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RESEARCH PAPER

Antimicrofouling activities of marine macroalga *Dictyota dichotoma* from the Red Sea

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الأنشطة المضادة للحشف لأحد أنواع الطحالب البحرية (*Dictyota dichotoma*) من البحر الأحمر

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Abstract. Marine organisms produce a variety of secondary metabolites mainly for achieving the defence against the competitors and predators. These compounds could be used as natural product antifoulants for the management of biofouling growth on marine structures. To understand the antifouling defence strategies of marine macroalgae, the macroalga *Dictyota dichotoma* was collected from the Obhur Creek of Red Sea, Saudi Arabia and extracted using methanol. Surface and total extraction methods were performed and tested against a bacterial strain isolated from the microfouling assemblages. The extracts obtained from the macroalgal samples have strong antibacterial and antibiofilm activities against the bacterial strain isolated from the marine microfouling assemblages. The percentage of growth varied significantly between the bacterial culture treated with extracts and control. The total extracts showed strong bacterial growth inhibitory activities in culture plate method. In microtitre plate assay, surface extract showed higher biofilm inhibitory activity than total extract. GC-MS analysis indicated considerable variations in the metabolic profile of the surface and total extracts with higher number of compounds in the surface extract. This study revealed the importance of surface-associated compounds in antifouling defence mechanism of the marine macroalgae.

Keywords: Seaweeds; biofouling; antifouling; bioactive compounds; biofilms; chemical defence; Red Sea

المستخلص: تنتج الكائنات البحرية مجموعة متنوعة من المنتجات الثانوية بشكل رئيسي لتحقيق الدفاع ضد المنافسين والمفترسين. ويمكن استخدام هذه المركبات كمضادات لتكوين الحشف وإدارة نمو الكائنات الحشفية على الهياكل البحرية. يهدف هذا العمل الي فهم استراتيجيات الدفاع المضادة للحشف من الطحالب البحرية التي تم جمعها من البحر الأحمر. تم جمع أحد الطحالب الكبيرة وهو *Dictyota dichotoma* من خور أبحر في البحر الأحمر ، المملكة العربية السعودية واستخلاص هذه المواد الثانوية باستخدام الميثانول. تم إجراء هذا الاستخلاص من كل من الطبقة السطحية ومن الطحلب الكلي واختبارها ضد سلالة بكتيرية معزولة من مجموعات حشفيه. تحتوي المستخلصات التي تم الحصول عليها من العينات الكلية لهذا الطحلب على أنشطة قوية مضادة للبكتيرياوكذلكضد السلالة البكتيرية المعزولة من الطبقة الحيوية المكونة للحشف.تفاوتت نسبة النمو بشكل كبير بين البكتيريا التي تمت معالجتها بالمستخلصات والمعاملة المرجعية. أظهرت المستخلصات الكلية فعالية مثبطة لنمو البكتيريا في طريقة الزراعة على الأطباق. بينما باستخدام طريقة الاختبار باستخدام اطباق الإليزا، أظهر المستخلص السطحي فعالية مثبطة للطبقة السطحية أعلى من المستخلص الكلي للطحلب. وقد أظهر تحليل كروماتوجرافيا الغاز المقترن بمطياف الكتلة GC–MS تباينات كبيرة في شكل وتركيب المركبات الثانوية الناتجة عن عملية الأيضلكل من السطح والمستخلصات الكلي للطحلب مع عدد أكبر من المركبات الناتجة من الطبقة السطحية. . كشفت هذه الدراسة عن أهمية المركبات السطحية المستخرجة من الطحالب البحريةفي آلية الدفاع المضادة للحشف.

الكلمات المفتاحية: الكلمات الدالة:الطحالب البحرية، المضادة للحشف، مركبات نشطة، الدفاع الكيميائي، البحر الاحمر

Introduction

In the marine environment, biofilm development on hard surfaces is the initial process, which subsequently will lead to the attachment of marine organisms, a process commonly known as biofouling growth on substrates (Satheesh et al. 2016). The attachment of bacterial communities will help the settlement of higher organisms on the surfaces (Hadfield and Paul 2001; Huang et al. 2012). Biofouling, involve all settling 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 which needs frequent cleaning and antifouling measures (Armstrong et al. 2000; Qian et al. 2009; Satheesh et al. 2016). For example, biofilm formation and subsequent biofouling may increase 14% of the fuel cost in ships and 8%–29% loss in propulsive power has also been attributed to due to mature marine biofilms (Schultz et al. 2004; Salta et al. 2013).

