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Antimicrobial activity and lipid profile of seaweed extracts from the North Portuguese Coast

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Abstract

Seaweeds or marine macroalgae are a potential renewable resource in the marine environment and can represent a source of new natural compounds for human nutrition. This experimental work focused on the selection of a suitable extraction method, using *Gracilaria vermiculophylla* as a model, followed by the characterization of the antimicrobial activity of different solvent extracts of *Gracilaria vermiculophylla*, *Porphyra dioica* and *Chondrus crispus*, both from wild and from an integrated multi-trophic aquaculture system (IMTA), collected in the north of Portugal. Additionally, the fatty acid profile of the seaweed extracts was also investigated. Extraction tests included the definition of the physical state of the seaweed, temperature and type of solvents to be used during the process. Results revealed that test organisms (Gram negative and Gram positive bacteria as well as one yeast species) were more sensitive to extracts obtained with dried algae, processed continuously at higher temperatures. Results from antimicrobial activity of wild and IMTA seaweed extracts showed stronger antimicrobial activity in extracts of ethyl acetate when compared with those from methanol and diethyl ether; furthermore, among the type of microorganisms tested, there was tendency for inhibition of the Gram positive ones. In general, there appears to be a higher antimicrobial activity for the microorganisms under study in extracts obtained from aquaculture species, when compared with the wild ones. The fatty acid profile of ethyl acetate extracts revealed a predominance of saturated fatty acids (SFA), especially palmitic acid (16:0), followed by polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA). A higher content of fatty acids in aquaculture extracts of *Gracilaria vermiculophylla* and *Porphyra dioica* was also observed.

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Introduction

It is known that algae are part of the diet of Asian countries such as China and Japan since ancient times (Sachindra *et al.*, 2009). In these locations, there is evidence that the incidence of some types of cancer, such as breast and prostate cancers, is lower due to regular consumption of seaweeds, the main reason being their richness in bioactive compounds that may protect against those diseases (Vijayavel and Martinez, 2010; Kumar *et al.*, 2011). In fact, algae have large amounts of vitamins, minerals, fibers, proteins with great nutritional value and compounds with antioxidant and antimicrobial properties, which can inhibit the growth of some pathogenic bacteria, as well as some yeasts (Taskin *et al.*, 2007). The use of marine algae as potential sources of cosmetic and pharmaceutical agents as well as an important source of food has been increasing recently since they contain bioactive compounds rich in carotenoids, essential fatty acids, polysaccharides (e.g. alginates, carrageenan and agar) and antioxidants with potential to replace synthetic compounds such as BHA (Butylated hydroxyanisole) and TBHQ (tert-Butylhydroquinone) considered less healthy for people (Patra *et al.*, 2008).

In recent years there has been an increase of the resistance of microorganisms to antibiotics that are usually used in the treatment of some diseases. To overcome this problem, new therapeutic drugs from natural products have been explored (Sasidharan *et al.*, 2010). Thus, marine organisms appear as an efficient alternative source of new drugs and algae have been extensively documented for their capacity to provide a rich source of primary and secondary metabolites (Tuney *et al.*, 2006). Actually, there are several substances obtained from algae that are already in use in traditional medicine for a long time (Taskin *et al.*, 2007). For example, red algae (Rhodophyta) are able to synthesize halogenated metabolites such as ketones, low molecular weight hydrocarbons and phenols (Carvalho and

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Roque, 2000; Taskin *et al*., 2007), which have been associated with antibiotic – Gram negative and Gram positive strains, antiviral, antifungal or anti-inflammatory activities (del Val *et al.*, 2001).

Although there are numerous studies published on antimicrobial activity of algae extracts, they usually report results of algae collected from their natural habitats. In fact, to our best of knowledge, there is no data concerning the antimicrobial potential of algae from aquaculture systems. Since in this environment algae are placed in nutrient rich waters (organic and inorganic), likely with a higher microbial load, these conditions may modify their metabolic behaviour and lead to an increase in the production of such interesting compounds (Bansemir *et al.*, 2006).

Based on the above rationale this research study's objectives were: (i) to establish an improved procedure to obtain extracts from seaweeds and (ii) to test the antimicrobial activity of the prepared extracts on Gram positive and Gram negative bacteria, as well as on a yeast strain. It is important to notice that extracts were prepared from 3 species of seaweed collected from both the sea and from integrated multi-trophic aquaculture systems (IMTA). Furthermore, it was attempted to characterize the lipid profile of the seaweed extracts to try to understand if there is any relation between these components and the antimicrobial activity reported.

Materials and methods

Algae material

Seaweeds used in this study were provided by CIIMAR (Centre of Marine and Environmental Research of the University of Porto) and collected in the North of Portugal. Species studied were *Gracilaria vermiculophylla*, *Porphyra dioica* and *Chondrus crispus* (Rhodophyta), from both an IMTA system and collected along the coast during the period between December and March 2011.

Briefly, seaweeds from the IMTA system were produced in polyethylene circular tanks (volume 1200 L), with bottom aeration, in the facilities of A. Coelho e Castro, Lda. (Póvoa do Varzim, Portugal). A more detailed description of the IMTA system used can be found in Abreu *et al.* (2011). Wild seaweeds were collected from the intertidal zone, during low tide in Vila do Conde, Portugal (41º18'37''N; 8º44'32''W). All the samples were collected in plastic bags, transported at low temperatures in isothermal boxes and brought to the laboratory in order to be washed with fresh water, to remove all the epiphytes, necrotic parts and suspended materials.

Selection of the extraction procedure

Part of the *G. vermiculophylla* sample was dried at 37ºC for about 12 hours and shredded, whereas the remaining fresh part was immediately extracted.

Fresh and dried samples of *G. vermiculophylla* were tested at boiling and room temperatures (25 ± 1ºC), using ethyl acetate as extraction solvent. This solvent was selected because it had demonstrated the most promising results in previous extraction studies when compared with other solvents such as hexane and acetone (data not shown). Extracts at room temperature (Troom) were prepared by weighing 15 g of fresh or dried algae into a closed bottle wrapped with aluminium foil (to protect from light deterioration); 50 ml of ethyl acetate (Merck, Germany) were added and the mixture was kept under agitation for 72 hours. At the end, extracts were filtered using Whatman nº1 filter paper.

