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Antifouling Properties of Brown *Macroalgae Dictyota* Dichotoma Collected from the Red Sea; Comparison of Surface and Whole Thallus Extracts

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Abstract. The metabolites produced by the marine macroalgae are considered as a source for the natural product antifoulants. In this study, the surface and whole thallus extracts of the marine macroalgae *Dictyota dichotoma* was tested against a biofilm-forming bacterial strain *Vibrio harveyi* and barnacle larval settlement to understand the best extraction strategies for antifouling screening bioassays. The surface extracts were prepared in different time duration by dipping the algal samples for 10, 20 and 30 seconds in hexane. The results indicated that the extracts (both surface and whole thallus) obtained from the macroalgal samples have strong antifouling activities against the bacterial strain *V. harveyi* and barnacle larvae settlement. The extraction duration of the surface extracts strongly affected the antifouling activity. In all the assays, the surface extract obtained by 30 sec. dipping in the solvent showed high inhibitory activity than whole thallus extract. In conclusion, this study indicated the necessity for the inclusion of surface extraction methods for antifouling screening assays using marine macroalgae as a source for natural products.

Keywords: Biofouling, Biofilms, Barnacle larvae, Natural products, Bioactive compounds, seaweeds; Red Sea.

1. Introduction

Biofouling is a serious annoyance that results in significant economic losses for marine infrastructure and maritime industries such as pipelines, desalination water intake systems, ship hulls, probes and sensors, construction materials, and filters. (Chambers *et al.,* 2006; Hellio and Yebra, 2009; Schultz *et al*., 2011). The maritime industries are using various antifouling measures for controlling fouling on marine underwater structures (McCloy and De Nys, 2000; Carver *et al*., 2003; Lodeiros and Garcia, 2004; Satheesh *et al*. 2016). Among them, antifouling paints have long been the most effective method to prevent biofouling, where toxic biocides such as organotin (e.g. tributyltin fluoride, tributyltin oxide), and copper (e.g. Cuprous thiocyanate, cuprous oxide) are released from the coatings and inhibit the attachment of fouling organisms (Alberte *et al*., 1992; Thomas *et al*., 2001). Although biocide based antifouling coatings were efficacious against fouling, the long-term usage of these biocides posed environmental

risks to non-target creatures (Gibbs *et al*., 1990; Callow and Callow, 2002). For instance, TBT is highly toxic to non-target organisms such as mussel (larvae mortality), oyster (shell malformation), and gastropod (imposex) (Alzieu, 2000; Sonak, 2008; Thomas and Brooks, 2010). Because of environmental concerns about the use of TBT as a biocide, the International Maritime Organization (IMO) and the Marine Environment Protection Committee (MEPC) prohibited the use of TBT for antifouling applications (Satheesh *et al*. 2016). Alternatively, natural products from both marine and terrestrial organisms are considered eco-friendly antifouling compounds due to their properties such as biodegradability, low-toxicity and broad-spectrum antifouling property at very low concentrations (Turk *et al*., 2007).

Among the marine organisms, marine macroalgae produce lots of biologically active compounds that can act as a chemical barrier against pathogenic microorganisms, herbivores and biofouling organisms (Charles and Victoria, 2005; Paul *et al*., 2006). Over 3000 marine macroalgal derived natural products mainly belong to polysaccharides, lipids, fatty acids, sterols, phenolics and carotenoids have been documented (Perez *et al*., 2016). The antifouling action of chemicals obtained from marine macroalgae has been widely described (Piazza *et al*. 2011; Cho 2013). For instance, Chapman *et al*. (2014), found the antifouling activity of the compound 3-bromo-5-(diphenylene)-2(5H)-furanone isolated from the green algae *Ulva rigida*. Whereas, Munoz *et al*. (2013) isolated the antifouling acyclic linear diterpenoids from the brown algae *Bifurcaria bifurcate.* Similarly, Umezawa *et al*. (2014) isolated the antifouling Omaezallene from the red algae *Laurencia* sp. Besides, the production of the antifouling compounds from various macroalgae such as *Laurencia* sp., *Ulva* sp., *Galdieria* sp., *Pterocladiella* sp., *Asparagopsis* sp. etc. have been reported (Dahms and Dobretsov, 2017).

