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Potent Antifouling Metabolites from Red Sea Organisms

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Forty two marine macro-organisms include; algae, corals and sponges were collected from the Saudi Red Sea territorial water. The methanolic extracts of all the samples were evaluated towards their antifouling effects cyprid larvae of *Balanus amphitrite*. Fifteen crude extracts showed antifouling activity at 10 µg/mL, eleven of which considered to be promising for isolation targets from the results of low mortality. Two marine species of sponge *Siphonochalina siphonella* and red alga *Laurencia obtusa* were selected further chemical investigation.The extract of *S. siphonella*, was partially fractionated and led to isolation of two known triterpenoidal metabolites; sipholenol A (**1**) and sipholenone A (**2**). Three compounds; 2,10-dibromo-3-chloro-7-chamigrene (**3**) and 12-hydroxyisolaurene (**4**) and (12*E*)-*cis*-maneonene-E (**5**), were isolated from *L. obtusa*. All the compounds (**1-5**) were evaluated for their antifouling activities. Compounds **1**, **3** and **4** showed potent effects with 48 h EC_{50}, 0.2, 0.14 and 1.34 µg/mL, respectively. Compounds **1**and **3** were more potent than the positive control $CuSO_4$ (EC_{50} = 0.34 µg/mL), while compound **2** showed moderate activity at 10 µg/mL.

Keywords: Red Sea, Antifoulants, Algae, Soft corals, Sponge, *Siphonochalina siphonella*, *Laurencia obtusa*.

INTRODUCTION

Biofouling is a natural processes in many cases responsible for the increasing of the cost of marine transportation. These organisms could be responsible for the biodeterioration[1]. Antifouling activities are necessary in order to avoid the deposition on the surfaces by bio-foulers and consequently the high costs relative to transport delays, hull repairs, cleaning of desalination units and bio-corrosion, which estimated up to 150 billion USD per year[2-4]. On these bases, there is a need for the continuous development of new environmental friendly antifouling agent. An interesting and promising line of research is inspired by biomimetic solutions[2]. The soft bodies of marine organisms could play an important role as a defense against possible colonies, but do resist overgrowth by epibionts[5]. Marine organisms include sponges, corals and macroalgae and/or their associated microflora and/or symbionts, are considered potential sources for antifouling metabolites[6,7].

To date, natural products from marine organisms which were published more than 200 molecules with diversity of structures[8]. The unexplored marine species with different environment estimated by 1-2 million had novel biosynthetic capabilities[8]. A triterpene glycoside was obtained from the sponge *Eurylus formosus* and exhibited potent and broad-spectrum activities towards bacteria, fungi, macroalgae and invertebrates[9,10]. Many other compounds were isolated from sponges displayed activities against invertebrate's settlement or microbial growth only[11].

The study of macroalgae metabolites particularly Rhodophyceae and Phaeophyceae for antifouling activity was considered. Genus *Laurencia* were investigated yielded to production ca. 700 natural metabolites, particularly bioactive halogenated compounds. Investigation of *Bifurcaria bifurcate* led to isolation of diterpenes with significant antifouling activity[8,12]. Interesting compounds from *Sargassum tennerimum* were shown to interfere with larval settlement of *Hydroide selegans* and biofilm formation. The publications of antifouling metabolites from sponges' families (*e.g.* Axinellidae and Dictyonellidae) produce sesquiterpenes and diterpenes, isocyano, isothiocyano, thiocyano and formamide functionalities[13].

In continuation of projects for isolation and identification of bioactive metabolites from the Saudi marine sources; two

marine species of sponge *Siphonochalina siphonella* and red alga *Laurencia obtusa* were selected further chemical investigation based on the chemical finger print and productivity of the two species. Herein, we report the results of screening, isolation, identification and antifouling activities of metabolites from *S. siphonella* and *L. obtusa*.

EXPERIMENTAL

Optical rotations were measured on ATAGO POLAX-L 2 polarimeter. EI/MS analyses were carried out on a Shimadzu-QP 2010. GC/MS analyses were carried out usig RTX-1 column (30 m, 0.25 mm) was used. 1D and 2D NMR spectra were recorded on Bruker AVANCE III WM 600 MHz spectrometers and ^{13}C NMR at 150 MHz. Chemical shift values are given in δ ppm relative to TMS as internal standard. Thin layer chromatography was performed on silica gel (Kieselgel 60, F254) of 0.25 mm layer thickness. Gel filtration was carried out using Sephadex LH-20. Spots were detected by using ethanol/sulfuric acid as spray reagent.

