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(3β,6α)-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; *Ulva rigida*; *Gelidium microdon*; Isofucosterol; 7(3β,6α)-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 Spectrometry; SRB - Sulforhodamine B; MLC - 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 an open access journal, is extremely abundant at mid shore level. Consequently, *G. microdon* is common and abundant at mid and low shore levels whereas *U. rigida* is abundant and dominant at the Azorean intertidal bedrock areas, 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, 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 at 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, *Corresponding author:* Prof, Anake Kijjoo, ICBAS - Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal, Tel: +351-220428331; Fax: +351-220428090; E-mail: anakekijjoo@icbas.up.pt

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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.

**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...
Extraction and isolation of the constituents

Dried powdered material (U. rigida - 1472.5 g and G. microdon - 151.91 g). Treatment of the crude methanol extracts to remove the chlorophylls 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). The crude methanol extracts were chromatographed over a 0.2-0.5 mm Si Gel column (120 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, 1:4), frs 53-112 (petroleum ether-CHCl3, 1:4), frs 113-126 (petroleum ether-CHCl3, 1:4), frs 127-145 (CHCl3-Me2CO, 1:1), frs 212-243 (CHCl3-Me2CO, 3:7), frs 244-289 (CHCl3-Me2CO, 1:4), frs 290-303 (Me2CO). Frs 30 and 31 were combined (691.1 mg) and recrystallized in CHCl3 to give 66.9 mg of cholesterol (4) (MeOH). Frs 81-100 were combined (127.6 mg) and recrystallized in CHCl3 in ppm: 145.9 (C-9), 121.9 (C-29), 60.8 (C-17), 42.3 t (C-4 and C-13), 39.8 t (C-12), 39.5 t (C-12), 37.2 t (C-11), 36.5 t (C-10), 36.2 t (C-22), 35.8 d (C-20), 31.9 t (C-7), 31.9 t (C-8), 23.7 t (C-28), 23.7 t (C-27), 22.6 q (C-26), 21.1 t (C-11), 19.4 q (C-18), 18.7 q (C-21), 11.9 q (C-18).

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-H460 (non-small cell lung cancer) and A375-S (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 convert cell counts to absorbance measurements.

Table 1: NMR data for compound 2 in CDCl3 (1H 300.13, 13C 75.47 MHz).

| Position | δ2 (δ in ppm) | δ3 (δ in Hz) | COSY | HMBC |
|----------|---------------|--------------|-------|-------|
| 1        | 35.1, C       |              |       |       |
| 2α       | 46.6, CH3     | 1.64, dt (12.9, 1.8) | H-2β  |       |
| β        | 1.26, dt (12.9, 10.4) |            | H-2α  |       |
| 3        | 64.0, CH3     | 3.90, m      |       |       |
| 4α       | 40.5, CH3     | 2.39, ddd (14.5, 5.1, 1.7) | H-17  | C-3, 5, 6 |
| β        | 1.66, dd (14.5, 8.7) |              | H-2α  | C-2, 5 |
| 5        | 67.3, C       |              |       |       |
| 6        | 69.4, C       |              |       |       |
| 7        | 142.4, CH3    | 7.03, d (15.6) | H-8   | C-6, 8, 9 |
| 8        | 132.6, CH3    | 6.29, d (15.6) | H-7   | C-6, 7, 9 |
| 9        | 197.5, CO     |              |       |       |
| 10       | 28.3, CH3     | 2.28, s      |       | C-4, 6 |
| 11       | 19.8, CH3     | 1.19, s      |       | C-4, 8 |
| 12       | 25.0, CH3     | 0.98, s      |       | C-1, 2, 6, 13 |
| 13       | 29.3, CH3     | 1.19, s      |       | C-1, 2, 6, 12 |

Table 2: NMR data for compound 3 in CDCl3 (1H 300.13, 13C 75.47 MHz).

| Position | δ2 (δ in ppm) | δ3 (δ in Hz) | COSY | HMBC |
|----------|---------------|--------------|-------|-------|
| 1        | 41.4, C       |              |       |       |
| 2α       | 48.5, CH3     | 2.51, d (17.0) | H-2β  | C-1, 3, 11 |
| β        | 2.34, d (17.0) |              | H-2α  |       |
| 3        | 197.0, CO     |              |       |       |
| 4        | 127.8, CH3    | 5.96, brt (1.0) | H-2β  | C-2, 6, 13 |
| 5        | 160.4, C      |              |       |       |
| 6        | 79.3, C       |              |       |       |
| 7        | 145.0, CH     | 6.84, d (15.7) | H-8   | C-5, 6, 9 |
| 8        | 130.3, CH3    | 6.47, d (15.7) | H-7   | C-6, 9 |
| 9        | 197.4, CO     |              |       |       |
| 10       | 28.4, CH3     | 2.31, s      |       | C-8, 9 |
| 11       | 18.7, CH3     | 1.88, s      |       | C-4, 5, 6 |
| 12       | 24.3, CH3     | 1.03, s      |       | C-1, 2, 13 |
| 13       | 22.9, CH3     | 1.11, s      |       | C-1, 2, 12 |
Table 3: NMR data for compound 5 in CDCl₃ (¹H 300.13, ¹³C 75.47 MHz).