Secondary metabolites are extracted from macroalgae using a variety of extraction techniques and solvents. The majority of the previous studies used whole thallus extract for screening the bioactivity and separation of metabolites (e.g. Cho 2003; Medeiros *et al.* 2007; Salama *et al*., 2018). Some previous studies highlighted the role of surface-associated molecules in marine algal chemical defense (Nylund *et al.*, 2007; Sudatti *et al*., 2008; Thabard *et al.*, 2011; Rickert *et al*., 2016). During the extraction process from the whole thallus, there is a possibility of losing some of these metabolites due to the sample handling and drying process. Further, extraction of whole algal tissue may not provide the essential metabolites due to the presence of antifouling defence molecules on the surface of the thallus (Nylund *et al*., 2007). As a result, it is critical to analyze the bioactive role of chemicals linked with the surface of marine macroalgae. Hence, the objective of this study was to evaluate the antifouling activity of surface and whole thallus extracts of the brown macroalgal species *Dictyota dichotoma* collected from the Red Sea in Saudi Arabia. The findings of this investigation would contribute to our understanding of antifouling defence activity of marine macroalgae and also could provide a lead for the formulation of extraction strategies for the isolation of natural product antifouling compounds.

2. Materials and Methods

2.1 Collection of Algal Samples

The macroalgal species *D. dichotoma* was collected from the Obhur creek (21°42'33.52"N and 39° 5'45.71"E) on the Red Sea shore of Jeddah, Saudi Arabia. The collected macroalgal samples were transported to the laboratory in polythene bags containing seawater. The macroalgal samples were then rinsed with filtered seawater (Millipore, 0.45 µm) to remove any dirt and other attached epifauna. Following that, the samples were used for extraction.

2.2 Extraction of Metabolites

Hexane was used as the solvent to extract the secondary metabolites of the algal samples at the ratio of 1L hexane kg^{-1} (wet weight) of macroalgal samples (de Nys *et al*., 1998; Thabard *et al*., 2011). Hexane was selected as a solvent as it may extract the molecules both from the surface and associated biofilms (Thabard *et al*., 2011). Two different types of extracts were obtained from the alga viz. surface extracts and whole tissue extracts. The surface extracts were prepared in different time duration by dipping the algal samples (about 5 g in 5 ml of hexane) for 10, 20 and 30 seconds. The whole tissue extract was prepared by grinding the algal samples (5 g) in a pestle and mortar and extracted with 5 ml of hexane for 48 h. After extraction, the extracts were centrifuged initially at 3000 rpm for 10 min. at 4 °C to remove the debris. Following that the extracts were concentrated in a rotary evaporator under reduced pressure. The concentrated extracts were kept at -20°C for further studies.

2.3 Bioassays Against Biofilm-Forming Bacteria

The biofilm-forming bacterial strain *Vibrio harveyi* (NCBI GenBank accession number: KY266820) isolated from the aquaculture cage nets (Balqadi *et al*., 2018) was used as a target organism to test the antifouling activities of the macroalgal extracts.

2.4 Bacterial Growth Inhibition Assay

According to conventional microbiological techniques, the biofilm bacterial strain *V. harveyi* was inoculated into the marine nutrient broth (Himedia, India) and kept at an incubator at 30 °C. About 3 ml of overnight grown bacterial culture was taken in small test tubes and the macroalgal extracts (whole algal extract and surface extracts: 10 sec. 20 sec. and 30 sec.) were added. Three different concentrations (10, 30 and 50 µl ml^{-1}) of the extracts were used for the experiments. The OD of the bacterial culture was measured before the addition of extracts and immediately after the addition of the extracts at 630 nm in a spectrophotometer. The cultures were incubated at room temperature (28°C) for 3 h and after that, the OD was measured. The bacterial cultures without the addition of extracts were considered as a negative control and hexane were taken as a positive control. The experiment was repeated three times (n=2 for each experiment) and the percentage of bacterial growth inhibition was calculated using the following formula

$$\text{Biofilm growth inhibition (\%)} = \frac{\text{Control OD - Experiment OD}}{\text{Control OD}} \times 100$$

2.5 Biofilm Inhibition Assay-Microtitre Plate Assay

The microtitre plate biofilm development assay was used to investigate the effects of macroalgal extracts on biofilm growth inhibition according to the procedure described previously (O'Toole 2011) with some modifications. The overnight grown bacterial cultures (41×10^6 CFU ml^{-1}) were taken (300 µl in each well) in microtitre plate wells. To this, 20 µl of the extract was added with triplicates in each concentration. In addition, the controls were maintained with hexane (20 µl). The extract concentration was selected based on the results of the bacterial growth inhibition assay. The microtitre plates were kept in an incubator for 24 h at 28°C. After the incubation period, the plates were inverted to remove the bacterial cultures and rinsed with filtered seawater. The microtitre plate wells were then stained with 150 µl of 0.1% crystal violet solution. The plates were kept for 10 min. for adequate staining of the bacterial cells attached to the surfaces and after that, the stain was removed. The wells were rinsed again with filtered seawater and 150 µl of glacial acetic acid was added to each well. After 10 min. the O.D was measured using a plate reader (Biotek) at 630 nm.