Marine samples: The samples were collected from different places around Jeddah coast at depths 1-20 m by different techniques such as skin and Scuba diving. The collected species were sort out and.

Algae: The algal collection belongs to different genus. After collection, the samples were washed with sterile filtered seawater to remove associated debris and large epiphytes. A 10 min washing with 5 % ethanol was performed to clean the surface from microflora. The cleaned material was lyophilized and then the samples were extracted.

Corals: The collected corals species represent ≅ 24 % of the total organisms collected from the Saudi Red Sea coat. The samples were kept in ice boxes and transported to the laboratory for lyophilization then extraction.

Sponge: Sponge species were also collected from different depth and places of the Saudi Red Sea water, by different techniques such as skin and Scuba diving. The sponges were washed with freshwater transported to the laboratory for lypholization then extraction.

Preparation of crude extracts for screening: The freeze-dried samples were macerated and percolated by the organic solvents (MeOH) for 24 h at 22 °C. The crude extracts were dried under nitrogen gas and prepared for bioactivity assays.

LC-ESIMS analysis: The HPLC system consisted of an Agilent 1200 system, a solvent delivery module, a quaternary pump, an autosampler, a diode-array detector (DAD) and a column compartment (Agilent Technology, Germany). The column effluent was connected to an Agilent 6320 Ion Trap HPLC-ESI-MS. The column heater was set to 25 ± 2 °C. The control of the HPLC system and data processing were performed using ChemStation (Rev. B.01.03 SR2 (204)) and 6300 Series Trap Control version 6.2 Build No. 62.24 (Bruker Daltonik GmbH). The analytes were separated using an Agilent Zorbax Extend-C_{18} column (80 Å, 150 mm length X 4.6 mm, i.d., 5 µm) an Agilent-Zorbax Extend-C_{18} pre-column (Agilent Technologies, Palo Alto, CA, USA). Positive auto-MSn mode; the mobile phase was prepared by mixing 700 mL of 0.1 % formic acid in water with 300 mL acetonitrile and was pumped at a flow rate of 0.5 mL/min. While, negative auto-MSn mode; the mobile phase was prepared by mixing 700 mL of 0.3 % ammonia solution 25 % in water with 300 mL acetonitrile and was pumped at a flow rate of 0.5 mL/min.

Antifouling assay: *Balanus Amphitrite*, attached to bamboo poles were procured from oyster farms in Lake Hamana, Shizuoka, Japan and maintained in an aquarium at 20 °C by feeding on *Artemia salina* nauplii. Broods released I-II stage nauplii upon immersion in seawater after being dried overnight. Nauplii thus obtained were cultured in 80 % filtered seawater (filtered seawater diluted to 80 % by deionized water) including penicillin G (20 µg/mL, ICN Biochemical) and streptomycin sulfate (30 µg/mL, Wako Pure Chemical Industries, Ltd.) at 25 °C by feeding with the diatom *Chaetoceros gracillis* (40×10^4 cells/mL). Larvae reached the cyprid stage in 5 days. The cyprids were collected and then stored at 4 °C until use. Test samples were dissolved in ethanol. Aliquots of the solution were supplied to wells of 24 well polystyrene tissue culture plates and air dried. To each well were added 2 mL of 80 % filtered seawater and six 1 day-old cyprids. Four wells were used for each concentration. The plates were kept in the dark for 48 h at 25 °C and the number of larvae that attached, metamorphosed, died, or did not settle were counted under a microscope. For first screening tests of crude extracts, samples were evaluated at 2 concentration sets (10, 100 µg/mL). In the case of pure compounds, each concentration (0.03, 0.1, 0.3, 1.0, 3.0, 10.0 µg/mL) was repeated 3 times. The antifouling activity of compounds was expressed as an EC_{50} value, which indicated the concentration that reduces the larval settlement to 50 % of the control. The EC_{50} values were calculated by a probit analysis. When compounds could not apply probit analysis, the EC_{50} values of their were estimated by straight-line graphical interpolation.

