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ANTIBIOFILM ACTIVITIES OF EXTRACTS OF THE MACROALGA HALIMEDA SP. FROM THE RED SEA

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Key words: antifouling, biofouling, biofilm, chemical defence, seaweed, bioactive compounds.

ABSTRACT

Marine macroalgae generally keep their surface free from fouling organisms by their antifouling defence. Antifouling activity has been attributed to the production of secondary metabolites. In the present study, antifouling performance of the macroalga *Halimeda* sp., collected from the Red Sea coastal waters, was assessed using extracts prepared through various methods, including surface extraction, wet sample extraction and dried sample extraction. The solvents methanol and hexane were used to assess the effect of solvents on bioactivity. Results indicated that extracts of the macroalga inhibited growth and settlement of a biofilm-forming bacterial strain. Though the extracts prepared using different methods and solvents showed inhibitory activities, considerable variation was observed between different assays. Hexane extracts showed strong bacterial growth inhibitory activities in the spectrophotometric assay. The extract of dried algal samples also showed strong growth inhibitory activity, while the extract obtained from fresh algal samples exhibited strong antibiofilm activity. GC-MS analysis showed variations in the biochemical profile of the crude extracts prepared by different methods. In conclusion, results of the present study indicated that the potential bioactive metabolites present in the crude extracts of the marine macroalga *Halimeda* sp. could be used as a natural antibiofilm and as an antifouling compound.

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I. INTRODUCTION

All substrates submerged in the aquatic environment are covered by a layer of microbial film commonly called as "biofilms" (Viano et al., 2009; Satheesh and Wesley, 2010). In the marine environment, biofilm development on hard surfaces leads to a process called biofouling (Satheesh et al., 2016). The attachment of bacterial communities helps settlement of higher organisms on the surfaces. Biofouling includes all attaching organisms such as microfoulers (bacteria, fungi, protozoans, microalgae) and macrofoulers such as barnacles, tube worms, mussels and bryozoans (Stoodley et al., 2002; Satheesh and Wesley, 2008). Generally, biofouling is a main concern for underwater man-made structures and a costly problem for marine technology sectors as frequent cleaning and antifouling measures are needed (Armstrong et al., 2000; Satheesh et al., 2016). Presently, most of the available antifoulants are mainly toxic chemicals and applications of these compounds have many harmful effects on the environment (Bellas, 2006; Thomas and Brooks, 2010). After the ban of tributyltin (TBT) for marine applications, research interest for searching non-toxic or less toxic antifouling compounds has gained momentum with the discovery of many new compounds from natural resources (Satheesh et al., 2016).

Natural products from many organisms have been reported to prevent biofilm formation and subsequent development of biofouling on hard substrata (Qi et al., 2008; Viju et al., 2013; Satheesh et al., 2016; Viju et al., 2016). For example, marine organisms produce an array of secondary metabolites (those metabolites which are not essential for normal growth of the organisms) mainly for defence purposes. Macroalgae are one of the most important groups of marine organisms for natural occurring antifouling compounds as evidenced from studies of the antibiofilm and antifouling activities of macroalgal species (Nylund et al., 2007; Viano et al., 2009; Saha and Wahl, 2013; Othmani et al., 2016). For screening antifouling activities of marine macroalgae, different extraction methods and solvents were used previously (Yi et al., 2001; Nylund et al., 2007; Neoh et al., 2016; Norra et al., 2016; Olivares-Molina and Fernandez, 2016). The extraction methods and solvent types were also

reported to affect the outcome of screening assays conducted in the laboratory (Manilal et al., 2009; Cox et al., 2010). Extraction method and choice of solvent have been known to influence the physical and chemical properties of extracts (for a review, see Shannon and Abu-Ghannam, 2016). A comparative account of the inhibitory activities of macroalgal extracts obtained from different methods and solvents against biofilm-forming bacteria is clearly lacking. Hence, in the present study, antibiofilm activities of extracts of *Halimeda* sp., collected from the Red Sea coast, were assessed using different laboratory assays. Specifically, the questions addressed in this study were: (1) Do different extraction methods such as total extraction and surface extraction affect activity of macroalgal extracts against bacteria? (2) Do different solvents used for the extraction have any influence on antifouling screening assays? and (3) Has extraction using wet samples more advantages than using dried samples? Results obtained in this study not only improve our knowledge of antifouling defence of the Red Sea macroalgae but also provide useful information for designing appropriate extraction methods in antifouling screening assays using marine organisms.

II. MATERIALS AND METHODS

Samples of *Halimeda* sp. were collected from the Obhur Creek of Jeddah coastal waters (near KAU Obhur marine station, N21°42.551′, E 039°05.763′) and transported to the laboratory. In the laboratory, the macroalgal samples were rinsed with filtered seawater (Millipore, 0.47 µm) to remove debris and other attached organisms and used for extraction. The macroalgal samples were extracted separately using methanol (laboratory reagent, Sigma-Aldrich) and hexane (laboratory reagent, Sigma-Aldrich) solvents.

