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Chemical Study and Biological Activity Evaluation of Two Azorean Macroalgae: Ulva rigida and Gelidium microdon

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Abstract

The green macroalgae Ulva rigida C. Agardh (Chlorophyta) and the red macroalgae Gelidium microdon Kützing (Rhodophyta), collected from the Azorean archipelago, were investigated for their secondary metabolites and their in vitro growth inhibitory effect on three human tumor cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma), as well as for their antifungal and antibacterial activities. The methanol extract of U. rigida furnished isofucosterol (1), 7(E)-3β-hydroxy-5α,6α-epoxymegastigmane (2) and (+)-dehydrovomifoliol (3) while the methanol extract of G. microdon yielded cholesterol (4) and lumichrome (5). The crude extracts of both macroalgae were found to be moderately active against the three cell lines whereas compound 1 showed a weak effect and compound 2 was inactive. The crude extracts of the two macroalgae were found to be moderately active against some fungi and bacteria while compounds 1 and 2 were inactive against all microorganisms tested.

Keywords: Azores; Macroalgae; Ulvragrida; Gelidium microdon; Isofucosterol; 7(E)-3β-hydroxy-5α,6α-epoxymegastigmane; (+)-dehydrovomifoliol; Lumichrome; Antitumor; Antimicrobial

Abbreviations: MeOH - Methanol; Me2CO - Acetone; δ - Chemical Shift in ppm; DMSO - Dimethyl Sulphoxide; HR-ESIMS - High Resolution Electrospray Ionization Mass Spectroscopy; SRB - Sulfurhodamine B; MIC - Minimal Inhibitory Concentration; MLC - Minimal Lethal Concentration

Introduction

The marine environment is an exceptional reservoir of bioactive compounds, many of which exhibit structural/chemical features not found in terrestrial natural products. This is easily understood since the Ocean, which covers almost 71% of the Earth’s surface and represents a uniqueness of the physical and chemical conditions, is considered as a very promising source of Natural Products covering a wide range of bioactivities [1-6]. Therefore, marine Natural Products continue to play a major role in drug discovery.

Since the Azorean archipelago is located in the warm temperate region of the North East Atlantic, approximately 1200 km from Europe, the marine fauna and flora of this group of islands appear to be a mixture of species which can be found both in the Atlantic and the Mediterranean [7]. Marine macroalgae are abundant and structuring organisms of the coastal area of the entire Azores archipelago, some having a markedly seasonal pattern and others being present during the whole year in the Azorean coasts [8-10]. The geographical distribution of these macroalgae is related to the temperature regime of the region where they grow, reproduce, and survive. However, the diversity and abundance of these organisms depend on many other biological factors [11], leading to production of different secondary metabolites of the species from different geographical locations [12]. Macroalgae produce myriads of secondary metabolites which are synthesized at the end of the growth phase and/or due to metabolic alterations induced by environmental stress conditions [13]. These metabolites have been targets of the drug discovery program and some of these bioactive compounds such as sulfated polysaccharides, steroids and diterpenes have found their applications in the pharmaceutical industry [14,15].

During our on-going project aiming at exploiting bioactive secondary metabolites from macroalgae of the Azorean archipelago for added-value products, we have conducted phytochemical studies of the green alga Ulva rigida C. Agardh and the red alga Gelidium microdon Kützing, and evaluation of the in vitro antitumor and antimicrobial activities of the crude extracts of these two macroalgae as well as their isolated metabolites. The main reasons for selection of these two species were based on the fact that Ulva and Gelidium species are well-recognized sources of industrially important biopolymers and the organic crude extracts of these two species had been previously found to exhibit a promising in vitro cytotoxicity on cancer cell lines and antioxidant activity [16]. Furthermore, they are abundant in the Azorean intertidal areas [8,17]. Although both species are locally abundant and dominant in the Azorean intertidal bedrock areas, U. rigida is common and abundant at mid and low shore levels whereas G. microdon is extremely abundant at mid shore level. Consequently,