Siphonochalina siphonella

Extraction and isolation of compounds 1 and 2: The freeze-dried sponge (94.9 g) was extracted two times with 6 L of MeOH for 24 h at 22 °C, a viscous dark reddish oil was obtained (22.7 g). This extract was fractionated on NP-Silica (5 × 75 cm, 500 g, Merck 7739), employing gradient elution from petroleum ether to EtOAc. Fractions of 50 mL were collected. TLC was carried out by employing silica-gel chromatoplates, appropriate solvent system and 50 % sulfuric acid in methanol as spraying reagent. Fractions containing a single compound were combined and further purified by preparative TLC of glass supported silica gel plates (20 cm × 20 cm) of 250 mm thickness. The fraction (F-A) which was eluted with Pet. ether -EtOAc (6:4, v/v, 117 mg, F-A) was further purified by PTLC employing benzene -EtOAc (7:3) yielded **2**. The fraction (F-B) which eluted with pet. ether-EtOAc (5:5, v/v, 10 mg was purified by PTLC employing Pet. ether -EtOAc (5:5, v/v), yielded **1** (10 mg).

Sipholenol-A (1): White amorphous powder (10 mg, 0.010 %); R_f = 0.50 (silica gel, pet. ether-EtOAc 8:2, brownish spot upon spraying with a solution of 50 % sulfuric acid in methanol); IR (λ_{max}, film, cm^{-1}): 3615, 3599, 3422, 2967, 2933, 2861, 1462, 1439, 1374, 1291, 1250, 1160, 1128, 1077, 1050, 987, 931, 909, 840, 753, 663; ^{13}C NMR ($CDCl_3$, 150 MHz) and ^1H NMR ($CDCl_3$, 600 MHz) spectroscopic data; HRFABMS *m/z* = 499.3755 [M^+ + Na]. (Calculated *m/z* = 499.3763 for $C_{30}H_{52}O_4Na$)[14].

Sipholenone-A (2): Colourless oil (17 mg, 0.0179 %); R_f = 0.33 (Si gel, benzene -EtOAc 9:1, reddish brown spot upon spraying with a solution of 50 % sulfuric acid in methanol); IR (λ_{max}, film, cm^{-1}): 3457, 2860, 2932, 2865, 1709, 1463, 1375, 1294, 1253, 1169.73, 1129, 1082, 1044, 911, 842, 815, 751; ^{13}C NMR ($CDCl_3$, 150 MHz) and ^1H NMR ($CDCl_3$, 600 MHz) spectroscopic data[14].

Laurencia obtusa

Extraction and isolation of compounds 3-5: Algal material was washed with water and dried in the shade at room temperature. The dried material of the red alga *Laurencia obtusa* (270 g) was exhaustively extracted with methanol (2 × 6 L, 24 h for each batch) at room temperature. The residue (7.5 g) was partitioned between ether and water, the organic layer (5.4 g) was chromatographed on NP (Merck, 60G) column chromatography employing *n*-hexane/diethyl ether mixtures with increasing polarity. The fraction (F-A) which was eluted with pet. ether (100 %, 98 mg, F-A) was further purified by PTLC employing pet. ether (100) yielded **3** (13 mg). The fraction (F-B) which eluted with pet. ether-ether (9:1, v/v, 85 mg was purified by PTLC employing pet. ether-ether (9:1, v/v), yielded **5** (7 mg). The fraction (F-C) which eluted with pet. ether-ether (8:2, v/v, 120 mg was purified by PTLC employing pet. ether-ether (7:3, v/v), yielded **4** (15 mg).

2,10-Dibromo-3-chloro-7-chamigrene (3): Colourless oil (13 mg, 0.048 %); R_f = 0.95 (Si gel, Pet. ether, violet spot upon spraying with a solution of 50 % sulfuric acid in methanol); GCMS (rel. int.): 402, 400, 398, 396 (1:3:4:2) [M, $C_{15}H_{23}Br_2Cl]^+$, 321, 319, 317 (2:10:5) $[M-Br]^+$, 264 (14), 261 (7), 201 (18), 159 (35), 147 (39), 145 (91), 109 (70); ^{13}C NMR ($CDCl_3$, 150 MHz) and ^1H NMR ($CDCl_3$, 600 MHz) spectroscopic data[15].