1. Extraction of Fresh Algal Samples

Fresh macroalgal samples (10 g for each solvent) were macerated (immediately after collection) using a pestle and mortar and used for extraction. The macerated sample was kept in 25 ml of the respective solvents (separately in methanol and hexane) for 5 h at room temperature (28°C) in the dark and centrifuged at 2000 × *g* for 15 min. The extract was concentrated under vacuum in a rotary evaporator at 40°C. The concentrated crude extract was adjusted to 5 ml by adding respective solvents and maintained at -20°C until further analysis.

2. Extraction of Dried Algal Samples

Macroalgal samples were air-dried under shade for one week at room temperature (28°C). The dried samples were powdered using a grinder and stored in polythene bags (28°C) without direct exposure to light. The dried macroalgal powder was extracted using solvents (hexane and methanol) for 5 h. Thereafter, the extract was collected, centrifuged at 2000 × *g* for 15 min. and concentrated as described above. The crude extract was adjusted to 5 ml by addition of the respective solvents and stored at -20°C for further assays.

3. Surface Extraction

The surface extraction method reported by de Nys et al. (1998) for seaweeds was followed in this study. In brief, surface molecules from the macroalga were extracted by dipping the fresh algal (10 g) samples for 10 seconds in 25 ml of either methanol or hexane. The extract was collected and centrifuged at 2000 × *g*. The extract was concentrated to 5 ml under vacuum in a rotary evaporator at 40°C and stored at -20°C for further assays.

4. Antibacterial and Antibiofilm Assays

A marine biofilm-forming bacterial strain *Vibrio harveyi* (NCBI GenBank accession number: KY266820) available at the Department of Biology, King Abdulaziz University, was used as the test bacterium in the antibacterial and antibiofilm assays. This biofilm-forming bacterial strain was isolated from artificial substrata submerged in Obhur Creek waters of the Red Sea. The bacterial strain was cultured using marine agar (marine agar 2216, Difco) and maintained in marine broth (Difco) for antibiofilm assays.

5. Bacterial Growth Inhibition Assay (Spectrophotometric Assay)

The biofilm bacterial strain (*V. harveyi*) was inoculated into marine broth and log phase culture (approximately 1.7×10^8 CFU ml^{-1}) was used for the growth inhibition study and other experiments. The bacterial broth (3 ml) was transferred to test tubes and 50 µl macroalgal extract (surface extract, fresh and dried algal extracts) was added to each tube. In control tubes, 50 µl solvents (methanol and hexane) were used instead of extracts. The optical density of the bacterial broth was measured before the start of the experiment and at 1 h intervals at 670 nm (modified from 660 nm used by Pan et al., 2014) in a spectrophotometer. The experiment was conducted over a period of 5 h in triplicate (for each extract) and the mean ± standard deviation values were recorded. The percentage of bacterial growth/inhibition was calculated using the following formula:

$$Growth\ Rate\ (\%) = \frac{Final\ OD\ Value - Initial\ OD\ Value}{Initial\ OD\ Value} \times 100$$

6. Effects of Algal Extracts on Extracellular Polymeric Substance Production in Biofilm-Forming Bacteria

The bacterial strain was inoculated into marine broth (25 ml) and algal extract (50 µl ml) was added to the broth. The culture without any algal extract treatment was considered as the control. Control cultures with the addition of hexane or methanol solvents were also maintained. The cultures were incubated at 37°C for 24 h. After the incubation, the broth was centrifuged at 3000 × *g* for 15 min. The supernatant was collected, mixed with equal volume of ice-cold ethanol (absolute) and kept for 24 h at room temperature (Satheesh et al., 2012). After 24 h, the precipitate was collected and stored at 4°C. The precipitated extracellular polymeric substances (EPS) was filtered through

a membrane filter and diluted with a known volume of distilled water. The carbohydrate concentration of the EPS was measured using the phenol sulphuric acid assay (Dubois, 1956).

7. Biofilm Inhibition Activity of Macroalgal Extracts: Microtitre Plate Assay

The biofilm inhibition assay for all extracts was determined by the 96-well microtitre plate method described by Coffey and Anderson (2014). Overnight culture of the biofilm-forming bacterium (100 µl) was added to each well and 5µl extracts were added. Two types of controls were also maintained: one without any extract and the other with solvents (methanol and hexane respectively) instead of extracts. The microtitre plates were incubated at room temperature (28°C) for 24 h. After incubation, the plate was inverted to remove the suspending cells and rinsed with sterilized water. Crystal violet (0.1%) in a volume of 150 µl was added to each well of the microtitre plate. After 10 min., the plate was inverted again to remove the crystal violet stain and rinsed with sterile water. Finally, 150 µl of glacial acetic acid was added to each well and kept for 10 min. The plate was read at 630 nm (Costa et al., 2014) in a Biotek plate reader. The whole experiment was repeated (n = 3) and the mean OD values for each treatment was calculated.