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their abundance and easy access for collection can guarantee their quantity for further biotechnological exploitation in the future. Furthermore, as these two species are annual and intertidal, they do not present any significant variations of the concentrations of their secondary metabolites, which can be influenced by their age and depth of the collection site. Although both Ulva and Gelidium species have been extensively investigated as sources of biotechnologically relevant biopolymers, their secondary metabolites have never been fully exploited for value-added products. While Ulva species are an important source of ulvan, a natural sulfated polysaccharide which has been extensively investigated for development of novel drugs and functional foods [18], Gelidium species are one of the main sources of phycocolloids, such as agar [19,20]. Several types of secondary metabolites such as bromophenol [21-23], sesquiterpenes [24,25], and steroids [23,26] have been previously reported from the macroalgae of the genus Ulva; however, there are only few reports on the chemical constituents of the genus Gelidium. While gelidene, a polyhalogenated monocyclic monoterpene, was isolated from G. sesquipedale [27], jasmonic acid was reported from G. latifolium [28].

Due to the pristine environment of the Azorean archipelago, we have elaborated the project aiming to exploit the potential of the macroalgae of this region. The collections of these two species were carried out in May and October in order to allow us to study their chemical compositions in different seasons, i.e. spring and autumn, as well as of two different reproductive stages. We now report the chemical study together with the antitumor and antimicrobial activities evaluation of the first collection (May 2011) of the green macroalga U. rigida and the red macroalga G. microdon from S. Miguel Island which is considered to be one of the environmentally healthy habitats and rich in algal communities of the Azorean Sea. Examination of the methanol extract of U. rigida led to isolation of isofucosterol (1), 7(E)-3β-hydroxy-5α,6α-epoxymegastigmane (2) and (+)-dehydrovomifoliol (3), while the methanol extract of G. microdon yielded cholesterol (4) and lumichrome (5) (Figure 1). The crude extracts of both macroalgae, together with isofucosterol (1) and 7(E)-3β-hydroxy-5α,6α-epoxymegastigmane (2), were evaluated for their in vitro growth inhibition on three tumor cell lines: MCF-7, NCI-H460 and A375-C5, as well as for their antifungal and antibacterial activities. Figure 1.

Material and Methods

General experimental procedures

Melting points were determined on a Bock monoscope and are uncorrected. Optical rotations were determined on an ADP410 Polarimeter. ¹H and ¹³C NMR spectra were recorded at ambient temperature on a Bruker Advance instrument operating at 300.13 and 75.4 MHz, respectively. High resolution mass spectra were measured with a Waters Xevo QToF mass spectrometer coupled to a Waters Aquity UPLC system. A Merck silica gel GF 254 was used for preparative TLC, and a Merck Si gel 60 (0.2-0.5 mm) was used for analytical chromatography.

Biological material

U. rigida and G. microdon were collected in May 2011 from the...
coast of S. Miguel island - Azores archipelago, and the samples of both macroalgae were deposited at the Department of Biology of University of Azores (Vouchers: SMG-11-49 and SMG-11-30, respectively).

Extraction and isolation of the constituents

Dried powdered material (U. rigida - 1472.46 g and G. microdon - 1293.4 g) was percolated with MeOH, at room temperature until exhaustion. The resulting solutions were filtered with filter paper (Whatman no 1) and concentrated under reduced pressure to yield crude extracts of U. rigida (154.49 g) and G. microdon (151.91 g). Treatment of the crude methanol extracts to remove the chlorophylls [29], furnished 14.22 g of U. rigida and 4.74 g of G. microdon purified extracts.

U. rigida - The purified extract (14.22 g) was chromatographed over a 0.2-0.5 mm Si Gil column (180 g) and eluted with mixtures of petroleum ether, CHCl3, Me2CO, and MeOH, 250 ml fractions were collected as follows: frs 1-2 (petroleum ether-CHCl3, 9:1), frs 3-52 (petroleum ether-CHCl3, 4:1), frs 53-112 (petroleum ether-CHCl3, 1:4), frs 113-126 (petroleum ether-CHCl3, 1:4), frs 127-145 (CHCl3), frs 146-167 (CHCl3-Me2CO, 9:1), frs 168-211 (CHCl3-Me2CO, 4:1), frs 212-237 (CHCl3-Me2CO, 1:1), frs 238-280 (CHCl3-Me2CO, 9:1), frs 281-294 (CHCl3-Me2CO, 3:7), frs 295-300 (Me2CO). Frs 30 and 31 were combined (691.1 mg) and recrystallized in petroleum ether to give 32.0 mg of compound 1. Frs 93-106 were combined (121.9 mg) and purified by TLC (Si Gel, CHCl3-EtOAc-Me2CO). Frs 81-100 were combined (127.60 mg) and recrystallized from petroleum ether-CHCl3 to yield 2.7 mg of lumichrome (5).