12-Hydroxyisolaurene (4): Colourless oil (15 mg, 0.055 % yield); R_f = 0.75 (Si gel, *n*-hexane-Et_2O, 8:2, violet appearance under UV, while yellow orange spot developed upon spraying with 50 %-sulfuric acid in methanol); $[\alpha]_D^{22}$ = 41.7 ($CHCl_3$; C = 0.01). UV (MeOH) 212, 273, 279 nm; IR (λ_{max}, neat, cm^{-1}): 3298, 2925, 1637, 1580, 1510 nm; HREI-MS data *m/z* 215.1423 $[M]^+$ (calculated for $C_{15}H_{20}O$, 215.1436); ^{13}C NMR ($CDCl_3$, 150 MHz) and ^1H NMR ($CDCl_3$, 600 MHz) spectroscopic data[16].

(12*E*)-*cis*-Maneonene-*E* (5): Colourless oil (7 mg, 0.026 % yield); R_f = 0.65 (Si gel, *n*-hexane-Et_2O, 9:1, yellow spot developed upon spraying with 50 % sulfuric acid in methanol); IR (λ_{max}, film, cm^{-1}): 3285, 2970, 2929, 2100, 1686, 1454, 1438, 1344, 1326, 1267, 1204, 1182, 1128, 1117, 1086, 1044, 1027, 990, 974, 959, 916, 889, 829, 816, 797, 776, 724, 655; GCMS (70 eV) *m/z* (rel. int.): 346 (4), 344 (12), 342 (8), $[M]^+$ ($C_{15}H_{16}BrClO_2$); ^{13}C NMR ($CDCl_3$, 150 MHz) and ^1H NMR ($CDCl_3$, 600 MHz)[17].

RESULTS AND DISCUSSION

The total extracts (42 samples) were tested towards their antifouling activity using cypris larvae of barnacle. Fifteen samples showed antifouling activity at 10 µg/mL, eleven were promising and four had potent antifouling effects (*Siphonochalina siphonella*, *Sarcophyton glaucum*, *Sinularia leptoclades* and *Hyrtios* species). They showed 90 % settlement inhibition against barnacle cyprids larvae (10 µg/mL). Especially, *Hyrtios* sp. inhibited larval settlement at 10 µg/mL completely and did not kill any larva even (100 µg/mL) suggesting environmentally benign and potent antifouling compounds could be isolated.

The major number of the bioactive extracts was found between the soft coral samples, followed by sponge samples and the lowest number was found between the algal extracts (Table-1). These results indicated that the potent antifouling compounds are included on two soft corals (*Sarcophyton glaucum* and *Sinularia leptoclades*) and two sponges (*Siphonochalina siphonella* and another belongs to the genus *Hyrtios*). Extensive investigation of the literatures indicated that these are productive species. Soft corals, belonging to the genus *Sarcophyton* and *Sinularia* are recognized as a rich source of macrocyclic cembrane-type diterpenoids and *bis*cembranoids. Up to date more than 300 natural cembranoid derivatives were isolated. Cembrane-type diterpenoids are a large family with diversity of functionality and obtained from both terrestrial and marine organisms. They are usually exhibit cyclic ether, lactone, or furan moieties around the cembrane framework. The cembranes derivatives play an important role in the biomedical perspective[18-20]. *Hyrtios* sp. is rich source of biologically active metabolites. These include sesterterpenes sesquiterpenes macrolides, indole and β-carboline alkaloids[21,22]. Indole containing metabolites have diversity of biological activities[23]. It is a speculation that the antifouling activity could be due to any metabolites manufactured inside the biological factory of these species. To prove this speculation, three things must be done extensive LCESIMS analysis, chromatographic processes and antifouling assay of the isolated compounds. In addition, we added *Laurencia obtusa* for further investigation, because *Laurencia* is one of the richest source of biologically active compounds in our screened species. In the current phase we did the LCESIMS of the most of the samples and two species were subjected to partial chromatographic isolation. The remaining potent extracts and/or chemically interested one will be subjected for isolation and biological assay of the pure compounds.