Due to the economic loss, various antibiofilm and antifouling strategies are currently practiced in the marine technology sector (Satheesh et al. 2016; Dahms and Dobretsov 2017). The most common antifoulants are

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Table 1. Compounds identified from the total extract of *D. dichotoma* based on the peaks observed in the GC-MS spectrum

Sl. No	Retention Index	Compound name	Molecular weight (g mol-^{1})
1	1937	Ethyl pentyl phthalate	264
2	2573	4-Methoxy-3-[(2,4,6-trichlorophenoxy)methyl] benzaldehyde	344
3	659	2-Butynol	70
4	2434	Dihexyl phthalate	334
5	2235	Phthalic acid, butyl hexyl ester	306
6	2633	1,2-Benzenedicarboxylic acid, butyl decyl ester	362
7	2832	Phthalic acid, butyl dodecyl ester	390
8	2171	Pthalic acid butyl ioshexyl ester	306
9	3589	Propanoic acid, 3,3'-thiobis-didodecyl ester	514
10	3986	Propanoic acid, 3,3'-thiobis-ditetradecyl ester	570
11	2011	Octadecyl trifluroacetate	366
12	1710	Undecane, 3-cyclohexyl	238
13	4085	Hexadecane,1,1-bis (dodecyloxy)	594

mainly based on heavy metals (copper, zinc) or tributyltin (TBT) and applications of these compounds creates many harmful problems to the environment (Bellas 2006; Thomas and Brooks 2010). These chemical coatings are not only toxic to fouling organisms but also toxic to other non-target organisms (Fingerman 1998; Hoch 2001). TBT is considered as one of the most toxic materials discharged into the marine environment due to antifouling applications (Evans et al. 1995). International Maritime Organization (IMO) banned the use of TBT coatings because of the toxic effects to marine organisms and environment (Satheesh et al. 2016).

Among marine organisms, macroalgae attracted much attention for the natural product research (de Nys et al. 1995; Sudatti et al. 2008). This was evidenced by the large number of publications available in the scientific domain (see reviews: Perez et al. 2016; Dahms and Dobretsov 2017). Marine macroalgae are particularly exposed to many epiphytes (those organisms settling on it) in the environment. To avoid the settlement of organisms on their surfaces, the macroalgae are reported to produce chemically active metabolites (Plouguerné et al. 2008). These compounds are of interest due to many drug leads that are isolated from the macroalgae. Some studies have analysed the effects of macroalgal secondary metabolites on the planktonic bacterial community (Nylund and Pavia 2005; Paul et al. 2006; Dubber and Harder 2008; Lam et al. 2008). Lu et al. (2008) observed that macroalgae such as *Ulva clathrata* have an inhibitory effect on *Vibrio anguillarum,* a fish and mussel pathogen.

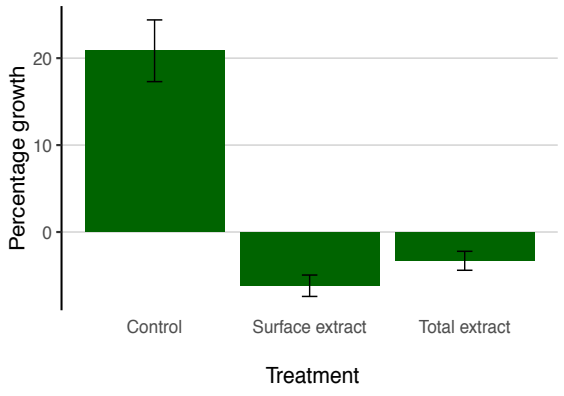

Figure 1. Percentage of bacterial growth by *D. dichotoma* extracts. Bacterial growth was measured using spectrophotometric method for 5 h. A negative percentage indicates inhibition of bacterial growth in compariton to the control. Control growth is that observed without extract treatment. Error bars indicate the standard deviation of the mean (n=3).