Extracts of *G. vermiculophylla* at higher temperature (hot extracts) were obtained by weighing the same amounts of algae as for Troom extracts (fresh and dried), and refluxing 200 mL of ethyl acetate in a Soxhlet apparatus for about 18 hours using filter paper for the cartridge. Final extracts were evaporated under reduced pressure with a roto-evaporator and re-dissolved in Dimethylsulfoxide - DMSO (Sigma-Aldrich, Missouri, USA).

Preparation of algae extracts for antimicrobial activity tests

Seaweed samples were dried at 37ºC for about 8-12 hours (depending on the algae morphology), shredded with a food processor and kept in the dark until further use. Extracts from dried *G. vermiculophylla*, *P. dioica* and *C. crispus*, both from aquaculture and wild regimes, were prepared using solvents with different polarity: diethyl ether (P'=2.8), ethyl acetate (P'=4.4) and methanol:H_2O (1:1) (P'=5.1:9.0) (Sigma-Aldrich, Missouri, USA). Briefly, 15 g of each seaweed sample were extracted in 200 mL (in duplicate) of each solvent, using a Soxhlet apparatus, during approximately 18 hours using filter paper for the cartridge. At the end of each extraction procedure the solvents were evaporated, and the dry extract was re-dissolved in DMSO and stored at -30ºC until use, as described by Lekameera *et al*. (2008). Concentrations of all the extracts prepared were ca. 500 g/L.

Antimicrobial activity determination

To evaluate the antimicrobial activity of the seaweed extracts, the following microorganisms from food origin were tested: Gram negative - *Escherichia*

coli (ATCC 8739), *Salmonella enteritidis* (ATCC 3076), *Pseudomonas aeruginosa* (ATTC 10145); Gram positive - *Listeria innocua* (NCTC 11286), *Bacillus cereus* (ATCC 11778), *Enterococcus faecalis* (LMG S 19456 5002), *Lactobacillus brevis* (LMG 6906), *Staphylococcus aureus* (ATCC 6538). A strain of methicillin-resistant *Staphylococcus aureus* from Instituto Português de Oncologia (IPO) and the yeast *Candida* spp. (CCUG 49242), both from clinical origin, were also tested.

All bacteria were cultivated and stored in Brain Heart Infusion broth (BHI) or agar (BHI agar) (BD, Maryland, USA) except for *Enterococcus faecalis* and *Lactobacillus brevis* that were cultivated and stored in Nutrient Broth (NB) or Nutrient Agar (NA) (Oxoid, England), and for Candida spp. that used either Sabouraud Broth or Agar (Difco, Michigan, USA), according to requirements. The agar diffusion method was used to assess the antimicrobial activity of the extracts: overnight cultures of the microorganisms were adjusted to 0.5 of McFarland standard (1.5 x 10^8 CFU/mL) before spreading 100 µL of the culture broth on the respective culture medium plate; then, seaweed extracts were applied directly on seeded agar plates using the drop method (20 µL). Negative control included was performed with DMSO whereas positive controls on the plate were checked with ampicillin at 1000 µg/mL (Sigma-Aldrich, Missouri, USA) for *L. innocua*, cycloheximide at 1000 µg/mL (Sigma-Aldrich, Missouri, USA) for *Candida* spp. and chloramphenicol at 1000 µg/mL (Sigma-Aldrich, Missouri, USA) for the remaining microorganisms. Lactic acid 30% (Fluka, Missouri, USA) was also used as a complementary positive control for all the microorganisms.

All tests were performed under sterile conditions and each combination microorganism/extract was prepared in quadruplicate. Plates were incubated at 37ºC for 16 hours and examined for growth inhibition zones around the drop points. Antibacterial activity was evaluated by measuring the diameter (in mm) of inhibition zones against the test microorganisms.

Determination of lipid profile

To determine the lipid profile of the samples Lepage and Roy method (1984), modified by Carvalho *et al*. (2006), was used for sample derivatization for gas chromatography (GC). Thus, 1 mg of internal standard (heptadecanoic acid) and about 200 mg of algae extract were added to a Teflon-capped Pyrex tube. Then, 2 mL of a freshly prepared mixture of acetyl chloride in dried methanol (5:100, v/v) was also added. Nitrogen was injected into each tube to remove oxygen; tubes were closed, protected from the light with aluminium foil and heated in a heating block at 90-100ºC for 1 hour. After cooling to 30 - 40ºC in the dark, 1 mL of hexane (with 0. 01% BHT) was added and mixed in vortex for a few seconds. Subsequently, 1 mL of pure water was added, the tubes were mixed gently to allow the phases to separate, and the upper phases were filtered and collected into GC vials. Each extract sample was tested in duplicate.

Analysis of fatty acid methyl esters was performed in a gas chomatograph AutoSystem XL from HP (California, USA), equipped with a flame ionization detector. The separation was performed in a Supelcowax-10 (60 m, 0.32 mm and 0.25 µm) column from Supelco (Pennsylvania, USA). The temperature was programmed to increase from 170 to 220ºC at a rate of 1ºC min^{-1}; the injector and detector temperatures were 250 and 270ºC, respectively. Injections were performed under splitless mode, using helium as a carrier gas. Calculations of the peak areas were performed according to the AOCS official method Ce 1b-89. Pure standards (Sigma-Aldrich, Missouri, USA) were used for fatty acid identification, which was based on a comparison of peak retention times between samples and standards.

Statistical analysis

To evaluate the normality of the distributions, the Kolmogorov-Smirnov test was used. The One Way ANOVA test in association with Scheffe's test was used when a normal distribution was observed between the differences in sample groups. The differences were considered statistically significant at a 5% confidence degree level. All statistical analysis was performed using IBM SPSS Statistics v.19.0.0 (New York, USA) software.