8. GC-MS Analysis of Macroalgal Extracts

Three extracts of *Halimeda* sp. (hexane and methanol extract of fresh algal samples and surface extraction using hexane) were analysed through GC-MS to understand the difference in the chemical composition of extracts prepared using different solvents and methods. Macroalgal extracts were partially purified using silica column and used for GC-MS analysis. The GC-MS analysis was carried out using a Shimadzu GC-MS QP 2010 available at the Faculty of Meteorology, Environment and Arid Land Agriculture, King Abdulaziz University. The compounds were separated using a capillary silica column (30 m × 0.25 mm × 0.25 µm) with helium as carrier gas at a flow rate of 1.5 ml/min. The mass spectrometer was operated in the electron impact (El) mode at 70 eV in the scan range of 40-700 m/z, split ratio: 1:10; injected volume: 1 µl; injector temperature: 250°C; oven temperature: 70°C for 3 min and then increased to 250°C at 14°C min^{-1} (total run time 41 min). Peak identification of crude seaweed extracts was performed by comparison with those available in NIST 11-Mass Spectral Library.

9. Statistical Analysis

One-way analysis of variance (ANOVA) was used to test the significant difference at $P < 0.05$ using Microsoft Excel program (Excel 2013 version).

III. RESULTS

1. Bacterial Growth Inhibition

The results showed that all extracts of *Halimeda* sp. inhibited growth of the marine biofilm-forming bacterium. The biofilm bacterial culture without extract showed an increase in growth of 20.82% during the 5-h incubation (Fig. 1). However, biofilm-forming bacterial cultures treated with the algal extracts showed negative growth during the 5-h period. Cultures treated with the surface extract (hexane solvent) showed higher growth inhibitory activity (-29.26%) followed by the extract obtained from dried samples (23.51%). In fresh algal samples, the hexane extract showed strong growth inhibitory activity (21.05%) against the biofilm-forming bacterial strain. The extracts prepared using methanol from fresh samples and surface extract also showed negative effects but their inhibitory activity was not as pronounced as the other extracts (Fig. 1). One-way ANOVA results revealed a significant variation (F = 15.21; df = 7, 23; P = 0.000) in the bacterial growth inhibitory activity of the different extracts.

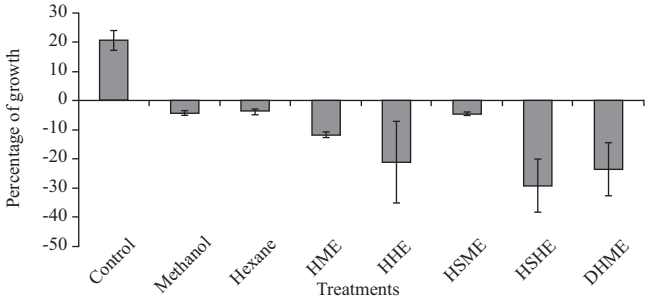

Fig. 1. Percentage of bacterial growth inhibition (mean ± SD, n = 3) by *Halimeda* sp. extracts. Bacterial growth inhibition was measured using the spectrophotometric method for 5 h. Control = without extract; HME = *Halimeda* methanol extract; HHE = *Halimeda* hexane extract; HSME = *Halimeda* surface methanol extract; HSHE = *Halimeda* surface hexane extract; DHME = dried *Halimeda* methanol extract.

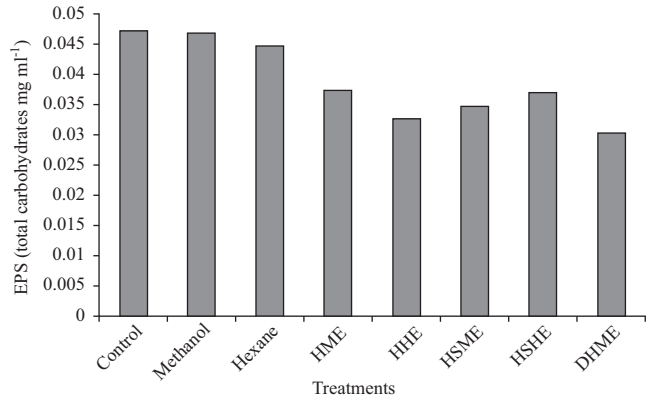

Fig. 2. Effects of *Halimeda* extract on extracellular polymeric substance production in biofilm-forming bacterial culture. Control = without extract; HME = *Halimeda* methanol extract; HHE = *Halimeda* hexane extract; HSME = *Halimeda* surface methanol extract; HSHE = *Halimeda* surface hexane extract; DHME = dried *Halimeda* methanol extract.