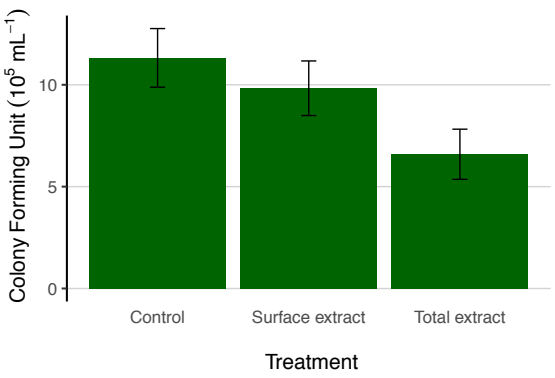

Figure 2. Numbers of colony forming units (CFUs) in the presence and absence of extracts (total and surface) of *D. dichotoma*. Growth inhibitory activity was studied by traditional culture plate method. Error bars indicate the standard deviation of the mean (n=3). Control=without extract treatment.

Table 2. Compounds identified from the surface extract of *D. dichotoma* by GC-MS analysis (continued on next page)

Sl. No	Retention Index	Compound name	Molecular weight (g mol-^{1})
1	1117	**Azetidin-2-one 3,3-dimethyl-4-(1-aminoethyl)-**	142
2	1517	**2-Formyl histamine**	139
3	1744	**Benzene ethanamine, 2,5-difluoro- beta.,3,4-trihydroxy-N-methyl-**	219
4	931	**Octodrine**	129
5	1081	**1-(5-Bicyclo[2.2.1] heptyl) ethylamine**	139
6	1662	**Benzene propanoic acid, .alpha.-(1-aminoethyl)-, [R-(R*,R*)]-**	193
7	2135	**Propanamide, 3-(3,4-dimethylphenylsulfonyl)-**	241
8	1171	**Benzene ethanamine, .alpha.-methyl-**	135
9	1716	**Benzene methanol, 2-(2-aminopropoxy)-3-methyl-**	195
10	907	**p-Xylene**	106
11	907	**O-Xylene**	106
12	2220	**4-Oxatricyclo[4.2.1.0(3,7)]nonane-9-carboxamide, 5-oxo-N-(phenyl-methyl)-**	271
13	1190	**Benzene ethanol, .alpha.,.beta.-dimethyl-**	150
14	824	**1,3-Cyclopentadiene, 5-(1-methylethylidene)-**	106
15	1369	**3-Pyridine carboxaldehyde, O-acetyloxime, (E)-**	164
16	1500	**Piperidine, 3-phenyl-**	161
17	1307	**Cathinone**	149
18	2135	**Propanamide, 3-(3,4-dimethylphenylsulfonyl)-**	241
19	1913	**N-Methyl-N-(toluene-4-sulfonylmethyl)-acetamide**	241
20	1334	**Phenyl propanolamine**	151
21	806	**Methylpent-4-enylamine**	99
22	1294	**1-Methyl decylamine**	171
23	2190	**Cystine**	240
24	1787	**dl-Alanyl-dl-norleucine**	202
25	1891	**Nitro-L-arginine**	219
26	1451	**N(Epsilon)-methyl-1-lysine**	160

In this study, the macroalga *Dictyota dichotoma* collected from the Jeddah coastal waters of Red Sea was screened for antimicrobial and antibiofilm activities against bacterial strain isolated from marine biofilms. The main objective of this study was to analyse the role of surface-associated molecules in achieving the chemical defence of macroalgae against the microfouling organisms. Result obtained in this study will improve our knowledge of the chemical defence strategies of macroalgae and lead to the isolation of novel metabolites from the Red Sea macroalgae that can be used as natural product antifoulants.