Results and Discussion

Selection of the extraction procedure

Considering that the properties of the extracts depend on the conditions in which they were prepared, the selection of the extraction procedure was the first logical step to be performed. Therefore, since most of the consulted literature referred extractions with organic solvents, this was the line of research followed in this study (Patra *et al.*, 2008; Demirel *et al.*, 2009).

Preliminary tests were performed with *G. vermiculophylla* from an IMTA system, testing simultaneously two parameters: temperature (room temperature or boiling temperature of the solvent) and physical state of the seaweed (fresh or dried). The type of solvent was not under scrutiny within

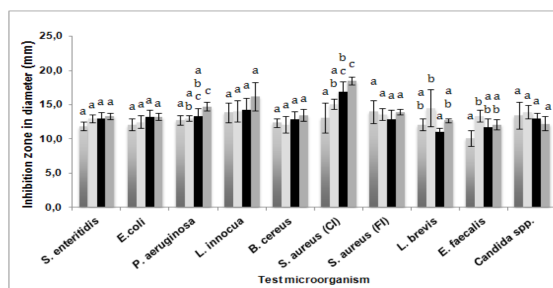
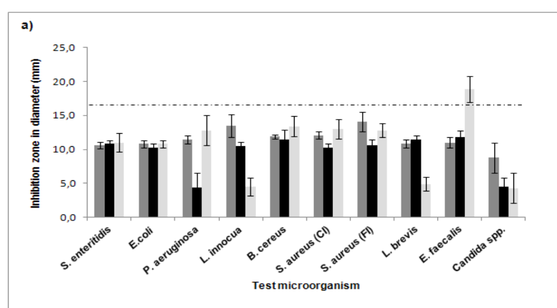


Figure 1. Effect of type of processing and physical state (▪ T_{room}, Fresh extraction; ▪ Hot, Fresh extraction; ■ T_{room}, Dry extract; ▪ Hot, Dry extract) of *G. vermiculophylla* extracted with ethyl acetate on test microorganisms. Inhibition zones marked with the same letter are not significantly different ($p > 0.05$). (CI = Clinical Isolate; FI = Food Isolate).



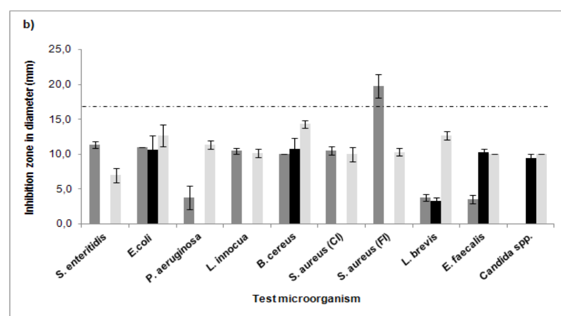
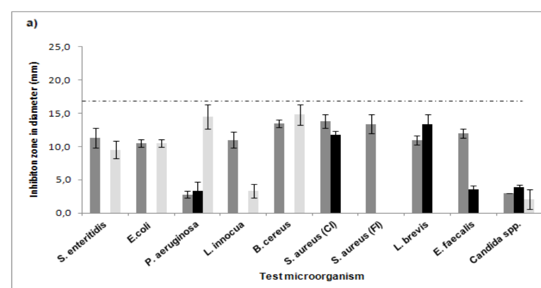


Figure 2. Effect of ethyl acetate extracts of (▪) *G. vermiculophylla*, (■) *P. dioica* and (▪) *C. crispus* from (a) IMTA and (b) wild regimes on the inhibition of test microorganisms (the absence of columns indicates that the specific extract had no inhibitory action on corresponding test organism). The dotted line represents the average inhibition zone of the positive control (lactic acid at 30% (v/v) - 17 mm).

this step and all the extractions were obtained with ethyl acetate. When drying process was employed, the maximal temperature used was 37ºC, as it has been described that at higher temperatures (e.g. 60ºC) little or no activity against some microorganisms was shown (Mtolera and Semesi, 1996). Results revealed that the most effective extracts were those prepared with dried form of the seaweed, since these presented a larger microbial growth inhibition zone (data not shown). These results were not in accordance with Tuney *et al.* (2006), who observed less or no effects of dried seaweed extracts (i.e *G. gracilis*) on bacteria, when compared to the fresh counterparts. However,



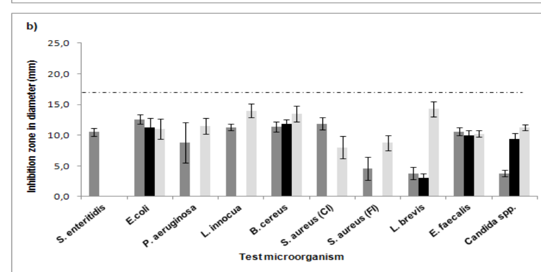
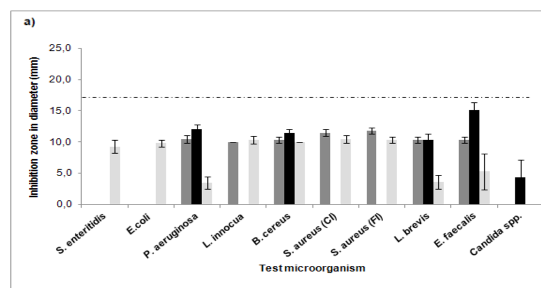


Figure 3. Effect of diethyl ether extracts of (▪) *G. vermiculophylla*, (■) *P. dioica* and (▪) *C. crispus* from (a) IMTA and (b) wild regimes on the inhibition of test microorganisms (the absence of columns indicates that the specific extract had no inhibitory action on corresponding test organism). The dotted line represents the average inhibition zone of the positive control (lactic acid at 30% (v/v) - 17 mm).



