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

Marín-Álvarez, A.[1], J. I. Murillo-Álvarez [1], M. Muñoz-Ochoa[1] & G. M. Molina-Salinas[2]

[1]Centro Interdisciplinario de Ciencias Marinas. Instituto Politécnico Nacional (CICIMAR), Avenida Instituto Politécnico Nacional s/n, Col. Playa Palo de Santa Rita, La Paz, Baja California Sur 23096, Mexico. [2] Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, Avenida San Luis Potosí y 2 de Abril. Col. Independencia, Monterrey, Nuevo León. 64720, México. email: amarinal@ipn.mx

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 ^{1}H 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

Constituyentes químicos y bioactividad de *Codium amplivesiculatum*

RESUMEN: Con el propósito de descubrir sustancias bioactivas a partir de organismos marinos encontrados en México, se fraccionó el extracto crudo etanólico de *Codium amplivesiculatum* en columnas cromatográficas de sílica gel 60 Å (230-400 de malla) utilizando mezclas de solventes de polaridad creciente. Todas las fracciones se sometieron a ensayos antibacterianos. El principal metabolito de la fracción activa antituberculosis (MIC = 100 µg mL^{-1}), fue purificado e identificado como 1-octodecanol (1). La actividad antituberculosis, basada en reportes previos, se atribuyó al compuesto 1. Además, se obtuvo clerosterol (2) por cristalización de una fracción activa frente a *Staphylococcus aureus* y *Vibrio parahaemolyticus* (MIC = 125 y 250 µg mL^{-1}, respectivamente). Las dos estructuras fueron inferidas mediante interpretación y comparación de datos obtenidos por espectroscopía de IR-ATR y ^{1}H RMN. En contraste con otros estudios, el compuesto 2 mostró una citotoxicidad no significativa contra la línea celular PC-3 (% IC = 21.05 ± 0.3 a 50 µg mL^{-1}). Hasta donde sabemos, estos metabolitos se reportan por primera vez en *C. amplivesiculatum* y 1-octadecanol es un reporte muy raro de alcohol de cadena larga aislado de clorofitas.

Palabras clave: 1-octodecanol, clerosterol, antituberculosis, alcohol graso.

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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 thalli 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

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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_2Cl_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_2Cl_2 (1:1), followed by the same amount of CH_2Cl_2, CH_2Cl_2:EtOH in a polarity gradient from (97:3), (93:7) to (9:1), and mixtures of CH_2Cl_2:EtOH:H_2O (7:3:1), (6:3:1), (6:4:1), EtOH, and H_2O. 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_2Cl_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_2Cl_2 (1:1), followed by equal volumes of CH_2Cl_2 to CH_2Cl_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_2Cl_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_2Cl_2:EtOH (97:3) was found to be an amorphous solid which, after several crystallization steps in hot methanol, yielded a crystalline solid (compound

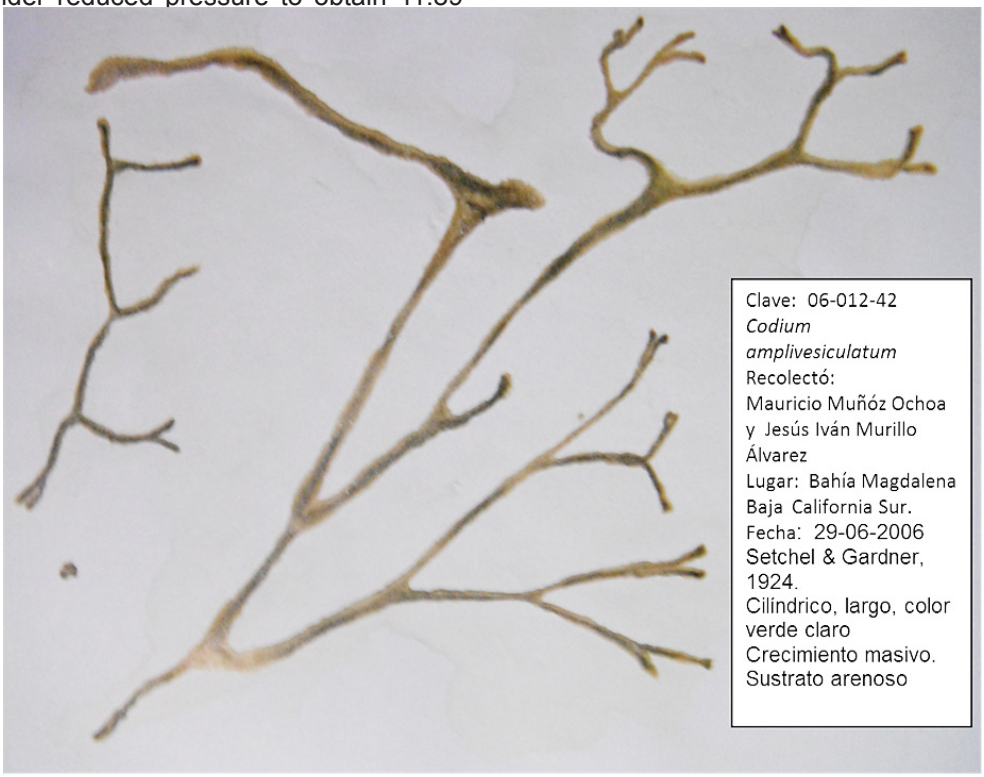

Figure 1. *Codium amplivesiculatum* from Margarita Island, BCS, México.