Materials and Methods

The macroalga *Dictyota dichotoma* (Hudson) J.V. Lamouroux (Class: Phaeophyceae) was collected from the Obhur Creek on the Jeddah coast (near King Abdulaziz University Marine Station, Obhur) and transported to the laboratory. In the laboratory, macroalgal samples were rinsed with filtered (Millipore, 0.47µm) seawater to remove the debris and other attached organisms and used for extraction. Total extraction and surface extraction were followed in order to extract as much as active compounds from the algal samples. Methanol was the solvent used for both types of extraction. About 10 g fresh algal samples were used for both surface and total extraction. In total extraction, the fresh macroalgal samples were extracted using methanol (5 ml) after grinding the wet samples using pestle and mortar. The macerated samples were kept in methanol for 5 h at room temperature in dark and after that centrifuged at 3000 rpm to remove the solid part. The solvent part was collected and maintained at -20°C until further analysis. The surface molecules were extracted by dipping the algal samples in methanol (5 mL) for 10 seconds. After that, the extract

Table 2 (continued). Compounds identified from the surface extract of *D. dichotoma* by GC-MS analysis

Sl. No	Retention Index	Compound name	Molecular weight (g mol-^{1})
27	1904	5-(Prop-2-enoyloxy) pentadecane	282
28	1693	Imidazole, 2-amino-5-[(2-carboxy) vinyl]-	153
29	1673	Pentafluoropropionic acid, pentadecyl ester	374
30	1613	Tetradecyl trifluoroacetate	310
31	1773	Pentafluoropropionic acid, hexadecyl ester	388
32	1435	Heptafluorobutyric acid,n-tridecyl ester	396
33	1669	4-Heptafluorobutyryloxy hexadecane	438
34	1440	Dimethyl phthalate	194
35	1508	Benzoic acid, 2-(1-oxopropyl)-, methyl ester	192
36	1555	Phenol, 2,6-bis(1,1-dimethylethyl)-	206
37	2255	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	306
38	1484	1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethanone	206
39	1555	Phenol, 3,5-bis(1,1-dimethylethyl)-	206
40	1549	3-Trifluoroacetoxytetradecane	310
41	1639	Diethyl Phthalate	222
42	1729	Phthalic acid, allyl ethyl ester	234
43	1937	Phthalic acid, ethyl pentyl ester	264
44	2235	Phthalic acid, butyl hexyl ester	306
45	2434	1,2-Benzenedicarboxylic acid, butyl octyl ester	334
46	2037	Dibutyl phthalate	278
47	1973	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	278
48	1341	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	430
49	1580	Benzoic acid, 3-methyl-2-trimethylsilyloxy-, trimethylsilyl ester	296
50	1710	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	578
51	1526	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	504
52	1339	Silane, trimethyl[5-methyl-2-(1-methylethyl)phenoxy]-	222

was centrifuged and maintained at -20°C for antibiofilm assays.

Bacterial growth inhibition assay (Spectrophotometer assay)

The bacterial strain *Vibrio harveyi* (NCBI GenBank accession number: KY266820) isolated from the marine biofilm developed on nylon nets submerged in the Jeddah coastal waters of the Red Sea (Balqadi et al. 2017) was used as a target bacteria for antibiofilm assays. Before starting the antibiofilm assays, the bacterial strain was inoculated into marine nutrient broth (Zobell marine broth, HiMedia, India). The broth was kept at 28°C in an incubator for 48 h and after the incubation, the optical density (OD) of the culture was adjusted to 1 at 530 nm (Sayem et al. 2011) to maintain equal bacterial cells. Three millilitres of bacterial culture was taken in test tubes and 50 µL macroalgal extract (both surface and whole tissue extracts) was added and the control tubes were maintained without any extracts. Methanol control was also maintained and no growth inhibition activity was observed. The optical density of the bacterial culture was measured at 670 nm (modified from Pan et al. 2014) in a spectrophotometer for a period of 5 h (OD was measured at one-hour intervals for 5 h duration). The experiment was carried out in replicate (n=3) and the mean ± standard deviation values were considered. The percentage of bacterial growth/inhibition was calculated using the following formula (Viju et al. 2016).

$$GR(\%) = \frac{OD_{final}}{OD_{initial}} 100 \quad \text{Eq.(1)}$$

where GR is the growth rate and OD is optical density at the beginning ($OD_{initial}$) and at the end of the experiment