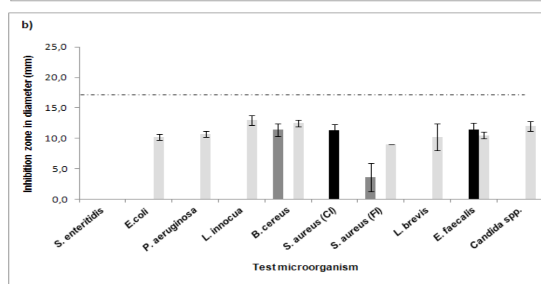


Figure 4. Effect of methanol:H_2O extracts of (▪) *G. vermiculophylla*, (■) *P. dioica* and (▪) *C. crispus* from (a) IMTA and (b) wild regimes on the inhibition of test microorganisms (the absence of columns indicates that the specific extract had no inhibitory action on corresponding test organism). The dotted line represents the average inhibition zone of the positive control (lactic acid at 30% (v/v) - 17 mm).

this result could probably be related to the loss of volatile antimicrobial compounds present in fresh algae (hydrogen peroxide, terpenoid and bromo-ether compounds and volatile fatty-acids) during the drying process at high temperatures employed.

Secondly, in order to pinpoint the extraction

temperature to be used Troom and high temperature extractions with ethyl acetate of fresh and dried *G. vermiculophylla* (also from IMTA regime) were tested. Results of these tests are presented in Figure 1 and show that extracts obtained with dried algae processed at high temperatures (with Soxhlet apparatus) presented wider inhibition zones for some of the tested microorganisms: *S. enteritidis*, *P. aeruginosa*, *L. innocua* and both clinical (CI) and food isolates (FI) of *S. aureus*. However, there were no significant differences between the four tested methods. Unlike results reported by Lima-Filho *et al.* (2002) who stated that antimicrobial activity can be influenced by the physical state of the different seaweed. Based on the preliminary tests' results, the extraction method chosen for the subsequent tests included high temperature (Soxhlet apparatus) and dried form of which parallels algae, the extraction method of Lekameera *et al.* (2008); the reasoning for such choice was based not only on these results but also on evidence published in the scientific literature, where different authors stated that higher temperatures increased the permeability of cell membranes facilitating the solvent passage through cells and cellular organelles and thus increasing the amount of extracted compounds (Liu *et al*., 2003; Franco *et al.*, 2007).

Antimicrobial activity

Upon selection of the most suitable extraction method, three extraction solvents of different polarity (diethyl ether<ethyl acetate<methanol:H_2O) were tested, using the drop method as previously described. Results obtained in these tests are synthesized in Figures 2, 3 and 4. Considering these figures, it is possible to observe that, in general, ethyl acetate extracts (Figure 2), mainly from IMTA system (a), showed a higher inhibition activity when compared with the other extraction solvents and cultivation regime for the majority of the tested organisms. Besides, for many of these organisms, there was no inhibition zone in the diethyl ether and in the methanol extracts. For example, the lack of inhibition can be demonstrated in diethyl ether extracts of aquaculture *P. dioica* (Figure 3-a) against *S. enteritidis*, *E. coli*, *L. innocua*, *B. cereus* and *S. aureus* (FI), and of wild *P. dioica* (Figure 3-b) against *S. enteritidis*, *P. aeruginosa*, *L. innocua* and *S. aureus* (FI and CI); diethyl ether extracts of *C. crispus* from IMTA regime (Figure 3-a) against *S. aureus* (FI and CI), *L. brevis* and *E. faecalis* and of wild *C. crispus* (Figure 3-b) against *S. enteritidis.* With regard to the methanol:H_2O extract of IMTA algae (Figure 4-a) this lack of inhibition, in the case of *G. Vermiculophylla*, can be seen against *S. enteritidis*, *E. coli* and *Candida* spp.; for *P. dioica* in the cases of *S. enteritidis*, *E. coli*, *L. innocua* and *S. aureus* (FI and CI); and for *C. crispus* it can be seen against *Candida* spp. In what concerns the (Figure 4-b) wild methanol:H_2O extracts, no inhibition can be found in the case of *G. vermiculophylla* for almost all the microorganisms tested with the exception of *B. cereus* and *S. aureus* (FI); *P. dioica* only could inhibit *S. aureus* (CI) and *E. faecalis*, and in what concerns *C. crispus*, it could inhibit all microorganisms tested with the exception of *S. enteritidis* and *S. aureus* (CI).

It should also be noted that, with the exception of the IMTA *C. crispus* (Figure 3-a) and wild *G. vermiculophylla* (Figure 3-b), both extracted with ethyl acetate, no extract tested exceeded the inhibition of the general positive control (lactic acid at 30% (v/v)), which was placed on average at 17 mm. Besides, statistical analysis showed that although there were some differences between the inhibition values, such differences were not statistically significant (data not shown).

The present experimental results are in agreement with those stated by Salem *et al.* (2011) that determined that ethyl acetate was the best solvent for isolation of antimicrobial compounds when compared with methanol. However, Tuney *et al*. (2006) stated that diethyl ether was the best solvent for extracting antimicrobial compounds from seaweed when compared with methanol, acetone and ethanol. These different positions indicate that the extraction method as well as the solvent used, do affect the isolation of bioactive compounds. The differences observed between results may be due to the different seaweed species tested, time/season and place of sample collection, differences within the method used and its capability of extracting active metabolites, and possible differences on the susceptibilities of the microbial strains used (Salem *et al.*, 2011).

However, some extracts had no inhibitory effects on some of the tested microorganisms (e.g. some *P. dioica* extracts). This result could be explained by the possible insolubility of certain bioactive compounds in some solvents. As ethyl acetate was the solvent with an intermediate polarity in this study, it was probably the one that could extract more bioactive compounds. Organic solvents have been commonly used to extract the lipid-soluble active compounds of seaweeds since it is stated that they can provide a significant increased efficiency when compared to water-based solvents (Stirk *et al*., 2007). Nevertheless, organic solvents themselves also present an inhibitory effect. For that reason, after the extraction and evaporation, extracts were re-dissolved in DMSO, which was then

used as a negative control in the antimicrobial tests. This compound is a polar aprotic solvent, miscible in a wide range of organic solvents as well as in water. In addition, one of the most important characteristics is that it is not toxic to cells, so it will not act as an inhibitor (Pope and Oliver, 1966).

