coast, yet only a handful of them have been targeted in academic studies, and fewer have been explored from a biotechnological perspective. Addressing the bioactivity potential of these species may prove useful in order to change the current perspective most people hold towards seaweed exploration. We believe that this work complements a previous assessment we conducted regarding these species, which addresses their primary composition and pigments [107] and offers motivation and opportunities to study them further. Moreover, we are developing small-scaled farming protocols targeted toward a number of these species that are not yet explored from a commercial culture perspective, to contribute to sustainable exploitation in the future.

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