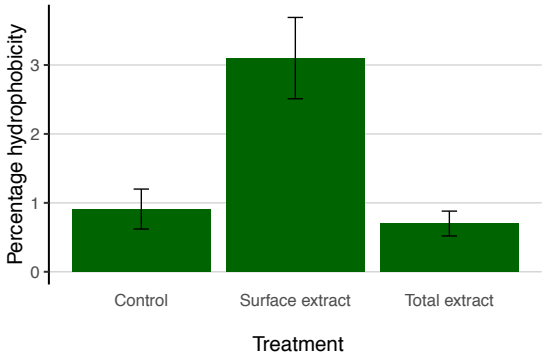

Figure 3. Effect of *D. dichotoma* extracts (total and surface extracts) on hydrophobicity of biofilm forming bacterial cells. Control=without extract treatment. Error bars indicate the standard deviation of the mean (n=3).

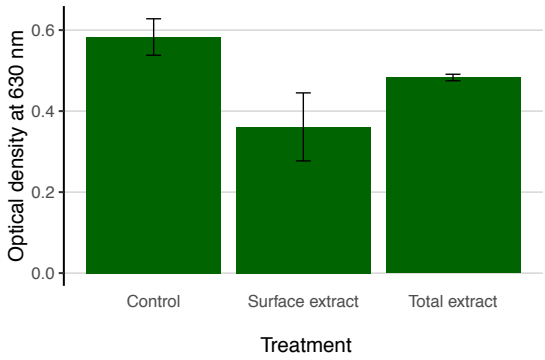

Figure 4. Antibiofilm activity of *D. dichotoma* extracts (total and surface extracts) against *Vibrio harveyi*. Error bars indicate the standard deviation of the mean (n=3).

(OD_{final}).

Bacterial growth inhibition assay (Culture plate method)

The bacterial culture (OD_{530}=1) was also used to study the growth inhibition activities of macroalgal extracts by culture plate method. The bacterial culture (3 mL) was taken in a test tube and 50 µL algal extract was added. Bacterial culture without macroalgal extract treatment was considered as a control. The test tubes with bacterial cultures treated with macroalgal extracts and controls (in replicates, n=3) were then incubated for 24 h at 28°C. After 24 h, 100 µL of bacterial culture from the test tube was spread on marine agar plates and the culture plates were incubated at 28°C in an incubator for 24-48 h. The bacterial colonies developed in each plate was counted and mean (± standard deviation) colony counts were expressed as colony forming units, CFU, mL^{-1}.

Effects of macroalgal extracts on cell surface hydrophobicity of bacteria

The bacterial cell surface hydrophobicity was measured according to the MATH (Microbial Adhesion to Hydrocarbons) assay described by Rosenberg et al. (1980).

About 3 mL of bacterial culture was taken in test tubes and 50 µL of macroalgal extract was added. After 1 h the OD of the culture was measured at 530 nm in a spectrophotometer. To this culture, 300 µL of hexane was added and vortexed for 1 min. The culture was then left for 10 min. to allow the separation of two phases. The aqueous phase of the culture was separated and used for OD measurement as above. The percentage of hydrophobicity (HB(%) was calculated using the following formula.

$$HB(\%) = \left(1 - A/A_0\right)100 \qquad \text{Eq.(2)}$$

Where HB(%) is the percentage hydrophobicity as measured by bacterial cell adherence to hexane and A is the Optical Density at 530 nm, before (A_0) and after (A) washing the cell suspension with hexane.

Biofilm growth inhibition activity of macroalgal extracts - microtiter plate assay

The anti-settlement activity of macroalgal extracts against the bacteria was determined by the 96-well microtiter plate method described by Coffey and Anderson (2014). The bacterial culture (OD adjusted to the OD 1.0 at 530 nm) was used for the antibiofilm assay. The wells of the microtitre plate were filled with 100 µL of bacterial culture and 5 µL of the extracts were added. The controls were maintained without adding any extract. The plates were incubated at 28°C for 24 h. After incubation, the plate was inverted to remove the unattached bacterial cells and rinsed with sterile water. After that, 150 µL of 0.1% crystal violet was added to each well of the microtiter plate. The plates were kept for 10 min. for staining of the cells and after 10 min. the plate was inverted to remove crystal violet stain. The wells were rinsed again with sterile water to remove the excess stain. Finally, glacial acetic acid (150 µL) was added to each well and kept for 10 min. After 10 min., the plates were read at 630 nm in a Biotek plate reader (Winooski, USA).