Overall, it can be stated that the ethyl acetate extracts appear to have a higher effect on all microorganisms tested (as stated by Patra *et al*., 2008; Salem *et al*., 2011) and the extracts from the IMTA regime *G. vermiculophylla* present, in general, a higher inhibitory effect followed by IMTA *C. crispus.* This effect appears to be more evident against Gram positive bacteria than against Gram negative ones, which is in agreement with that described by several authors (Demirel *et al.*, 2009; Salem *et al*., 2011); *Candida* spp. showed, in general, smaller inhibition zones for almost all types of extracts tested, which is consistent with the findings of Mtolera and Semesi (1996). The higher susceptibility of Gram positive bacteria to the seaweed extract when compared to Gram negative strains could be due to the differences in the cell wall structure and composition, since the outer membrane and the thick murine layer of Gram negative bacteria act like a barrier, preventing the entrance of environmental substances such as antibiotics and inhibitors (Salem *et al*., 2011).

Furthermore, in general, there seemed to be wider inhibition zones for the microorganisms under study in extracts obtained from aquaculture species, when compared with the wild counterparts. It seems that the extracts from IMTA regime appeared to contain more compounds with the capacity to inhibit the growth of the tested microorganisms. This may be due to the fact that seaweed in integrated aquaculture systems are subjected to particular conditions, since they are in contact with a large amount of compounds, both inorganic nutrients and organic compounds, arising from the breeding fish tanks. Other conditions that can have an effect on the seaweed may be the constant water motion through bottom aeration in the tanks or the exposure to higher light intensities during larger periods of time. This fact could affect seaweed metabolism and may cause the production of different amounts of metabolites, or even new ones, particularly secondary metabolites, which include antimicrobial compounds (Bansemir *et al.*, 2006).

Lipid profile of the extracts

Seaweeds, in general, and red marine algae, in particular, are a rich source of bioactive compounds such as specific fatty acids, e.g. polyunsaturated fatty acids (PUFA) from ω-3 and ω-6 series; consequently, they could be an alternative valuable source of these compounds for human and animal health (Gerasimenko *et al.*, 2010; Khotimchenko, 2005). Although lipid content in marine algae is usually low (less than 4%) (Gressler *et al.*, 2011), their PUFA content is superior to that of terrestrial vegetables (Kumari *et al*., 2010). Some PUFA, such as arachidonic (AA) and eicosapentaenoic (EPA) acids, have an enormous interest because they are precursors for the biosynthesis of regulating/signalling molecules like prostaglandins, thromboxans and other bioregulators of many cellular processes (Khotimchenko, 2005) and docosahexaenoic acid (DHA) is essential for visual and neurological development (Kumari *et al*., 2013). Furthermore, some fatty acids such as palmitic and eicosapentaenoic acids are described as possessing antimicrobial properties (Shin *et al*., 2007).

In order to characterize the lipid compounds present in the seaweed extracts under study, their fatty acid profile was evaluated. Chromatograms from methanol and diethyl ether extracts were very poor in terms of fatty acids (data not shown), so fatty acid extract analysis was concentrated mainly on ethyl acetate extracts. Results are presented in Table 1 where fatty acids were organised into saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids groups for better understanding.

From analysis of Table 1, it is noticeable that in general, all algae extracts presented higher amounts of saturated fatty acids (SFA) when compared with the other two fatty acids groups. These results are in agreement with the findings of Bhaskar *et al.* (2004), Kumar *et al.* (2011) and Pereira *et al.* (2012).

Ethyl acetate extracts of wild *C. crispus* had the highest quantity of total fatty acids (21942. 4 µg/g sample) followed by IMTA and wild *G. vermiculophylla* and IMTA *C. crispus*, in descending order of magnitude. The lowest values correspond to *P. dioica*, both from wild and IMTA system origins. In general, the IMTA algae had higher total fatty acid values than the wild types except for *C. crispus* where the opposite correlation was observed. These results do not show a clear trend with respect to the different regime of algal culture, although it appears that seaweed extracts from IMTA origin generated the highest quantity of total fatty acids if we exclude the wild *C. crispus.* This follows the trend observed previously in the antimicrobial activity where IMTA extracts demonstrated a higher activity. This tendency may be related with the fact that algae cultured in aquaculture regime are subjected to particular conditions that may lead to the production of larger quantities of different compounds in order to protect themselves from external dangers (Bansemir *et al*., 2006).

Table 1. Fatty acid content (µg/g sample) of the different ethyl acetate algae extracts from both IMTA and wild regimes