2. Effects of Algal Extracts on Extracellular Polymeric Substance Production in Biofilm-Forming Bacteria

The algal extracts affected extracellular polymeric substance

Table 1. Compounds identified from the methanol extract of *Halimeda* sp.

No.	Retention index	Compound name	Molecular formula	Molecular weight
1	2220	4-Oxatricyclo[4.2.1.0(3,7)nonane-9-carboxamide, 5-oxo-*N*-(phenylmethyl)-	$C_{16}H_{17}NO_3$	271
2	1787	1-Benzyl-1-(1-cyclopropyl-ethyl)-urea	$C_{13}H_{18}N_2O$	218
3	3238	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, trans-	$C_{28}H_{44}O_4$	444
4	888	1-Cyclohexene, 1-ethynyl-	C_8H_{10}	106
5	2774	6,9,12-Octadecatrienoic acid, phenylmethyl ester, (Z,Z,Z)-	$C_{25}H_{36}O_2$	368
6	1450	3-Trifluoroacetoxytridecane	$C_{15}H_{27}F_3O_2$	296
7	1216	Decyltrifluoroacetate	$C_{12}H_{21}F_3O_2$	254
8	1613	Tetradecyltrifluoroacetate	$C_{16}H_{29}F_3O_2$	310
9	1549	3-Trifluoroacetoxytetradecane	$C_{16}H_{29}F_3O_2$	310
10	1421	5-Tetradecene, (E)-	$C_{14}H_{28}$	196
11	1818	5-Octadecene, (E)-	$C_{18}H_{36}$	252
12	1556	n-Tridecan-1-ol	$C_{13}H_{28}O$	200
13	1669	4-Heptafluorobutyryloxyhexadecane	$C_{20}H_{33}F_7O_2$	438
14	1855	Carbonic acid, methyl tetradecyl ester	$C_{16}H_{32}O_3$	272
15	1457	1-Dodecanol	$C_{12}H_{26}O$	186
16	1440	Dimethyl phthalate	$C_{10}H_{10}O_4$	194
17	1508	Benzoic acid, 2-(1-oxopropyl)-,methyl ester	$C_{11}H_{12}O_3$	192
18	1555	Phenol, 2,6-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206
19	1484	1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethanone	$C_{13}H_{18}O_2$	206
20	2255	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	$C_{19}H_{30}O_3$	306
21	2259	Dichloroacetic acid, 4-hexadecyl ester	$C_{18}H_{34}C_{12}O_2$	352
22	1854	1-Hexadecanol	$C_{16}H_{34}O$	242
23	1954	n-Heptadecanol-1	$C_{17}H_{36}O$	256
24	1639	Diethyl phthalate	$C_{12}H_{14}O_4$	222
25	1729	Phthalic acid, allyl ethyl ester	$C_{13}H_{14}O_4$	234
26	1773	Pentafluoropropionic acid, hexadecyl ester	$C_{19}H_{33}F_5O_2$	388
27	1450	4-Trifluoroacetoxytridecane	$C_{15}H_{27}F_3O_2$	296
28	2037	Dibutyl phthalate	$C_{16}H_{22}O_4$	278
29	2235	Phthalic acid, butyl hexyl ester	$C_{18}H_{26}O_4$	306
30	1973	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	$C_{16}H_{22}O_4$	278
31	1580	Benzoic acid, 3-methyl-2-trimethylsilyloxy-, trimethylsilyl ester	$C_{14}H_{24}O_3Si_2$	296
32	1341	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	$C_{12}H_{38}O_5Si_6$	430
33	1339	Silane, trimethyl[5-methyl-2-(1-methylethyl)phenoxy]-	$C_{13}H_{22}OSi$	222
34	1049	Silicic acid, diethyl bis(trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$	296
35	3227	Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]-, 17-[O-(phenylmethyl)oxime], (3.alpha.,5.alpha.)-	$C_{29}H_{43}NO_3Si$	481

(EPS) production in the biofilm-forming bacterium (Fig. 2). The total amount of EPS (measured as the concentration of carbohydrates) produced by the bacterial culture without extract was 0.048 mg ml^{-1}. The biofilm bacterial culture treated with methanol extract of dried algal sample showed a higher reduction in EPS production (0.028 mg ml^{-1}) followed by the methanol extract of fresh algal samples (0.032 mg ml^{-1}) and the hexane surface extract (0.034 mg ml^{-1}). The surface extract (methanol) and the extract of fresh alga (hexane) showed a slight reduction in EPS production (0.037 mg ml^{-1}). One-way ANOVA indicated an insignificant variation in the EPS production by the bacterial culture under the different extract treatments (F = 2.73; df = 7, 15; P = 0.09).