Isolation and identification of the pathogen

The bioactive compounds present in surface and total extracts of *D. dichotoma* was analysed by GC-MS (Shimadzu GC-MS QP 2010, Japan, Faculty of Meteorology, Environment and Arid land agriculture, King Abdulaziz University). The protocol described by El-Din and El-Ahwany (2016) for macroalgal extracts was used for the GC-MS analysis. The crude algal extracts were partially purified through silica gel column and analysed in a capillary silica column (30 m ×0.25mm×0.25µm) using helium as carrier gas (1.5 mL for 1 min). The mass spectrometer was operated in the electron impact (El) mode at 70 eV in the scan range of 40–700 m/z. The following MS conditions were used for getting the spectrum: split ratio: 1:10, injected volume: 1 µL, injector temperature: 250°C, the oven temperature: initially 70°C for 3 min and

then increased to 250°C at 14°C min^{-1}. The peaks observed in the GC-MS spectrum where compared with those available in NIST 11 – Mass Spectral Library for the identification of the compounds.

Statistical analysis

The data obtained from biofilm bacterial growth inhibition assay was analysed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's test. Student's 't' test was used to analyse the difference in biofilm inhibition activity of algal extracts and control. For all statistical tests $P<0.05$ was considered as significant.

Results

Bacterial growth inhibition assay- spectrophotometric method.

The biofilm bacterial culture treated with *D. dichotoma* extracts showed negative growth during the 5 h period. The biofilm bacterial culture without any treatment showed a growth of 20.82 % during 5 h period under laboratory conditions (Fig 1). The total extract treated bacterial culture showed a negative growth of -3.11% during the experiment period (5 h). The bacterial culture treated with the surface extract of the *D. dichotoma* also revealed negative growth (-6.152 %) compared to control. One-way ANOVA showed a significant variation in bacterial growth percentage between control and extract treated cultures ($F = 127$; $df = 2, 8$; $P < 0.05$). However, the bacterial growth inhibiting activity of total extract and surface extract did not differ significantly (Post hoc Tukey test: total extract vs surface extract, $p=0.3$).

Bacterial growth inhibition-culture plate method

In traditional culture plate method, the total extract exhibited strong growth inhibitory activity than the surface extract. The bacterial culture without any treatment showed 1,140,000 CFU, mL^{-1} in culture plates (Fig. 2). About 665,000 CFU, mL^{-1} colonies were observed on the culture plates containing the bacteria treated with the total extract of *D. dichotoma*. In surface extract treatment, the number of colonies observed was comparatively higher than the total extract treatment (988,000 CFU, mL^{-1}).

Effects of macroalgal extracts on hydrophobicity of biofilm–forming bacterial cells

The bacterial cell surface hydrophobicity of the biofilm-forming bacteria under normal conditions (control, without any algal extract treatment) was 0.9% (Fig. 3). However, when treated with algal extracts the bacterial cell hydrophobicity was either reduced or increased. The total extract of *D. dichotoma* reduced the bacterial cell surface hydrophobicity by 0.7%. However, treatment with surface extract showed an increase in bacterial cell surface hydrophobicity (3.1%).

Quantification of biofilm prevention by macroalgal extracts: Microtitre plate assay

The results of microtitre plate assay revealed that the macroalgal extracts inhibited the biofilm formation (Fig. 4). The inhibition of biofilm growth was evidenced by the low OD values observed from the cultures treated with the extracts. The bacterial cultures treated with total extract showed an OD value of 0.48 and for surface extract treatment the OD was observed as 0.36. Also, the OD values were significantly lower than the control (Student's-t test: total extract, $t = 4.3$, $df = 2$, $p = 0.02$; surface extract $t = -3.47$, $df = 2$; $p = 0.03$).