| Position | δ₁, type | δ₁, (J in Hz) | HMBC |
|----------|-----------|---------------|-------|
| 1        | 130.2, C  | 130.2, C      |       |
| 2        | 146.5, C  | 146.5, C      |       |
| 3        | 138.4, C  | 138.4, C      |       |
| 4        | 128.7, CH | 128.7, CH     | 7.92, s | C-7, 13, 15 |
| 5        | 138.9, C  | 138.9, C      |       |
| 6        | 144.7, C  | 144.7, C      |       |
| 7        | 125.8, CH | 125.8, CH     | 7.71, s | C-6, 12, 16 |
| 8        | 141.6, C  | 141.6, C      |       |
| 9        | 150.1, CO | 150.1, CO     | 11.84, brs | C-2 |
| 10       | 11.8, brs | 11.8, brs     | C-2   |
| 11       | 160.7, CO | 160.7, CO     |       |
| 12       | 19.6, CH₂ | 19.6, CH₂     | 2.47, s | C-11, 12, 13 |
| 13       | 20.2, CH₂ | 20.2, CH₂     | 2.49, s | C-9, 12, 13 |

Antifungal assays

Broth microdilution methods based on Clinical and Laboratory Standards Institute (CLSI) reference protocols M7-A7 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]. Candida albicans ATCC 10231, Aspergillus fumigatus ATCC 46645 and dermatophytes: Epidermophyton floccosum FF9, Microsporum canis FF1, Microsporum gypseum FF3, Trichophyton mentagrophytes FF7, and Trichophyton rubrum FF5 were used as test organisms. The yeast cell suspensions were prepared in 0.85% NaCl with Tween 20 and the cell density adjusted at 20-250 conidia/square (hemocytometer) for A. fumigatus and 20-60 conidia/square for dermatophytes. To achieve an inoculum size of 0.4-5 × 10⁵ CFU/mL for A. fumigatus and 1-3 × 10⁶ 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 29253, and Methicillin Resistant Staphylococcus aureus (MRSA), clinical isolate, were used as test organisms. The cell suspensions were prepared in 0.85% NaCl and the transmittance of cell density adjusted to that produced by a 0.5 McFarland standard to achieve an inoculum size of 10⁵ 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 antibacterial 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) data as well as comparison of their NMR data with those reported in the literature.
chemical shift values of compound 1 were compatible with those of
isoucosterol [36,37]. Isoucosterol is a common phycoester and it has
been previously reported from several macroalgae [38].

The 1^1^C 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 sp^2 (δ 132.6, 142.4), two oxoquaternary sp^2 (δ 69.4, 67.3),
one quaternary sp^3 (δ 35.1), one oxymethine sp^3 (δ 64.0), two methylene
sp^3 (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-oxo-
butenyl side chain which linked to C-6 of the cyclohexenol moiety was
supported by the HMBC correlations of the methyl protons signals at δ
1.19s (δ C 127.8) to the carbon signals at δ C 138.4, 141.6, 144.7, 146.5, two methine
sp^2 (δ 125.8, 128.7) and two methyl (δ 19.6, 20.2) carbons. The HMBC spectrum
displayed cross peaks of the amide proton signals at δ
H 5.96, brt, (J= 15.7 Hz) to C-9 (δ C 138.4, 144.7, C-10 (δ C 141.6) and CH3-15 (δ C 19.6),
the proton signal at δ 7.71s (δ 125.9) showed HMBC correlations to C-5 (δ C 138.4), C-7 (δ C 138.9) and CH1-16 (δ C 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].

The effect of the extracts of *U. rigida* and *G. microdon* (before and
after removal of the chlorophylls), isoucosterol (1) and (7E)-3β-hydroxy-5α,
6α-epoxymegastigmane (2) were evaluated for their capacity to inhibit in vitro
growth of three tumor cell lines: MCF-7, NCI-H460 and A375-C5. The results showed that the crude
extracts were moderately active against the three cell lines; however,
isoucosterol (1) was found to be less active than the crude extract of
*U. rigida*, while (7E)-3β-hydroxy-5α,6α-epoxymegastigmane (2) was inactive (Table 4).

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 dermatophytes
*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, *G. microdon* 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 isoucosterol (1) and (7E)-3β-hydroxy-5α,6α-
epoxymegastigmane (2) were inactive against all the tested organisms
(Table 5).

**Table 4: Growth inhibitory effect crude methanol extracts of *U. rigida* and *G. microdon*, compounds 1 and 2, in different cell lines**.
control was performed by testing the inhibitory activity of gentamicin against the lowest concentration of the crude extract or compound causing bacterial death. All experiments were performed in duplicate and repeated at least three times. Quality control for the young researcher scholarship under the PTDC/MAR/100482/2008 project. LA0015/2011 and QOPNA-PEst-C/QUI/UI0062/2011. Madalena Silva thanks FCT Ciência e a Tecnologia (FCT), COMPETE, QREN, FEDER, MCTES, and partially showed activity against all the strains of tested organisms (Table 6).

-3β-hydroxy-5α,6α-epoxymegastigmane (2)

E isofucosterol (1) nor 7(1)

and that sensitivity increases removal of the chlorophylls showed a weak activity against S. aureus, however removal of the chlorophylls caused S. aureus, and MRSA. The results (Table 6) showed that the crude methanol extract of U. rigida (before removal of chlorophylls) 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 isofucosterol (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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