CHEMICAL CONSTITUENTS AND BIOACTIVITY OF Codium amplivesiculatum SETCHELL & N. L. GARDNER (CHLOROPHYTA; BRYOPSIDALES)

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ABSTRACT: In search of bioactive substances from Mexican marine organisms, crude ethanol-extract from the marine alga Codium amplivesiculatum was fractionated in chromatographic columns of silica gel at 60 Å (230–400 mesh) using solvent mixtures of increasing polarity. All the fractions were submitted to antibacterial assays. The major metabolite from an anti-tuberculosis fraction (MIC = 100 μg mL⁻¹) was purified and identified as 1-octodecanol (1). The anti-tuberculosis activity was attributed to 1 with bases in previous reports. In addition, clerosterol (2) was obtained by crystallization from an active fraction against Staphylococcus aureus and Vibrio parahaemolyticus (MIC = 125 and 250 μg mL⁻¹, respectively). Both structures were established by interpretation and comparison of infrared and 1H NMR spectroscopic data. In contrast with other studies, 2 showed a non-significant cytotoxicity against the cell line PC-3 (% GI = 21.05 ± 0.3 at 50 μg mL⁻¹). To our knowledge, these metabolites are reported for the first time from C. amplivesiculatum, and this is one of very rare reports of saturated long-chain alcohols isolated from chlorophytes.

Keywords: 1-octodecanol, clerosterol, anti-tuberculosis, fatty alcohol

INTRODUCTION

Marine alga and the former brown algae are recognized for synthesizing secondary metabolites that enable them to survive the physical and chemical conditions of the marine environment and respond to the pressures of competition, predation, epiphytes, and defense (Kovganko & Kashkan, 1999; Matsubara et al., 2003; Smit, 2004; Chou, 2006; Blunt et al., 2006; Amsler, 2008; Blunt et al., 2012). Among the organisms traditionally recognized as macroalgae, chlorophytes have been the least investigated as a source of bioactive compounds (Hay & Steinberg, 1992; Blunt et al., 2010; Munro & Blunt, 2005). Among them, Codium species are characterized by a spongy thallus and having a cosmopolitan distribution. Also for their ability to biosynthesize steroidal compounds clerosterol-type, glycoacylglycerides, glycosides derivatives and cyclic and acyclic terpenes that are of interest for their biological activity (Ahmad et al., 1992; Ali et al., 2001; Ali et al., 2002; He et al., 2010). In terms of biological activity, particularly interesting are clerosterol and its oxygenated derivatives isolated from C. arabicum due to their potent cytotoxic activity against several cancer cell lines (Sheu et al., 1995). Our aim is to determine the potential of the Baja California coast as a source of bioactive compounds, particularly the antibacterial activity of crude extract from C. amplivesiculatum (Setchell & Gardner, 1924; Muñoz-Ochoa et al., 2010). In the East coast off Margarita Island, C. amplivesiculatum is the most abundant. It is distributed through broad zones apparently hosting few epiphytes. For the first time, the antibacterial activity of
fractions isolated from *C. amplivesiculatum* are reported.