GC-MS analysis of macroalgal extracts

GC-MS analysis of macroalgal extracts revealed considerable differences between total and surface extracts (Fig 5). The total extract of *D. dichotoma* revealed 13 compounds in the GC-MS spectrum (Table 1). Most of the compounds are identified as phthalic acid derivatives. The compounds identified include ethyl pentyl phthalate, 4-methoxy-3-[(2,4,6-trichlorophenoxy) methyl] benzaldehyde, dihexyl phthalate, phthalic acid butyl dodecyl ester and phthalic acid butyl isohexyl ester. About 52 compounds (Table 2) were identified from the surface extract of *D. dichotoma* which include azetidine-2-one 3,3-dimethyl-4-(1-aminoethyl), 2-formyl histamine, benzene ethanamine, 2,5-difluoro- beta.,3,4-trihydroxy-N-methyl, -oxatricyclo [4.2.1.0(3,7)] nonane-9-carboxamide, 5-oxo-N-(phenylmethyl), 1,3-cyclopentadiene, 5-(1-methylethylidene) and piperidine, 3-phenyl.

Discussion

Marine macroalgae are abundant in coastal ecosystems and play key roles in ecosystem functioning (Ba-akdah et al. 2016). As herbivory is high in marine ecosystems, the algae possess several defence systems against herbivores (Paul et al. 2001). Macroalgae also show antifouling defence by different ways such as the use of chemical and physical defences (Burns et al. 2003; da Gama et al. 2014) or symbiotic relationships between epibionts (e.g., bacteria) (Satheesh et al. 2016). Due to these chemical defence, macroalgae are one of the most important groups for natural product antifouling research (Nylund et al. 2007; Hellio et al. 2009; Viano et al. 2009; Saha and Wahl 2013; Othmani et al. 2016; Carvalho et al. 2017; Salama et al. 2017).

In the present study, surface and total extracts of the macroalgae *D. dichotoma* was tested against three important properties growth, cell surface hydrophobicity and biofilm formation (attachment) of the marine bacterial strain isolated from marine microfouling assemblages. The growth of the bacteria was inhibited by both surface and total extracts, though a higher percentage

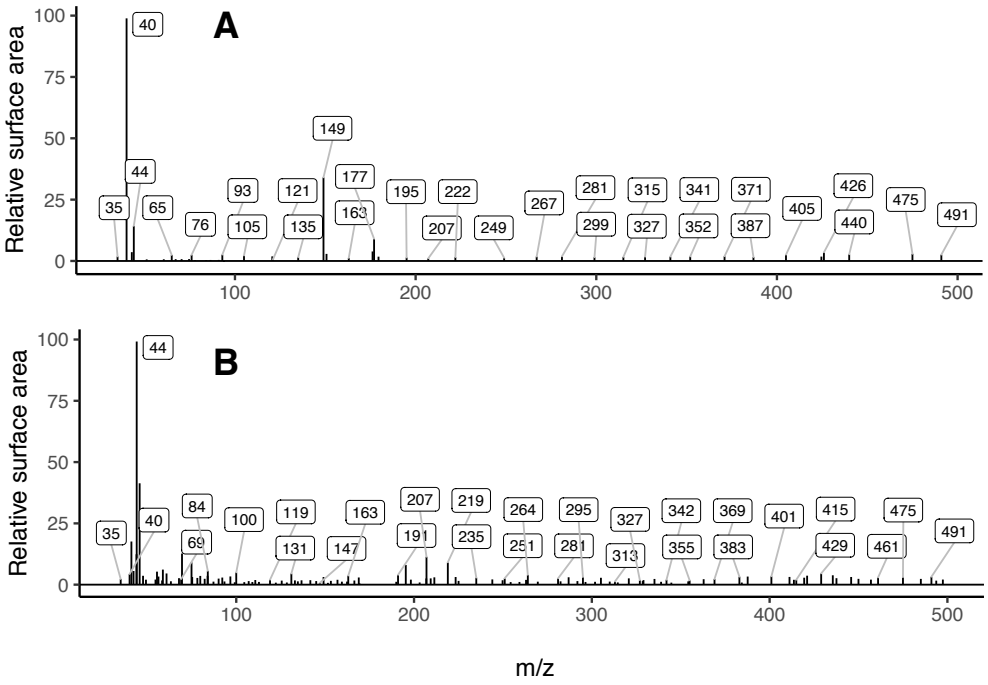

Figure 5. GC-MS analysis of *D. dichotoma* extracts. A). GC-MS spectrum obtained for total extract, B). GC-MS spectrum of surface extract.