TABLE-1
ANTIFOULING RESULTS OF THE COLLECTED MARINE ORGANISMS

Marine classes	Tested samples	Active sample[a]	Promising sample[b]
Algae	11	1	0
Soft corals	10	5	5
Sponges	19	7	4
Others	2	2	2
Total	42	15	11

[a]Those which showed fewer than 10 % larval settlement rates at 10 µg/mL; [b]Those which showed fewer than 10 % larval settlement rates and low toxicity at 10 µg/mL (< 15 % lethality)

Siphonochalina siphonella was fractionated by silica gel column chromatography and preparative TLC and led to isolation of two metabolites; sipholenol A (**1**) and sipholenone A (**2**) (Fig. 1). Sipholenol (**1**) showed wide range of biological activities especially towards different types of cancer for instance; it had antiproliferative effects towards PC-3 and A549 with IC_{50} = 7.9 ± 0.12 and 8.9 ± 0.010, respectively[24].

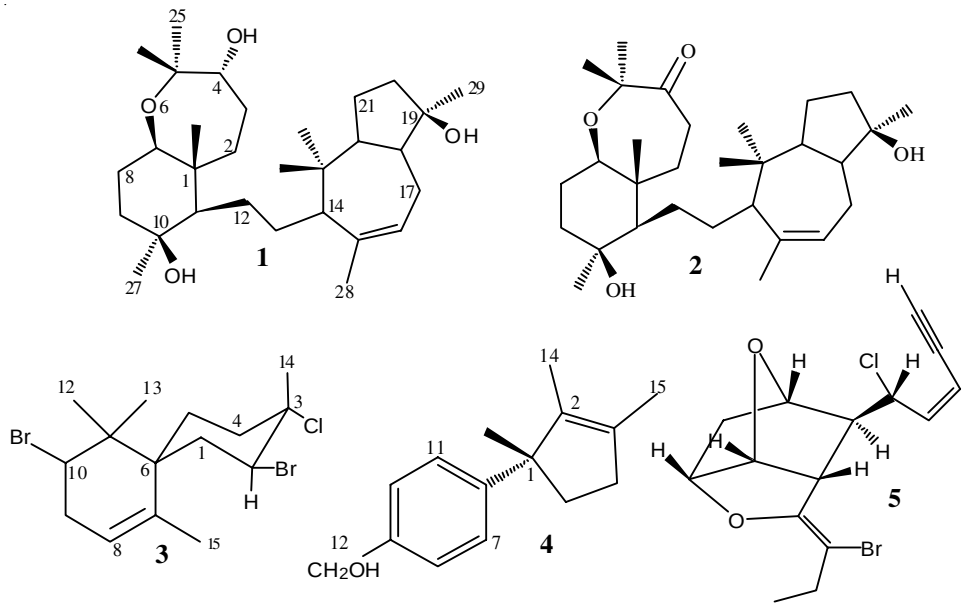

Fig. 1. Chemical structure of compounds **1-5** isolated and identified from *Siphonochalina siphonella* and *Laurencia obtusa*

Laurencia obtusa was also fractionated using chromatographic techniques (silica gel column chromatography and preparative TLC). Three metabolites isolated and these are 2,10-dibromo-3-chloro-7-chamigrene (**3**), 12-hydroxyisolaurene (**4**) and (12*E*)-*cis*-maneonene-*E* (**5**) (Fig. 1). The structures were determined by comparing the measured NMR and MS with the published data.

The identified compounds (**1-5**) were evaluated against cyprids of *B. Amphitrite* and only 3 compounds (**1**, **3** and **4**) showed potent effects (Table-1, Fig. 2).