3. Biofilm Prevention by Macroalgal Extracts: Microtitre Plate Assay

Results indicated that biofilm formation on the microtitre

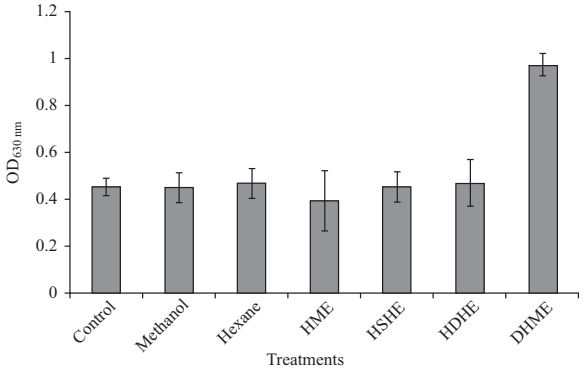

Fig. 3. Antibiofilm activity of *Halimeda* sp. extracts against *Vibrio harveyi*. The antibiofilm activity was measured using microtitre plate assay (OD values, mean ± SD, n = 3). Control = without extract; HME = *Halimeda* methanol extract; HSHE = *Halimeda* surface hexane extract; HDHE = dried *Halimeda* hexane extract; DHME = dried *Halimeda* methanol extract

Table 2. Compounds identified from the hexane extract of *Halimeda* sp.

No.	Retention index	Compound name	Molecular formula	Molecular weight
1	816	Octane	C_8H_{18}	114
2	1716	Benzenemethanol, 2-(2-aminopropoxy)-3-methyl-	$C_{11}H_{17}NO_2$	195
3	1171	Benzeneethanamine, .alpha.-methyl-	$C_9H_{13}N$	135
4	954	Acetamide, *N*-(1-methylpropyl)-	$C_6H_{13}NO$	115
5	1627	*N*-lsopropyl-3-phenylpropanamide	$C_{12}H_{17}NO$	191
6	1694	Ethyl N-isopropyl-3-phenylpropanimidate	$C_{14}H_{21}NO$	219
7	1474	Mexiletine	$C_{11}H_{17}NO$	179
8	2297	Benzylamine, *N*-(3-chloro-2,2-dimethyl-1-phenylpropylidene)-	$C_{18}H_{20}C_1N$	285
9	2007	Phenethylamine, *N*-methyl-.beta.,3,4-tris(trimethylsiloxy)-	$C_{18}H_{37}NO_3Si_3$	399
10	1103	1,2-Ethanediamine, *N*-(2-aminoethyl)-	$C_4H_{13}N_3$	103
11	2075	3,4-Dihydroxymandelic acid, ethyl ester, tri-TMS	$C_{19}H_{36}O_5Si_3$	428
12	1766	Butanamide, 2,2,3,3,4,4,4-heptafluoro-*N*-[2-[(trimethylsilyl)oxy]-2-[4-[(trimethylsilyl)oxy]phenyl]ethyl]-	$C_{18}H_{26}F_7NO_3Si_2$	493
13	1440	Dimethyl phthalate	$C_{10}H_{10}O_4$	194
14	1508	Benzoic acid, 2-(1-oxopropyl)-, methyl ester	$C_{11}H_{12}O3$	192
15	1639	Diethyl phthalate	$C_{12}H_{14}O_4$	222
16	1729	Phthalic acid, allyl ethyl ester	$C_{13}H_{14}O_4$	234
17	1993	Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)]bis[trimethyl-	$C_{20}H_{42}O_4Si_4$	458
18	1716	2-(2',4',4',6',6',8',8'-Heptamethyltetrasiloxan-2'-yloxy)-2,4,4,6,6,8,8,10,10-nonamethylcyclopentasiloxane	$C_{16}H_{48}O_{10}Si_9$	652
19	557	Formamide, *N*,*N*-dimethyl-	C_3H_7NO	73
20	937	2-Methylaminomethyl-1,3-dioxolane	$C_5H_{11}NO_2$	117
21	2434	1,2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	334
22	3364	Phthalic acid, isobutyl octadecyl ester	$C_{30}H_{50}O_4$	474
23	2136	Phthalic acid, hexyl propyl ester	$C_{17}H_{24}O_4$	292
24	1553	Tricyclo[4.3.1.1(3,8)]undecane-1-carboxylic acid	$C_{12}H_{18}O_2$	194
25	2668	Phthalic acid, isobutyl undecyl ester	$C_{23}H_{36}O_4$	376
26	3058	Carbamic acid, N-[10,11-dihydro-5-(2-methylamino-1-oxoethyl)-3-5H-dibenzo[b,f]azepinyl]-, ethyl ester	$C_{20}H_{23}N_3O_3$	353
27	1742	6-Dimethyl(trimethylsilyl)silyloxytetradecane	$C_{19}H_{44}OSi_2$	344
28	1746	Benzeneacetic acid, 3-methoxy-4-[(trimethylsilyl)oxy]-, ethyl ester	$C_{14}H_{22}O_4Si$	282
29	2543	Hexanedioic acid, dioctyl ester	$C_{22}H_{42}O_4$	370
30	1642	2-Dimethyl(trimethylsilyl)silyloxytridecane	$C_{18}H_{42}OSi2$	330

plates was inhibited by the methanol and hexane extracts of fresh alga and methanol and the hexane surface extracts (Fig. 3). A higher inhibition was observed in the treatment added with the surface extract of *Halimeda* sp. obtained using methanol sol-vent. One-way ANOVA showed a significant variation ($F = 20.00$; $df = 6, 20$; $p = 0.000$) in biofilm formation between the different treatments.