Fatty Acids	***G. verm.* IMTA**	***G. verm.* wild**	***P. dioica* IMTA**	***P. dioica* wild**	***C. crispus* IMTA**	***C. crispus* wild**
12:0	nd	nd	nd	11.2±0.5^{a}	16.4±0.2^{a}	138.0±17.0^{b}
14:0	1719.5±6.6^{a}	1318.9±45.7$^{a, d}$	457.5±384.2^{b}	111.5±1.9^{b}	874.9±1.4$^{b, c}$	1111.9±48.9$^{c, d}$
16:0	9859.7±67.7^{a}	8166.8±46.7^{a}	3141.0±833.5^{b}	3442.3±40.1^{b}	3493.0±11.5^{b}	8988.5±754.6^{a}
18:0	351.7±35.3^{a}	346.0±10.5^{a}	96.8±45.9^{b}	123.5±0.3^{b}	130.0±7.2^{b}	nd
20:0	19.1±4.5^{a}	19.2±1.3^{a}	9.3±5.6^{a}	4.3±0.3^{b}	14.7±0.7^{a}	33.1±35.7^{c}
21:0	nd	nd	13.0±5.7^{a}	6.7±0.7^{a}	9.7±0.6^{a}	nd
22:0	25.4±0.2^{a}	20.6±2.6^{a}	nd	11.5±0.4^{a}	nd	28.0±8.4^{a}
Total SFA	**11975.4**	**9871.5**	**3717.5**	**3711.0**	**4538.6**	**10299.5**
14:1	34.7±1.2^{a}	23.9±0.6$^{a, b}$	nd	15.3±0.5$^{a, b}$	15.3±0.2$^{a, b}$	27.2±0.5$^{a, b}$
16:1 (*n*-7)	585.5±31.5^{a}	166.8±24.4^{b}	201.3±15.2^{b}	22.6±1.7^{d}	305.0±21.8^{c}	182.0±7.2^{b}
18:1 (*n*-9) *cis*	807.8±21.7$^{a, b}$	845.6±38.2$^{a, b}$	149.2±63.8^{b}	133.5±24.8^{b}	323.6±20.2^{b}	1841.1±586.8^{a}
20:1 (*n*-9)	24.7±2.1$^{a, b}$	50.6±6.6^{a}	38.9±14.5^{a}	34.4±4.0^{a}	3.7±1.2^{b}	29.8±5.2$^{a, b}$
22:1 (*n*-9)	21.7±43.0^{a}	20.6±5.4^{a}	12.9±4.4^{a}	16.6±0.6^{a}	21.8±2.5^{a}	65.7±1.4^{b}
Total MUFA	**1474.5**	**1107.5**	**402.3**	**222.4**	**669.4**	**2145.9**
18:2 (*n*-6)	178.5±10.1^{b}	126.0±5.7^{a,b}	123.8±77.4$^{a, b}$	52.8±14.0$^{a, b}$	37.9±3.8^{a}	166.6±23.6^{b}
18:3 (*n*-6)	83.8±5.5^{a}	42.2±2.0^{b}	29.7±21.2$^{b, c}$	3.6 ±1.2^{c}	25.2±3.6^{b,c}	73.0±1.5^{a}
18:3 (*n*-3)	98.5±6.7^{a}	19.0±0.9^{b}	22.8±13.3^{b}	7.5±0.3^{b}	26.4±3.9^{b}	65.9±2.2^{c}
20:2 (*n*-6)	15.7±0.4$^{a, b}$	18.9±5.7$^{a, b}$	36.4±17.9^{a}	41.1±3.8^{a}	nd	17.0±5.5$^{a, b}$
20:3 (*n*-6)	224.4±19.6$^{a, b}$	366.2±28.3^{b}	121.8±98.4$^{a, c}$	35.8±10.8^{c}	10.8±0.8^{c}	56.8±0.8^{c}
20:3 (*n*-3)	3531.1±247.9^{a}	3484.1±269.8^{a}	333.8±2947^{b}	126.4±45.5^{b}	266.1±36.3^{b}	3277.2±90.0^{a}
20:4 (*n*-6)	nd	nd	4.4±1.7^{a}	16.4±3.8^{b}	nd	nd
20:5 (*n*-3)	75.3±5.8^{a}	23.4±2.5^{a}	nd	164.3±69.4^{a}	364.1±62.7^{a}	5801.5±202.9^{b}
22:2 (*n*-6)	nd	nd	nd	7.7±1.6^{b}	nd	nd
22:6 (*n*-3)	50.8±17.9^{a}	65.0±10.4^{a}	nd	15.8±6.9^{b}	7.8±1.1^{b}	39.0±23.7^{a}
Total PUFA	**4257.9**	**4144.7**	**672.8**	**471.5**	**738.3**	**9497.0**
Total	**17707.8**	**15123.6**	**4792.6**	**4404.9**	**5946.3**	**21942.4**

Values in the same line followed by the same letter (a–d) are not statistically different from each other ($p > 0.05$).

nd – not detected.

When analyzing the fatty acid profile of the extracts, in terms of saturated fatty acids, it was observed that palmitic acid (16:0) was the dominant fatty acid in all of the extracts tested, followed by myristic (14:0) and stearic acids (18:0), in descending order of magnitude; note that wild *C. crispus* reveals absence of stearic acid. These results are in accordance with the findings of Pereira *et al*. (2012) for Rhodophyta and of Bhaskar *et al.* (2004) who described the same tendency for these fatty acids for red algae, especially for *Gracilaria edulis* and *Gracilaria folifera* (although there appear to be no differences between these two algae). Norziah and Ching (2000) also observed, for *Gracilaria changgi*, that palmitic acid (16:0) was the main saturated fatty acid found.

Saturated fatty acids represent the majority of fatty acids found in the seaweed under study, a fact also described by Bhaskar *et al.* (2004) that reported that such high content of saturated fatty acids was probably due to the influence of water temperature, since in their experiments (similarly to those developed herein), water from the algae harvesting location had a moderate temperature.

Regarding MUFA, it was found that oleic acid (18:1 *n*-9) was the predominant MUFA present in all of the seaweed extracts tested (with the exception of aquaculture *P. dioica*), followed by palmitoleic acid (16:1 *n*-7) and eicosenoic acid (20:1 *n*-9). Wild *Chondrus crispus* and *Gracilaria vermiculophylla*, both from IMTA and wild systems, standout in terms of their high quantities. This predominance of 18:1 *n*-9 was in accordance with results reported by Li *et al.* (2002). In terms of PUFA, eicosatrienoic acid (20:3 *n*-3) was predominant in wild and IMTA *G. vermiculophylla* and IMTA *P. dioica.* Wild *C. crispus* also had a high content of this fatty acid, similar to that of *G. vermiculophylla* extracts although this was not the predominant PUFA in this extract. Once again, *G. vermiculophylla* stands out among all extracts together with wild *C. crispus*. In respect to eicosapentaenoic acid (EPA, 20:5 *n*-3), this situation changes as *G. vermiculophylla* reported the lowest amount of this fatty acid when comparing with the remaining extracts (not counting with IMTA extract of *P. dioica* where this fatty acid was not detected). Gressler *et al*. (2011), Kumar *et al*. (2011) and Pereira *et al*. (2012) also stated that 20:4 *n*-6 and 20:5 *n*-3 fatty acids were the predominant PUFA found in red algae. On the other hand, wild *C. crispus* had the highest quantity (5801.5 µg/g sample) of 20:5 *n*-3 fatty acid in the present study followed by aquaculture *C. crispus* and wild *P. dioica*. This result is not in accordance with the findings of Fleurence *et al*. (1994) who stated that *Porphya umbilicalis* had the highest value of 20:5 *n*-3 whereas *Chondrus crispus* was richer in 20:4 *n*-6, which in this study, this last fatty acid was not even detected in this extract. The other fatty acids detected in the algae extracts were present in smaller quantities, which was in accordance with the findings of Khotimchenko *et al.* (2002) that stated that the most predominant PUFA in red algae was 20:5 *n*-3 followed by 20:4 *n*-6. Furthermore, these authors sustained that palmitic (16:0), oleic (18:1 *n*-9),