of inhibition was observed in bacterial cultures treated with surface extracts (except in the culture plate method). Further, the extracts also possess antimicrobial activity that was evidenced by the negative growth percentages (indicating a decrease in bacterial counts from the beginning) observed in spectrophotometer assay. Antimicrobial activity of the genus *Dictyota* have been reported widely in the literature (Águila-Ramírez et al. 2012; Antonysamy et al. 2015).

The macroalgal extracts used in this study changed the bacterial cell hydrophobicity which is essential for attachment on surfaces. Bacterial cell surface hydrophobicity may play important role in biofilm formation on surfaces (Choi et al. 2013). This increase in cell surface hydrophobicity may be due to the release of outer membrane vesicles as an adaptive mechanism against the toxicity of extracts. Previous studies by Baumgarten et al. (2012) reported the release of membrane vesicles by the bacterium *Pseudomonas putida* strain DOT-T1E due to the toxicity of long-chain fatty alcohols, EDTA and heat. Generally, the if the hydrophobicity of the bacterial cell surface increases, then there will be a stronger attachment on hydrophobic surfaces and *vice versa* (Kochkodan et al. 2008; Giaouris et al. 2009; Krasowska et al. 2014). Hence, if the hydrophobicity is low then the bacteria prefer to settle on hydrophilic surfaces. In this study, the macroalgal extracts affected the normal hydrophobicity of the bacterial cells, but the attachment process may depend on other factors including surface characters and environmental conditions.

Chemical defence strategies of macroalgae and other organisms in the marine ecosystems are the topics of research in recent years (da Gama et al. 2014; Lopanik 2014; Rhode et al. 2015; Sacristan-Soriano et al. 2017). The difference between the bioactivities of surface and whole-tissue extracts of marine organisms have not been studied detail (Nylund et al. 2007). In this study, bacterial growth (spectrophotometer method) and biofilm inhibitory activity (microtitre plate assay) of the surface extract were higher than the total extract. The good performance of surface extract in antibiofilm assay raises the further question on the source of the secondary metabolites. Many studies indicated that the epibiotic microbial communities associated with marine organisms are providing the defence to the host against the colonizers and competitors (see review: Satheesh et al. 2016). Further, fresh algal samples were used for surface and total extraction in this study. Hence, the possibility for the presence of metabolites from epibiotic microbial sources cannot be ruled out in both surface and total extracts.

The concentration of metabolites in tissue extracts (total extract) may also be higher than the surface extracts (Sudatti et al. 2006). This difference may be due to the chemical defence mechanism in macroalgae that usually store the compounds in tissues and transported to the surfaces (see review: da Gama et al. 2014). However, results from the GC-MS analysis in this study confirmed an important aspect that the surface extract possess higher number of metabolites than the total extract. The good performance of surface extract in anti-settlement assay might be due to the presence of a large num-

ber of compounds, which need further studies. Some of the compounds identified from the surface extract such as azetidine-2-one, piperidine, propanamide and imidazole derivatives have potent pharmacological activities (Olgen et al. 2008; Noolvi et al. 2014; Sharma et al. 2016; Zhang et al. 2017). Imidazole and piperidines exhibited antifouling activities in previous studies (Majik et al. 2014; Huang et al. 2014). Among the compounds present in both surface extracts total extracts, phthalic acid derivate is reported to possess antifouling activities (Ganti et al. 2006).

In conclusion, the bioactive compounds present in the extract of *D. dichotoma* could be used as natural product antifoulants. The presence of many compounds in the surface extract confirmed the role of surface-associated molecules in antifouling chemical defence of marine macroalgae. While the source of surface-associated molecules needs further investigation, these metabolites could be used as a potential compounds for antifouling assays against invertebrate larvae and other organisms. In addition, the study emphasizes the role of surface extraction method in natural product antifouling screening assays.

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