Chemosterol (1): Crystals; mp. 129-131ºC. HR-ESIMS m/z 243.0918 [M+H]+ (calc for C12H11N4O2, 243.0882); 1H and 13C NMR (Table 3).

Growth inhibition of human tumor cell lines

The effect of the extracts and of compounds 1 and 2 were evaluated for their capacity to inhibit in vitro growth of three human tumor cell lines: MCF-7 (breast adenocarcinoma), NCI-H690 (non-small cell lung cancer) and A375-C5 (melanoma), according to the procedure adopted by the National Cancer Institute (NCI) in the “In vitro Anticancer Drug Discovery Screen” that uses the protein-binding dye SRB to

- (3)-Dehydrovomifoliol (3): White amorphous powder. 1H and 13C NMR (Table 2).

Table 3: NMR data for compound 5 in CDCl3 (1H 300.13, 13C 75.47 MHz).

<table>
<thead>
<tr>
<th>Position</th>
<th>δC, type</th>
<th>δH, (J in Hz)</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>160.7, C</td>
<td></td>
<td>C-2</td>
</tr>
<tr>
<td>2</td>
<td>196.6, CH</td>
<td>2.47, s</td>
<td>C-11, 12, 13</td>
</tr>
<tr>
<td>3</td>
<td>202.6, CH</td>
<td>2.49, s</td>
<td>C-9, 12, 13</td>
</tr>
</tbody>
</table>


Antifungal assays

Broth microdilution methods based on Clinical and Laboratory Standards Institute (CLSI) reference protocols M27-A3 and M38-A2 for yeasts (Candida albicans) and filamentous fungi (Aspergillus fumigatus and dermatophytes), respectively, were used to determine the MIC and MLC of the crude extracts and the isolated metabolites [34]. Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 25923, and Methicillin Resistant Staphylococcus aureus (MRSA), clinical isolate, were used as test organisms. The cell suspensions prepared in 0.85% NaCl and the transmittance of cell density adjusted at that produced by a 0.5 McFarland standard. To achieve an inoculum size of 0.4-10×10^6 CFU/mL for A. fumigatus and 1-3×10^4 CFU/mL for dermatophytes, the spore suspensions were diluted with RPMI 1640. The solutions of the extracts and compounds 1 and 2 were prepared in DMSO and added to the cell suspensions in order to obtain test concentrations ranging from 16 to 256 µg/mL. In addition, reference antifungal compound, fluconazole, was used as standard antifungal drug. Controls without crude extracts and isolated compounds, as well as sterility and DMSO control wells, were also included. The plates were incubated aerobically at 35°C ± 0.2°C for 24h/48h in atmospheric humidity (C. albicans and A. fumigatus) and at 25°C ± 0.2°C for 5 days in atmospheric humidity for dermatophytes. To evaluate the MLCs, 20 µL samples were taken from each negative well and the first well exhibiting growth (serve as a growth control), after MIC reading, spotted onto SDA (Sabouraud Dextrose Agar) plates and incubated at 35°C ± 0.2°C 24h/48h (C. albicans and A. fumigatus) or at 25°C ± 0.2°C for 7 days (dermatophytes).

Antibacterial assays

A broth microdilution method, based on CLSI reference protocol M7-A7, was used to determine the MIC and MLC of the crude extracts and the isolated metabolites [35]. Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 25923, and Methicillin Resistant Staphylococcus aureus (MRSA), clinical isolate, were used as test organisms. The cell suspensions prepared in 0.85% NaCl and the transmittance of cell density adjusted at that produced by a 0.5 McFarland standard. To achieve an inoculum size of 0.4-10×10^6 CFU/mL, the cell suspensions were diluted with MHB (Muller-Hinton Broth). The stock solutions of the extracts, and compounds 1 and 2 were prepared in DMSO and further diluted in serial two-folds with MHB to final concentrations ranging from 16 to 256 µg/mL. In addition, gentamicin was used as standard antibiotic drug and controls without crude extracts and isolated compounds, as well as sterility and DMSO control wells, were also included. The plates were incubated aerobically at 35°C ± 0.2°C for 16/20h in atmospheric humidity. To measure the MLCs, 20 µL samples were taken from each negative well and the first well exhibiting growth (serve as a growth control), after MIC reading, spotted onto MHA (Muller-Hinton Agar) plates and incubated at 35°C ± 0.2°C for 24h.