arachidonic (20:4 *n*-6) and eicosapentaenoic (20:5 *n*-3) acids together accounted for 76,9 - 89,1% of the total fatty acids found in red algae extracts, whereas the remaining percentage represented the other fatty acids present in lower concentrations.

As, to our best of knowledge, there are no existing studies comparing fatty acid profiles of algae cultivated under aquaculture and wild habitats, it was not possible to have a term of comparison for our data. Nevertheless, it is stated by many authors that the lipid content varies due to the environmental conditions especially temperature, harvesting season, habitat, geographical location, age or growth stage and alga species (Khotimchenko, 2005; Gressler *et al*., 2011). So, low growth temperature conditions typically result in high levels of unsaturated fatty acids (Gressler *et al*., 2011), and the warm water temperatures observed during the experiments probably were responsible for the high level of SFA found in the algae species assessed.

Although, antimicrobial activity has been more attributed to long-chain unsaturated fatty acids (C_{16}-C_{20}) such as palmitoleic, oleic and linolenic acids, long-chain saturated fatty acids fatty acids, including palmitic and stearic acids are known to have the same effect although to a lesser extent (Plaza *et al.*, 2010). Even though it was not possible to conclude that the large amounts of fatty acids, especially SFA observed in this study, were the responsible elements for the antimicrobial activity found, it cannot be forgotten that these compounds do play an important role in this activity and more work may be done in order to clarify this function by working with pure compounds and assessing their effect on test organisms.

Conclusions

The present study is the first to compare, to the best of our knowledge, the antimicrobial potential of extracts from seaweeds from both integrated aquaculture systems and natural habitat against several pathogenic agents. The test organisms were more sensitive to seaweed extracts obtained with dried algae processed at higher temperatures, registering a higher antimicrobial activity of the ethyl acetate extracts when compared with methanol and diethyl ether extracts. This was more significant especially for the extracts from algae cultivated under IMTA regime. Despite the broad spectrum of test microorganisms, the inhibition effect on Gram positive microorganisms appears to be higher than on Gram negative bacteria. Extracts appear to have little effect on the yeast studied. The lipid profile showed that the extracts were rich in fatty acids, in particular SFA, which may indicate their probable role in the antimicrobial activity found. Overall, results showed that the extracts of the studied algae need to undergo further research in order to deepen knowledge on the identification, isolation and characterization of the specific compounds responsible for the antimicrobial activities. The natural outcome of such research will be to further exploit those algae as potential sources of bioactive compounds for nutraceutical, pharmaceutical, food or cosmetic industries.

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References

Abreu, M.H., Pereira, R., Yarish, C., Buschmann, A. H. and Sousa-Pinto, I. 2011. IMTA with *Gracilaria vermiculophylla*: Productivity and nutrient removal performance of the seaweed in a land-based pilot scale system. Aquaculture 312:77-87.

Bansemir, A., Blume, M., Schroder, S. and Lindequist, U. 2006. Screening of cultivated seaweeds for antibacterial activity against fish pathogenic bacteria. Aquaculture 252(1):79-84.

Bhaskar, N., Kinami, T., Miyashita, K., Park, S.-B., Endo, Y. and Fujimoto, K. 2004. Occurence of conjugated polyenoic fatty acids in seaweeds from the Indian Ocean. Zeitschrift fur Naturforschung 59: 310-314.

Carvalho, A.P., Pontes, I., Gaspar, H. and Malcata, F.X. 2006. Metabolic relationships between macro- and micronutrients, and the eicosapentaenoic acid and docosahexaenoic acid contents of *Pavlova lutheri.* Enzyme and Microbial Technology 38(3-4):358-366.

Carvalho, L.R. and Roque, N.F. 2000. Fenóis halogenados e/ou sulfatados de macroalgas marinhas. Química Nova 23(6):757-764.

del Val, A.G., Platas, G., Basilio, A., Cabello, A., Gorrochategui, J., Suay, I., Vicente, F., Portillo, E., del Rio, M.J., Reina, G.G. and Peláez, F. 2001. Screening of antimicrobial activities in red, green and brown macroalgae from Gran Canaria (Canary Islands, Spain). International Microbiology 4:35-40.

Demirel, Z., Yilmaz-Koz, FF., Karabay-Yavasoglu, UN., Ozdemir, G. and Sukatar, A. 2009. Antimicrobial and antioxidant activity of brown algae from the Aegean Sea. Journal of the Serbian Chemical Society 74(6):619-628.

Fleurence, J., Gutbier, G., Mabeau, S. and Leray, C. 1994. Fatty acids from 11 macroalgae of the French Brittany coast. Journal of Applied Phycology 6:527-532.

Franco, D., Pinelo, M., Sineiro, J. and Núñez, M.J. 2007. Processing of *Rosa rubiginosa*: Extraction of oil and

antioxidant substances. Bioresource Techonology 98:3506-3512.