2, 34 mg) that was soluble in CH_2Cl_2 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 $K6F_{254}$ 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 cefalotine), *Escherichia coli* (BAA-196, resistant to ampicillin, ampicillin/sulbactam, cefazoline, gentamicin, piperacillin, tobramycin, and trimethoprim/clavulanic acid), *Streptococcus pyogenes* (BAA-946, resistant to erythromycin), *Vibrio parahaemolyticus* (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 seem, 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_2Cl_2:EtOH (97:3) was also active against *V. parahaemolyticus*. 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_2Cl_2 (1:1) and CH_2Cl_2 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 alkenyl 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_{28}–C_{32}) isolated as phytoconstituents from *Alpinia purpurata* showed inhibition of the same *M. tuberculosis* strain (Villaflores *et al*., 2010). Considering these reports, the anti-mycobacterial 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; Tlili *et al*., 2011) the occurrence of 1-octadecanol in *C.*

Table 1. Antibacterial activity of fractions and compounds from *Codium amplivesiculatum*

Inhibition zone in mm by the diffusion assay at 2 mg disc^{-1} and MIC in µg mL^{-1}

Sample	*Staphylococcus aureus* BAA-42	*Escherichia coli* BAA-196	*Streptococcus pyogenes* BAA-946	*Vibrio parahaemolyticus* 17802	*Vibrio alginolyticus* 17749	*Vibrio harveyi* 14126	*Mycobacterium tuberculosis* 27294
F1	–	–	–	–	–	–	–
F2	–	–	–	–	–	–	n/a(100)
F3	15 (125)	–	–	8 (>250)	–	–	–
F4	15 (>250)	–	17.5 (n/a)	–	–	–	–
F5	8 (>250)	–	11 (n/a)	–	–	–	–
F6	8 (>250)	–	14 (n/a)	–	–	–	–
F7	n/a	n/a	n/a	n/a	n/a	n/a	–
F8	9.5 (>250)	–	14 (n/a)	–	–	–	–
F9	–	–	7 (n/a)	–	–	–	–
F10	–	–	9.5 (n/a)	–	–	–	–
F11	–	–	7 (n/a)	–	–	–	–
F12	–	–	9.5 (n/a)	–	–	–	–
F13	–	–	7 (n/a)	–	–	–	–
F14	–	–	–	–	–	–	–
F15	–	–	–	–	–	–	–
F16	–	–	–	–	–	–	–
1-octadecanol	n/a	n/a	n/a	n/a	n/a	n/a	n/a
clerosterol	-		–	–	–	–	n/a

n/a = not assessed because of small amount. The negative sign means inactive. MIC = minimal inhibitory concentration. Fractions elution pattern: F1 [hex:CH_2Cl_2 (1:1)], F2 [hex:CH_2Cl_2 (1:1), CH_2Cl_2], F3 (CH_2Cl_2), F4 [CH_2Cl_2, CH_2Cl_2:EtOH (97:3)], F5 [CH_2Cl_2:EtOH (97:3), (93:7)], F6 [CH_2Cl_2:EtOH (93:7)], F7 [CH_2Cl_2:EtOH (93:7)], F8 [CH_2Cl_2:EtOH (93:7), (9:1)], F9 [CH_2Cl_2:EtOH (9:1)], F10 [CH_2Cl_2:EtOH: EtOH (9:1), CH_2Cl_2:EtOH:H_2O (7:2:1)], F11 [CH_2Cl_2:EtOH:H_2O (7:2:1)], F12 [CH_2Cl_2:EtOH:H_2O (6:3.5:0.5)], F13 [CH_2Cl_2:EtOH:H_2O (5:4:1)], F14 [CH_2Cl_2:EtOH:H_2O (5:4:1)], F15 (EtOH), F16 (H_2O).

amplivesiculatum 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 alage 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 ^{1}H-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 diptheriae*, *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 ED_{50} lev-

Table 2. Assignments of the IR espectra (ν in cm^{-1}, %T) and ^{1}H NMR (300 MHz, CDCl) (δ ppm). 1-Octadecanol (compound 1) of *Codium amplivesiculatum*.

White semi-solid at rt. IR: 3324 (75.3), 2974 (84.1), 2942 (82.1), 2831 (84.2), 1449 (85.9), 1414 (86.5), 1088 (84.1), 1024 (30.8), 880 (85.3), 737 (73.7), 623 (73.8). ^{1}H NMR: δ 0.89 (3H, t, J = 7.0 Hz, H-18), 1.20 (2H, br s, H-16), 1.28 (br s), 1.56 (2H, m, H-2), 3.64 (2H, t, *J* = 6.4 Hz, H-1).

Table 3. Assignments of the IR espectra (ν in cm^{-1}, %T) and ^{1}H NMR (300 MHz, CDCl) (δ ppm). Clerosterol (compound 2) of *Codium amplivesiculatum*.

Colorless crystal, mp: 131-132°C. IR 3327 (97), 2933 (87), 2850 (90), 1644 (96), 1452 (90), 1332 (94), 1244 (96), 1193 (95) 1108 (95), 1043 (83), 1022 (88), 884 (81), 839 (93), 800 (91), 739 (93), 626 (90), 558 (89). ^{1}H NMR δ 0.69 (3H, s, H-18), 0.82 (3H, t, *J* = 7.4 Hz, H-29), 0.92 (3H, d, *J* = 6.5 Hz, H-21), 1.02 (3H, s, H-19), 1.58 (3H, s, H-27), 3.51 (1H, m, H-3), 4.66 (1H, br.d, *J* = 2.3 Hz, H-26), 4.73 (^{1}H, br.dd, *J* = 2.3, 1.4 Hz, H-26), 5.36 (1H, br.d, *J* = 5.2 Hz, H-6).

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 keratinocyte cells (HaCaT) with a cell viability of 49% y 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-octodecanol 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.

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