**MATERIAL AND METHODS**

*Algal material collection, extract preparation and fractionation*

Specimens of *Codium amplivesiculatum* were collected during the summer of 2006 in Bahía Magdalena, Baja California Sur, Mexico (24°31' N and 111°11' W) and identified according to Setchell and Gardner (1924). The specimens were rinsed with tap water to remove sediments and detritus, then sun-dried, ground, and stored at −20°C until used. Specimens for reference (ID: 06–012) are kept in our collection at CICIMAR-IPN (Fig. 1). The crude extract was obtained by simple maceration of the ground *C. amplivesiculatum* (1.76 kg) with 3.5 L of ethanol (EtOH) for 8 days at room temperature (22–35°C). After this period, the mixture was filtered and the algal residue was re-extracted twice under the same conditions. The filtrates were combined and the EtOH was removed under reduced pressure at 40°C to yield 82.64 g EtOH extract. The extract was suspended in 250 mL dichloromethane (CH\(_2\)Cl\(_2\)) and distilled water (1:1) to initiate liquid–liquid extraction. The organic phase was sequentially extracted with water (8 × 50 mL) and concentrated to dryness under reduced pressure to obtain 11.89 g of a dark green gummy solid. Then 7.5 g of the organic phase was processed by column chromatography to maintain a 1:20 ratio of sample:adsorbent. The column (50 × 2.5 cm) was equilibrated and the elution was performed with 1 L of hexane:CH\(_2\)Cl\(_2\) (1:1), followed by the same amount of CH\(_2\)Cl\(_2\), CH\(_2\)Cl\(_2\):EtOH in a polarity gradient from (97:3), (93:7) to (9:1), and mixtures of CH\(_2\)Cl\(_2\):EtOH:H\(_2\)O (7:3:1), (6:3:1), (6:4:1), EtOH, and H\(_2\)O. Then 450 eluates were collected (~15 mL). The fractionation pattern was determined by comparison of relative composition of each eluate by thin-layer chromatography. Finally, 16 chromatographic fractions were collected. Thus, 0.172 g of fraction 2, eluted with CH\(_2\)Cl\(_2\), was subjected to column chromatography at a 1:145 ratio of sample:adsorbent. The column was eluted with 0.45 L of hexane:CH\(_2\)Cl\(_2\) (1:1), followed by equal volumes of CH\(_2\)Cl\(_2\) to CH\(_2\)Cl\(_2\):EtOH (98:2), (95:5), (90:10), (50:50), and finally EtOH. Five sub-fractions were obtained, from which the sub-fraction eluted with CH\(_2\)Cl\(_2\):EtOH (98:2) was the largest (compound 1, 78 mg). After been analyzed under various TLC conditions, it proved to be homogeneous in all cases. Furthermore, fraction 3 eluted with CH\(_2\)Cl\(_2\):EtOH (97:3) was found to be an amorphous solid which, after several crystallization steps in hot methanol, yielded a crystalline solid (compound...
2, 34 mg) that was soluble in CH\textsubscript{3}Cl, and homogeneous under several TLC conditions. All fractions and compounds were assayed for antibacterial activity, and compound 2 was assayed against three cell lines to determine cytotoxicity.

**General experimental procedure**

The melting point was determined in a digital melting point apparatus (Mel-Temp, Lab. Device, USA) and is uncorrected. \(^1\)H-NMR spectra were recorded on a Mercury 300 spectrometer. The chemical shifts are expressed as ppm, relative to TMS and assigned by comparison with published data (SDBS, 2003). The IR spectra were recorded on a Perkin-Elmer Spectrum Two equipped with an ATR scanner at a frequency from 350 to 4500 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\). All solvents were analytical grade (Fermont by Productos Químicos de Monterrey, Mexico) and were glass-distilled before use. Silica gel 60 Å, 230-400 mesh (Whatman International, England) was used for column chromatography. Pre-coated silica gel Partisil K6F\textsubscript{254} plates (Whatman International) were used for thin-layer chromatography and 10% sulphuric acid was used as the developing reagent.

**Antibacterial and cytotoxic assays**

The fractions and/or isolated compounds from *C. amplivesiculatum* were subjected to antibacterial assay by the agar-disc diffusion method (NCCLS, 2000). The target bacteria were *Staphylococcus aureus* (ATCC accession number: BAA-42), which is resistant to methicillin, penicillin, ampicillin/sulbactam, oxacillin, and cefalotin), *Escherichia coli* (BAA-196, resistant to ampicillin, ampicillin/sulbactam, ce-fazolone, gentamicin, piperacillin, tobramycin, and trimethoprim/clavulanic acid), *Streptococcus pyogenes* (BAA-946, resistant to erythromycin), *Vibrio parae-haemolyticus* (17802), *Vibrio alginolyticus* (17749), and *Vibrio harveyi* (14126). Samples were considered active if there was an inhibition zone around the discs. The minimal inhibitory concentration (MIC) of active fractions was determined by the broth dilution method. All assays were performed in duplicate (NCCLS, 2000). Anti-mycobacterial activity was determined against *Mycobacterium tuberculosis* HR37Rv strain (27294, sensitive to isoniazid, rifampicin, streptomycin, ethambutol, and pyrazinamide). The MIC against *M. tuberculosis* was determined by the microplate alamar blue assay. All tests were performed by duplicate (Molina-Salinas et al., 2006). The cytotoxicity against cell lines of human prostate cancer (PC-3), human colon carcinoma (HCT-15), and human breast adenocarcinoma (MCF-7) was determined by the sulforhodamine B method (Skehan et al., 1990). All determinations were performed in triplicate. The cytotoxic activity was tested at the concentration of 50 µg mL\(^{-1}\). Activity is reported as percent of cellular growth inhibition (% GI).