Gerasimenko, N.I., Chaykina, E.L., Busarova, N.G. and Anisimov, M.M. 2010. Antimicrobic and hemolityc activity of low-molecular metabolites of Brown seaweed *Laminaria cichorioides* (Miyabe). Applied Biochemistry and Microbiology 46(4):426-430.

Gressler, V., Fujii, M. T., Martins, A.P., Colepicolo, P., Mancini-Filho, J. and Pinto, E. 2011. Biochemical composition of two red seaweed species grown on the Brazilian coast. Journal of Science and Food Agriculture 91:1687-1692.

Khotimchenko, S.V. 2005. Lipids from marine algae *Gracilaria verrucosa*. Chemistry of Natural Compounds 41(3):285-288.

Khotimchenko, S.V., Vaskovsky, V.E. and Titlyanova, T.V. 2002. Fatty acids of marine algae from the pacific coast of north California. Botanica Marina 45:17-22.

Kumar, M., Kumari, P., Trivedi, N., Shukla, M.K., Gupta, V., Reddy, C.R.K. and Jha, B. 2011. Minerals, PUFAs and antioxidant properties of some tropical seaweeds from Saurashtra coast of India. Journal of Applied Phycology 23:797-810.

Kumari, P., Bijo, A.L., Mantri, V.A., Reddy, C.R.K. and Jha, B. 2013. Fatty acid profiling of tropical marine macroalgae: An analysis from chemotaxonomic and nutritional perspectives. Phytochemistry 86:44-56.

Kumari, P., Kumar, M., Gupta, V., Reddy, C.R.K. and Jha, B. 2010. Tropical marine macroalgae as potential sources of nutritionally important PUFAs. Food Chemistry 120: 749-757.

Lekameera. R, Vijayabaskar, P. and Somasundaram, S. T. 2008. Evaluating antioxidant property of brown alga *Colpomenia sinuosa*. African Journal of Food Science. 2:126-130.

Lepage, G. and Roy, C.C. 1984. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. Journal of Lipid Research 25: 1391-1396.

Li, X., Fan, X., Han, L. and Lou, Q. 2002. Fatty acids of some algae from the Bohai Sea. Phytochemistry 59: 157-161.

Lima-Filho, J.V.M., Carvalho, A.F.F.U., Freitas, S.M. and Melo, V.M.M. 2002. Antibacterial activity of extracts of six macroalgae from the Northeastern Brazilian coast. Brazilian Journal of Microbiology 33:311-313.

Liu, Y., Cao, T. and Glime, J.M. 2003. The changes of membrane permeability of mosses under high temperature stress. The Bryologist 106 (1): 53-60.

Mtolera, M.S.P. and Semesi, A.K. 1996. Antimicrobial activity of extracts from six green algae from Tanzania. Current Trends in Marine Botanical Research in East African Region 211-217.

Norziah, M.H. and Ching, C.Y. 2000. Nutritional composition of edible seaweed Gracilaria changgi. Food Chemistry 68:69-76.

Patra, J.K., Rath, S.K., Jena, K., Rathod, V.K. and Thatoi, H. 2008. Evaluation of Antioxidant and Antimicrobial Activity of Seaweeds (*Sargassum* sp.) Extract: A Study on Inhibition of Glutathione –S-Transferase Activity. Turkish Journal of Biology 32:119-125.

Pereira, H., Barreira, L., Figueiredo, F., Custódio, L., Vizetto-Duarte, C., Polo, C., Resek, E., Engelen, A. and Varela, J. 2012. Polyunsaturated Fatty Acids of Marine Macroalgae: Potential for Nutritional and Pharmaceutical Applications. Marine Drugs 10: 1920-1935.

Plaza, M., Santoyo, S., Jaime, L., García-Blairsy Reina, G., Herrero, M., Señoráns, F.J. and Ibáñez, E. 2010. Screening for bioactive compounds from algae. Journal of Pharmaceutical and Biomedical Analysis 51: 450-455.

Pope, D.C. and Oliver, W.T. 1966. Dimethyl Sulfoxide (DMSO). Canadian Journal of Comparative Medicine and Veterinary Science 30(1):3-8.

Sachindra, N.M., Airanthi, M.K.W.A., Hosokawa, M. and Miyashita, K. 2009. Radical scavenging and singlet oxygen quenching activity of extracts from Indian seaweeds. Journal of Food Science and Technology 47(1):94-99.

Salem, W.M., Galal, H. and Nasr El-deen, F. 2011. Screening for antibacterial activities in some marine algae from the red sea (Hurghada, Egypt). African Journal of Microbiology Research 5(15): 2160-2167.

Sasidharan, S., Darah, I. and Noordin, M.K.M.J. 2010. *In vitro* antimicrobial activity against *Pseudomonas aeruginosa* and acute oral toxicity of marine algae *Gracilaria changii.* New Biotechnology 27(4): 390-396.

Shin, S.Y., Bajpai, V.K., Kim, H.R. and Kang, S.C. 2007. Antibacterial activity of eicosapentaenoic acid (EPA) against foodborne and food spoilage microorganisms. LWT- Food Science and Technology 40(9):1515-1519.

Stirk, W.A., Reinecke, D.L. and van Staden, J. 2007. Seasonal variation in antifungal, antibacterial and acetylcholinesterase activity in seven South African seaweeds. Journal of Applied Phycology 19:271-276.

Taskin, E., Ozturk, M., Taskin, E. and Kurt, O. 2007. Antibacterial activities of some marine algae from the Aegean Sea (Turkey). African Journal of Biotechnology 6(24):2746-2751.

Tuney, I., Çadirci, B.H., Unal, D. and Sukatar, A. 2006. Antimicrobial activities of the extracts of marine algae from the coast of Urla (Izmir, Turkey). Turkish Journal of Biology 30:171-175.

Vijayavel, K. and Martinez, J.A. 2010. *In vitro* Antioxidant and Antimicrobial Activities of Two Hawaiian Marine Limu: *Ulva fasciata* (Chlorophyta) and *Gracilaria salicornia* (Rhodophyta). Journal of Medicinal Food 13(6):1494-1499.