4. GC-MS Analysis of Macroalgal Extracts

GC-MS analysis was performed for the hexane and methanol extracts of fresh algal samples and the surface extract obtained using hexane. GC-MS spectra of all three extracts are shown in Fig. 4. The GC-MS spectrum peaks observed in the methanol extract of the fresh algal samples showed 35 compounds (Table 1). The compounds identified include, 9-octadecenoic acid, 6,9,12-Octadecatrienoic acid, phenylmethyl ester, phenol, 2,6-bis (1,1-dimethylethyl), hexadecanol and heptadecanol. The hexane extract of the fresh algal samples showed 30 compounds in the GC-MS spectrum (Table 2). The compounds identified from this extract include benzenemethanol butanamide and hexanedioic acid dioctyl ester. Many phthalic acid derivatives were also observed in the hexane extract (Table 2).

The surface extract of *Halimeda* sp. prepared using hexane as the solvent revealed 32 compounds in GC-MS analysis (Table 3). Some of the compounds identified were piperidine 3-phenyl,

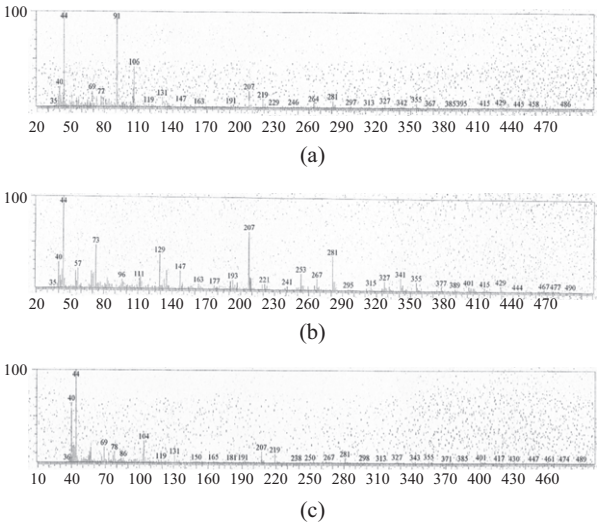

Fig. 4. GC-MS spectrum of extracts of *Halimeda* sp. samples. (a) Methanol extract of fresh algal samples; (b) hexane extract of fresh algal samples; and (c) surface extraction using hexane as solvent.

Table 3. Compounds identified from the surface extract of *Halimeda* sp., prepared using hexane as solvent.