Results and Discussion

The structures of the compounds were established mainly by 1D (1H and 13C NMR) and 2D (COSY, DEPT, HSQC and HMBC experiments) spectroscopic techniques as well as comparison of their NMR data with those reported in the literature.

Compound 1 was isolated as white crystals with mp 129-131°C. The 13C NMR, DEPT, and HSQC spectra revealed the presence of twenty-nine carbon signals which can be categorized as two quaternary sp3 (δC 145.9, 140.7), two methine sp2 (δC 121.7, 116.4), two quaternary sp2 (δC 42.3, 36.5), one oxymethine sp3 (δC 71.8), six methine sp2 (δC 56.7, 56.0, 50.1, 36.1, 31.9, 28.6), ten methylene sp2 (δC 42.3, 39.8, 37.2, 35.9, 31.9, 31.6, 28.2, 27.9, 24.3, 21.1) and six methyl (δC 21.1, 21.0, 19.4) carbons. The HMBC correlations of H-28 (δH 5.10, J= 6.8, dd, J= 4.4, 1.7, δC 121.7) to C-4 (δC 42.3), C-8 (δC 31.9), C-10 (δC 36.5) revealed the presence of a trisubstituted double bond between C-5 and C-6. That another trisubstituted double bond was on C-24 and C-28 was corroborated by the HMBC correlations of H-28 (δH 5.10, q, J= 6.8, δC 116.4) to C-25 (δC 28.6) and C-29 (δC 12.8). The coupling constants of H-3 suggested that the C-3 hydroxyl group was β. The 1H and 13C NMR data for compound 5 in CDCl3 (1H 300.13, 13C 75.47 MHz).
chemical shift values of compound 1 were compatible with those of isofucosterol [36,37]. Isofucosterol is a common phycoreovin and it has been previously reported from several macroalgae [38].

The 13C NMR spectrum of compound 2 displayed thirteen carbon signals which were categorized, by DEPT and HSQC experiments (Table 1), as one carbonyl of a conjugated ketone (δ 197.5), two methine sp3 (δ 132.6, 142.4), two oxayquaternary sp3 (δ 69.4, 67.3), one quaternary sp3 (δ 35.1), one oxymethine sp3 (δ 64.0), two methylene sp2 (δ 40.5, 46.6) and four methyl (δ 19.8, 24.9, 28.3, 29.3) carbons. The COSY spectrum displayed cross peak between the olefinic protons at δ 7.03 d (J=15.6) and δ 6.29 d (J=15.6), confirming the presence of a trans double bond. That this trans double bond was part of the 3-oxobutenyl side chain which linked to C-6 of the cyclohexanol moiety was supported by the HMBC correlations of the methyl protons signal at δ 3.22-s (δ 28.3) to the carbon signals at δ 132.6 (C-8), δ 142.4 (C-7) and δ 197.5 (C-9), as well as of the proton signal at δ 6.29. J=15.6 (δ 132.6) to the carbon signals at δ 197.5 (C-9), δ 142.4 (C-7), δ 69.4 (C-6). As the proton signals of the methyl groups at δ 0.98 (δ 25.0) and δ 1.19s (δ 29.3) gave cross peaks to the quaternary carbon signal at δ 35.1 (C-1) as well as to the carbon signals at δ 46.6 (C-2) and δ 69.4 (C-6), they were assigned for C-12 and C-13, respectively. These correlations led to the conclusion that the structure of compound 2 should correspond to 7(E)-3β-hydroxy-5α,6α-epoxymegastigmane (2), as was previously reported from several macroalgae [38].