**RESULTS AND DISCUSSION**

Table 1 summarizes the antibacterial activity of fractions/compounds, as determined by three different methods. As can be seen, five fractions from the organic crude extract from *C. amplivesiculatum* were active against *S. aureus* by the agar disc diffusion assay, from which fraction 3 eluted with CH\textsubscript{3}Cl/\textsubscript{2}EtOH (97:3) was also active against *V. parae-haemolyticus*. From the 16 fractions gathered, nine showed growth inhibition of *S. pyogenes*. None of the fractions were active against *E. coli*, *V. alginolyticus*, or *V. harveyi* at the tested concentration. *S. pyogenes* was the most sensitive of the target bacteria. Fractions 4 to 8 were active against *S. aureus* and *S. pyogenes*. Fraction 2 eluted with hexane:CH\textsubscript{3}Cl, (1:1) and CH\textsubscript{3}Cl was slightly active against *M. tuberculosis*, with a MIC = 100 µg mL\(^{-1}\). An additional separation step of fraction 2 by column chromatography was mainly composed of Compound 1 (Table No. 2). This was isolated as a low melting point solid. The IR spectrum of Compound 1 had absorption bands at 3324 and 1024 cm\(^{-1}\), characteristic of the hydroxyl function. Bands for carbonyl, aryl, and alkanyl or any other functional group were not observed. Based on these findings, the structure of an aliphatic alcohol was presumed and confirmed by the \(^1\)H-NMR data of Compound 1, which exhibited a triplet at δ 3.64 (J = 6.4 Hz) from geminal protons on a hydroxyl group. The remaining signals were all consistent with a saturated primary alcohol. The length of the hydrocarbonated chain of Compound 1 was estimated to be 18 by means of area integration of signals at δ 1.56, 1.28, 1.20, and 0.89. The experimental data was compared with the literature (SDBS, 2003) and Compound 1 was identified as 1-octadecanol. Very recently, Mukherjee et al. (2013) found that 1-alkanols with seven to ten carbons showed considerable anti-mycobacterial activity. Furthermore, a mixture of 1-alkanols of very long-chains (C\(_{16}-C_{32}\)) isolated as phytoconstituents from *Alpinia purpurata* showed inhibition of the same *M. tuberculosis* strain (Villaflores et al., 2010). Considering these reports, the antimycobacterial activity that we observed may be attributed to the presence of 1-octadecanol. In spite of free or conjugated long-chain alcohols known to be widely distributed in nature (Kaya et al., 2003; Boussaada et al., 2008; Tili et al., 2011) the occurrence of 1-octadecanol in *C. amplivesiculatum* is a novel finding.
Table 1. Antibacterial activity of fractions and compounds from *Codium amplivesiculatum*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibition zone in mm by the diffusion assay at 2 mg disc⁻¹ and MIC in μg mL⁻¹</th>
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<tbody>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
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<tr>
<td>F1</td>
<td>BAA-42</td>
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<tr>
<td>F2</td>
<td></td>
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<tr>
<td>F3</td>
<td>15 (125)</td>
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<td>F4</td>
<td>15 (&gt;250)</td>
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<td>F5</td>
<td>8 (&gt;250)</td>
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<td>F6</td>
<td>8 (&gt;250)</td>
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<td>F7</td>
<td>n/a</td>
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<tr>
<td>F8</td>
<td>9.5 (&gt;250)</td>
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<td>F9</td>
<td></td>
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<tr>
<td>F10</td>
<td>9.5 (n/a)</td>
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<td>F16</td>
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<td></td>
<td>1-octadecanol</td>
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</table>

n/a = not assessed because of small amount. The negative sign means inactive. MIC = minimal inhibitory concentration. Fractions elution pattern: F1 [hex:CHCl₃ (1:1)]; F2 [hex:CHCl₃ (1:1)]; CHCl₃; F3 (CHCl₃); F4 [CH₂Cl₂; CHCl₃; EtOH (97:3)]; F5 [CH₂Cl₂; EtOH (97:3), (93:7)]; F6 [CH₂Cl₂; EtOH (93:7)]; F7 [CH₂Cl₂; EtOH (93:7)]; F8 [CH₂Cl₂; EtOH (93:7), (9:1)]; F9 [CH₂Cl₂; EtOH (91:1)]; CHCl₃; EtOH; H₂O (7:2:1)]; F11 [CH₂Cl₂; EtOH; H₂O (6:3.5:0.5)]; F13 [CH₂Cl₂; EtOH; H₂O (5:4:1)]; F14 [CH₂Cl₂; EtOH; H₂O (5:4:1)]; F15 (EtOH); F16 (H₂O).