The only structural difference of **1** and **2** is that 4-hydroxy group in **1** is replaced by 4-ketone in **2**. However, **2** lost antifouling activity observed in **1**. Apparently 4-hydroxy group in **1** is important for antifouling activity. Although **4** was reported to

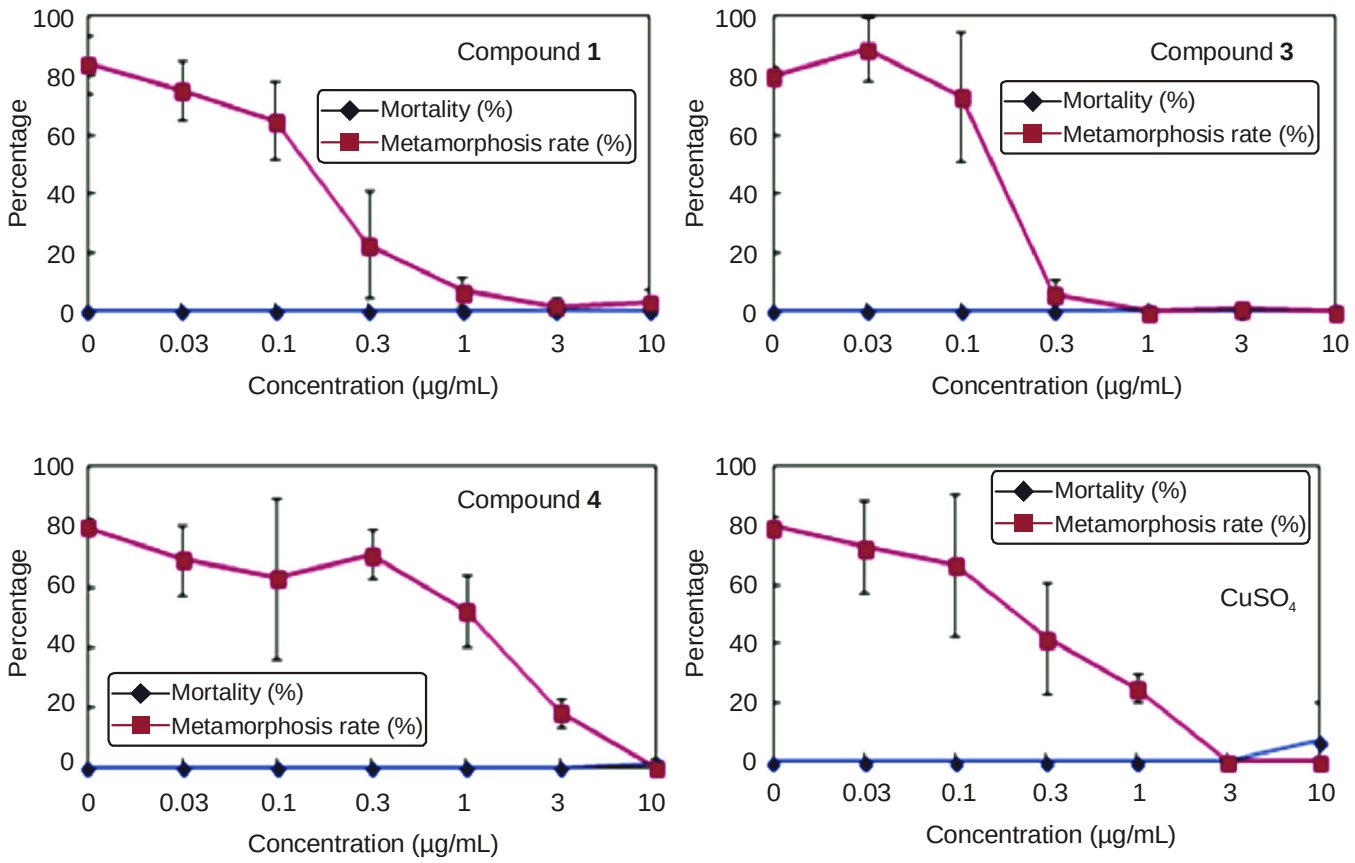

Fig. 2. Antifouling activity and mortality of compounds **1**, **3**, **4**, and $CuSO_4$ against barnacle cyprids after 48 h

show antifungal activity against *Candida albicans* (MIC = 70 mg/mL) and against *Aspergillus fumigatus* (MIC= 100 mg/mL)[16], **4** showed weak activity against the barnacle larvae.

Conclusion

Four of the collected Red Sea organisms extracts have potent effects and they showed 90 % settlement inhibition against barnacle cyprids larvae (10 µg/mL). Two compounds (**1** and **3**) isolated from *Siphonochalina siphonella* and *Laurencia obtusa* showed more potent than the positive control.

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