Sl. No	Retention Index	Compound name	Molecular formula	Molecular weight
1	1369	3-Pyridinecarboxaldehyde, *O*-acetyloxime, (E)-	$C_8H_8N_2O_2$	164
2	1500	Piperidine, 3-phenyl-	$C_{11}H_{15}N$	161
3	3273	2-(4,5-Dihydro-3-methyl-5-oxo-1-phenyl-4-pyrazolyl)-5-nitrobenzoic acid	$C_{17}H_{13}N_5O_5$	367
4	2330	3-tert-Butyl-5-chloro-2-hydroxybenzophenone	$C_{17}H_{17}C_1O_2$	288
5	931	2-Heptanamine, 5-methyl-	$C_8H_{19}N$	129
6	1312	6H-Pyrazolo[1,2-a][1,2,4,5]tetrazine, hexahydro-2,3-dimethyl-	$C_7H_{16}N_4$	156
7	1072	Piperazine, 2-methyl-	$C_5H_{12}N_2$	100
8	707	Pentanal	$C_5H_{10}O$	86
9	806	Hexanal	$C_6H_{12}O$	100
10	1440	Dimethyl phthalate	$C_{10}H_{10}O_4$	194
11	1508	Benzoic acid, 2-(1-oxopropyl)-, methyl ester	$C_{11}H_{12}O_3$	192
12	1639	Diethyl phthalate	$C_{12}H_{14}O_4$	222
13	1674	Phthalic acid, ethyl isopropyl ester	$C_{13}H_{16}O_4$	236
14	2235	Phthalic acid, butyl hexyl ester	$C_{18}H_{26}O_4$	306
15	2171	Phthalic acid, butyl isohexyl ester	$C_{18}H_{26}O_4$	306
16	2434	1,2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	334
17	2370	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	$C_{20}H_{30}O_4$	334
18	2037	Dibutyl phthalate	$C_{16}H_{22}O_4$	278
19	1185	Undecane, 2,4-dimethyl-	$C_{13}H_{28}$	184
20	893	Ethylbenzene	C_8H_{10}	106
21	907	p-Xylene	C_8H_{10}	106
22	1787	1-Benzyl-1-(1-cyclopropyl-ethyl)-urea	$C_{13}H_{18}N_2O$	218
23	2900	Pyrrolidine-2-carboxamide, 1-benzyloxycarbonyl-N-(4-tolyl)-	$C_{20}H_{22}N_2O_3$	338
24	2027	3-(1-Benzyl-1H-imidazol-2-yl)-5-methylisoxazole	$C_{14}H_{13}N_3O$	239
25	2220	4-Oxatricyclo[4.2.1.0(3,7)]nonane-9-carboxamide, 5-oxo-*N*-(phenylmethyl)-	$C_{16}H_{17}NO_3$	271
26	888	1-Cyclohexene, 1-ethynyl-	C_8H_{10}	106
27	907	o-Xylene	C_8H_{10}	106
28	3227	Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]-, 17-[*O*-(phenylmethyl)oxime], (3.alpha.,5.alpha.)	$C_{29}H_{43}NO_3Si$	481
29	2541	Benzimidazole, 2-benzylsulfonyl	$C_{14}H_{12}N_2O_2S$	272
30	2835	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-	$C_{22}H_{20}OS$	332
31	3863	3,4,3a,4,7,7a-Hexahydro-5,6-bis(methoxymethoxy)-2-phenyl-4-[3phenyl-1-(trimethylsilyloxy)propyl]-1H-isoindole-1,3-dione	$C_{30}H_{39}NO_7Si$	553
32	1815	1-benzylindole	$C_{15}H_{13}N$	207

6H-pyrazolo[1,2-a][1,2,4,5]tetrazine, piperazine-2-methyl, pentanal, hexanal, pyrrolidine-2-carboxamide, benzimidazole and 1-benzylindole .

IV. DISCUSSION

Many natural products are obtained from marine organisms mainly for pharmacological applications (Faulkner, 2001). Marine macroalgae are considered as important sources of secondary metabolites as many isolated compounds have been reported to possess strong biological activities (Kosanic et al., 2015; Khairy and El-Sheikh, 2015; Patra et al., 2015). For extraction of secondary metabolites or natural products from macroalgae, different solvents such as methanol, ethanol, chloroform, hexane, diethyl ether were used in previous studies (for a review, see Pérez et al., 2016). While selection of solvents for extraction of bioactive metabolites mainly depends on the type of compound being extracted; methanol is reported to be a more effective solvent (in terms of extracting bioactive metabolites) in many studies (Gupta et al., 2012; Al-Hazzani et al., 2014). In this study, methanol and hexane were used as solvents for the extraction of antibacterial metabolites from the macroalga. Though extracts prepared using both solvents showed strong activities, considerable variation was also observed between different assays. In particular, hexane extracts showed strong bacterial growth inhibitory activities in the spectrophotometric assay. In other antibiofilm assays, there was no unanimity on the choice of solvents as extracts obtained using both solvents revealed comparable effects against the biofilm-forming bacterium. This difference may be due to variations in the composition of compounds extracted from the algal samples by these solvents.

Previous studies on bioactivities of marine algae focused only on the crude extracts obtained from dried samples (Park et al., 2013; El-Shoubaky and Salem, 2014; Michalak et al., 2015). Extraction using fresh or frozen samples was also used by a few investigators (Ogasawara et al., 2016; Moubayed et al., 2017). Of the two types of extraction methods (fresh and dried alga) followed in this study, results indicated a strong antibacterial

activity of the extract derived from the dried samples using methanol as the solvent (growth inhibitory and EPS production). However, in the microtitre plate assay, crude extracts obtained from fresh algal samples showed higher antibiofilm activity than that from dried algal samples. The observed variations (on dry weight basis) between dried and fresh algal extracts are may be due to the difference in the composition of secondary metabolites. Particularly, during the drying process, there is a possibility of loss of volatile compounds such as terpenoids, fatty acids, etc. (Pérez et al., 2016).

Concerning activities of extracts obtained from algal surface and whole-tissue extracts, the surface extract (hexane) of *Halimeda* sp. showed higher bacterial growth inhibitory activity than other extracts. In addition, maximum biofilm growth inhibitory activity was observed in the treatment added with the surface extract prepared using hexane as the solvent. This indicates that the macroalga may have strong chemical defence on their surface layers than the internal tissues. Lachnit et al. (2013) revealed that two types of compounds are found on the surface of macroalgae: one group repels colonization of microorganisms and the other group favours attachment of beneficial bacteria.