*ampliesiculatum* is reported here for the first time. This may bear significance considering its rare occurrence. There are only a few reports of 1-alkanols isolated from marine algae belonging to the Rhodophyceae (Hayee-Memom et al. 1991).

Compound 2 (Table 3) was isolated by repeated crystallization of fraction 3 in hot MeOH. This compound was distinctly identified by comparison of the ¹H-NMR data as the (24S)-24-methylcholesta-5, 25-dien-3β-ol commonly named clerosterol. This compound has been previously isolated from *C. fragile* (Rubinstein & Goad, 1974), *C. iyengarii* (Ahmad et al., 1992), *C. decorticatum* (Anjaneyulu et al., 1991; Ahmad et al., 1993), *C. arabicum* (Sheu et al., 1995) and *C. dichotomum*, with a clerosterol proportion of up to 93% relative to the content of sterols (Kapetanovic et al., 2005). As afore mentioned, clerosterol was the main compound from fraction 3, which was active against *S. aureus* and *S. pyogenes*. However, compound 2 was inactive against all the target bacteria we tested.

In experiments with other bacteria, Saleem (2000) reported the antibacterial activity against *Corynebacterium diphtheriae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Shigella dysenteriae* and *Staphylococcus aureus* at a concentration of 2 μg mL⁻¹, with the cetonic clerosterol form on carbon-3 and esterified fucose on carbon-7, known as Iyengaroside-B, initially isolated of *Codium iyengarii*. And also a galactoside of clerosterol at concentration of 1 μg mL⁻¹, against: *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, but no antibacterial activity from pure clerosterol. Thus, confirming the results obtained in this study on inactivity with pure clerosterol from *Codium amplivesiculatum*.

The cytotoxicity of compound 2 was not significant against the human cancer cell lines PC-3, HCT-15, and MCF-7 (%GI = 21.05 ±0.3, 11.99 ±3.2, and 9.54 ±1.1) at the tested concentration. This contrasts with the finding of Sheu et al. (1995), who reported pure clerosterol and oxygenated clerosterols forms cytotoxicity on cell lines of human lung adenocarcinoma (A-549), colon (HT-29), human nasopharyngeal carcinoma (KB) and mouse lymphocytic leukemia cells (P-388), observing that the ED₅₀ lev-
els of oxygenated clerosterols were between 0.2 y 2.1 µg mL\(^{-1}\). The chemical form of clerosterol had no cytotoxicity on cell lines HT-29 and KB, as it had a higher ED\(_{50}\) at 40 µg mL\(^{-1}\). The cell lines A-459 and P-388 were 0.3 and 1.7 µg mL\(^{-1}\) respectively. This result shows that the pure clerosterol form may be less cytotoxic due to individual differences between the cell lines used.

Kim et al. (2013) showed that pure clerosterol inhibited growth of human melanoma cells (A2058) and human keratinocytes cells (HaCaT) at 49% and 73% respectively at IC\(_{50}\) of 150 µM for 24 hours and induced apoptotic cell death, in contrast to the results obtained in this study with high cell viabilities at the concentration tested.

In conclusion, to date this is the first report of the isolation of 1-octadecanol and clerosterol from the marine alga *C. amplivesiculatum*. More importantly, this article cites one of the very few cases of 1-octadecanol in the chlorophytes. We provide supporting evidence of the promise of marine algae as sources of compounds that may fight tuberculosis and other infectious diseases.

ACKNOWLEDGEMENTS

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canol, and Mercedez Cueto Prieto (Instituto de Productos Naturales y Agrobiología, Spain) identified clerosterol. We thank Instituto Politécnico Nacional of Mexico for financial support (SIP 20111014; SIP 20121143), Instituto Mexicano del Seguro Social for supporting the postgraduate studies. SEMS-COSDAC for supporting postgraduate studies.

REFERENCES