The 13C NMR, DEPT and HSQC spectra of the mixture revealed the presence of two amide carbonyls (δ 144.7) and C-10 (δ 141.6) and CH-15 (δ 19.6), the proton signal at δ 7.71s (δ 125.9) showed HMBC correlations to C-5 (δ 138.4), C-7 (δ 138.9) and CH-16 (δ 20.2). Thus, the structure of compound 5 is 7, 8-dimethylalloxazine or commonly known as lumichrome. Lumichrome, a derivative of the vitamin riboflavin, has been purified and chemically identified from culture filtrates of the alga Chlamydomonas as a Quorum Sensing (QS) signal-mimic compound capable of stimulating the Pseudomonas aeruginosa LasR QS receptor [40]. Bacteria, plants, and algae commonly secrete riboflavin or lumichrome, raising the possibility that these compounds could serve as either QS signals or as interkingdom signal mimics capable of manipulating QS in bacteria with a LasR-like receptor [40] (Table 3).

The crude methanol extracts of U. rigida and G. microdon (before and after removal of the chlorophylls) were also evaluated for their antifungal activity against C. albicans, A. fumigatus, and dermatomites E. floccosum, M. canis, M. gypseum, T. mentagrophytes, and T. rubrum. The results showed that removal of the chlorophylls caused an increase in antifungal activity of U. rigida against T. rubrum, T. mentagrophytes, M. canis, and E. floccosum. Whereas T. rubrum showed higher susceptibility, M. gypseum showed more resistance (MIC higher than 256 µg/mL). Removal of the chlorophylls also caused an increase in the activity of G. microdon crude extract against T. rubrum and E. floccosum. It was found that M. canis showed more susceptibility while T. mentagrophytes and M. gypseum showed higher resistance. Interestingly, both isofucosterol (1) and 7(E)-3β-hydroxy-5α,6α-epoxymegastigmane (2) were inactive against all the tested organisms (Table 5).

### Table 5

<table>
<thead>
<tr>
<th>Cell lines / GI50 (µM)</th>
<th>MCF-7</th>
<th>NCI-H460</th>
<th>A375-C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>U. rigida (before removal of chlorophylls)</td>
<td>44.5 ± 18.4</td>
<td>49.1 ± 14.0</td>
<td>40.8 ± 10.2</td>
</tr>
<tr>
<td>U. rigida (after removal of chlorophylls)</td>
<td>43.0 ± 10.3</td>
<td>41.9 ± 12.1</td>
<td>44.5 ± 7.6</td>
</tr>
<tr>
<td>G. microdon (before removal of chlorophylls)</td>
<td>75.9 ± 16.1</td>
<td>70.6 ± 20.1</td>
<td>36.3 ± 8.0</td>
</tr>
<tr>
<td>G. microdon (after removal of chlorophylls)</td>
<td>63.1 ± 14.1</td>
<td>64.9 ± 16.6</td>
<td>62.8 ± 15.9</td>
</tr>
</tbody>
</table>

*Results are given as the lowest concentrations causing 50% of cell growth inhibition (GI50) after a continuous exposure to the compounds for 48 hours and are expressed as means ± SEM of three independent experiments performed in triplicate. Doxorubincin was used as positive control; GI50: MCF-7 = 60.3 ± 1.2 nM; NCI-H460 = 19.6 ± 1.9 nM; A375-C5 = 130 ± 25.2 nM.*
The extracts of *U. rigida* and *G. microdon* (before and after removal of the chlorophylls) were evaluated for their activity against *E. coli*, *P. aeruginosa*, *S. aureus*, and MRSA. The results (Table 6) showed that the crude methanol extract of *U. rigida* (before and after removal of the chlorophylls) did not show any antibacterial activity against *E. coli*, *P. aeruginosa* and *S. aureus*, however removal of the chlorophylls caused a weak activity against MRSA. Similarly, the crude methanol extract of *G. microdon* did not show any activity against the test bacteria; however removal of the chlorophylls showed a weak activity against *S. aureus* and that sensitivity increases against MRSA. Interestingly, neither isoferucosterol (1) nor 7(E)-3β-hydroxy-5α,6α-epoxymegastigmane (2) showed activity against all the strains of tested organisms (Table 6).

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