GC-MS analysis indicated the presence of phenolic derivatives such as phenol 2, 6-bis (1, 1-dimethylethyl) and phenol 3, 5-bis (1, 1-dimethylethyl) in *Halimeda* sp. extracts. The biochemical profile of the crude extracts varied in relation to the extraction method and choice of solvents. For example, the methanol extract of *Halimeda* sp. showed the presence of decanol derivatives such as 1-dodecanol, 1-hexadecanol and n-heptadecanol. These fatty alcohols occur naturally in many plants and are reported to have antibacterial activities (Togashi et al., 2007; Mukherjee et al., 2013). Besides antibacterial activity, fatty alcohols also showed antifouling activities (Kang et al., 2016).

The other important compounds observed from *Halimeda* sp. extracts were phthalic acid derivatives and butanamide (hexane extract of *Halimeda* sp.). GC-MS results also revealed the presence of many interesting compounds with proven biological activities in the surface extracts of *Halimeda* sp. The compounds include piperazine derivative, pentanal and hexanal, pyrrolidine-2-carboxamide, benzylindole and imidazole derivatives. Most of these compounds were reported to possess antibacterial or antiviral activities by previous researchers (Verma et al., 2013; Netz and Opatz, 2015; Zhang et al., 2015). More importantly, previous studies confirmed the antifouling activities of butanamide, imidazole derivatives and diketopiperazines from different marine organisms (Yang et al., 2007; Li et al., 2008; Majik et al., 2014). Further, it is noteworthy to mention that some of the compounds identified based on the GC-MS spectrum may be from the environment that enter to the alga (e.g., Mexiletine, p-Xylene).

Antibacterial activity testing based on traditional disc diffusion assay could not give reliable quantitative data for the isolation of possible antimicrobial compounds (Jiang, 2011). Hence, a broad range of assays is required for preliminary screening of bioactive compounds. In the present study, different assays such as microbial growth inhibition, effects of macroalgal extracts on adhesion of the bacterium and effects of extracts on EPS production of bacterial cells were carried out to understand the antibacterial and antibiofilm activities of the macroalga. Results indicated that some extracts which possessed strong activity in one assay failed to exhibit the same in another assay. The methods used in this study have their own merits and demerits. For instance, the measurement of bacterial growth based on optical density (turbidity measurement) is a quick and simple method, but it quantifies both live and dead bacterial cells in the culture.

Generally, all fouling organisms including bacteria use adhesive material for the attachment to surface (Callow and Callow, 2002). EPS produced by the bacteria during colonization on the surface is one of the important adhesives involved in the biofilm process (Azeredo and Oliviera, 2000). EPS mainly consists of polysaccharides, protein, nucleic acids and lipids (Sutherland, 1972; Kachlany et al., 2001; Kumar et al., 2004; Hung et al., 2005). EPS helps the microbes to attach on surfaces and formation of biofilm. The carbohydrate concentration in the EPS produced by the bacterial strain was considerably reduced after treatment with the crude extract of the macroalga. This indicates that macroalgal extracts affected EPS production in the bacterial strain, which is considered as an essential step in the prevention of biofilm formation on surfaces.

Many previous investigators tested macroalgal extracts against marine biofilm-forming bacteria and reported strong inhibitory activities (Hellio et al., 2001; Bazes et al., 2006; Chambers et al., 2011). As biofouling is one of the major problems in ocean-bound ships and other sectors such as aquaculture and coastal power plants (Satheesh et al., 2016), natural products from marine algae could be used as less toxic or non-toxic eco-friendly antifoulants. Generally, compounds, which inhibit settlement of initial stage of biofouling, i.e., biofilm formation, may be considered as potential antifoulants for control of growth of other organisms (Satheesh et al., 2012). Though results obtained in this study revealed considerable growth inhibitory activities against the biofilm-forming bacterium, the extracts did not show significant biofilm inhibitory activities. This warrants additional experiments on other target microorganisms for screening natural antifouling compounds.

In conclusion, the results of the present study revealed that extracts of *Halimeda* sp. exhibited strong antimicrobial effects against the biofilm-forming bacterial strain *Vibrio harveyi* isolated from the Red Sea. The extraction methods and choice of solvents clearly affected the outcomes of the antibacterial and antibiofilm assays. Overall, hexane extract and algal surface extract showed strong antibacterial activities. The compounds such as butanamide derivative detected in hexane extract and piperazine derivative in surface extract (hexane solvent) may be responsible for the strong inhibitory activities observed in these extracts. Further studies on isolation and characterization of the bioactive fractions found in crude extracts may help us develop biologically active compounds as antibacterial agents in pharmacology and natural occurring antifoulants for biofilm

and biofouling management. As most of the Red Sea marine biota remains unexplored for natural products, bioprospecting of macroalgae from this region may provide more metabolites with biological activities.

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