2.6 Effects of Macroalgal Extract on Extracellular Polymeric Substance (EPS) Production in Biofilm-Forming Bacteria

This experiment was conducted to understand the effects of the algal extract on

EPS production in biofilm-forming bacterium *V. harveyi*. Overnight grown *V. harveyi* culture was inoculated into marine broth taken in a 250 ml conical flask. To this, the algal extracts (whole thallus and surface extracts) were added (100 µl). The control flasks were maintained without the addition of algal extracts. The flasks were kept in a shaker at room temperature (28 °C) for 5 days at 120 rpm. After the incubation period, the culture medium was centrifuged at 10000 rpm for 15 min. at 4 °C. The supernatant was collected in test tubes and added with an equal volume of cold ethanol. After that, the test tubes were kept at room temperature for 24 h for EPS precipitation. The white precipitate formed in the bottom of the tubes were collected, washed with distilled water and used for the quantification of carbohydrate and protein content. The carbohydrate concentration of the EPS was determined by the Phenol-Sulphuric acid method (Dubois *et al*., 1956). Lowry *et al*. (1951) method was used for measuring the protein concentration of the EPS produced by the biofilm-forming bacterium *V. harveyi*.

2.7 Collection of Barnacles and Larval Rearing

The barnacle *Amphibalanus amphitrite* adults were collected from the Obhur creek and maintained in a glass tank in the laboratory. The barnacles were kept in the tank with moderate aeration and provided a mixed diet consisting of *Artemia* nauplii and microalga (*Chaetoceros*). The nauplii released by the adults were collected and transferred to small tanks. The nauplii were reared up to the cypris stage (settling stage) using a mixed algal diet according to the procedure reported previously (Salama *et al*. 2018).

2.8 Barnacle Larval settlement Assay

The cypris larvae were used for settlement assays. The settlement assay was conducted in 6-well plates using 25 larvae in each well according to the procedure described earlier (Salama *et al*. 2018). The macroalgal extracts were added (20 µl ml^{-1}) to the wells. The control wells were maintained with 20 µl ml^{-1} hexane. The plates were kept in dark at room temperature and the number of cyprids settled was counted under a stereomicroscope after 24 and 48 h. The experiment was conducted in replicates (n=3) using a different batch of barnacle larvae.

2.9 Statistical Analysis

The variations in biofilm bacterial growth inhibitory and antibiofilm activities of the surface and whole thallus extracts were tested using one-way ANOVA (Analysis of variance). The anti-larval settlement activity of the extracts was tested using two-way ANOVA. The extract type and experiment duration were used as factors for two-way ANOVA. For the assays which showed significant variation between the extracts, the Tukey HSD test was conducted to understand the pair-wise interactions (between surface and whole thallus extracts). The statistical tests were carried out using Statistica (ver.13) and $P<0.05$ was considered as significant.

3. Results

3.1 Bacterial Growth inhibitory Activity of the Macroalgal Extracts

The macroalgal extracts inhibited the growth of the biofilm-forming bacterial strain *V. harveyi*. The degree of inhibition varied between the extract types (Fig. 1). A maximum of 63.36% of growth inhibition was noted in the bacterial culture treated with 30 µl of 30 sec. surface extract. The maximum percentage of growth inhibition observed in the bacterial culture treated with other extracts were 24 (10 sec.), 28.93 (20 sec.) and 33.72 (whole thallus extract). Notably, the surface extract obtained by dipping the alga for 30 sec. showed strong activity than other extracts including whole thallus extract. One-way ANOVA results revealed a significant variation in bacterial growth inhibiting activity between the extracts ($F=73.61$; $df=3,8$; $P>0.001$). Further, post-hoc Tukey test results showed significant variations between all the

extracts except 20 sec surface extract and whole thallus extract (Table 1).

3.2 Biofilm Inhibition of Macroalgal Extracts

The results indicated a reduction in biofilm formation on the microtitre plates due to the treatment of bacterial culture with macroalgal extracts (Fig. 2). The inhibition of biofilm growth on the plates was evidenced by the decrease in optical density (OD) of the wells after being treated with the extract. First of all, the extracts (both surface and whole thallus) treatment significantly ($P<0.05$) reduced the biofilm formation of the bacterium *V. harveyi* (*$F=92.213$; $df=4, 40$; $P>0.001$*). Among the extracts, the 30 sec. surface extract treatment showed a significant reduction in biofilm development than other extracts (Table 1).

3.3 Effects of Macroalgal Extract on EPS Production in Biofilm-Forming Bacteria

The carbohydrate and protein concentrations of the extracellular polymeric substance produced by the biofilm-forming bacterium *V. harveyi* were 81.78 and 29.83 µg ml^{-1} respectively. However, a reduction in total carbohydrate (*$F=4311.41$; $df=4,10$; $P<0.001$*) and protein (*$F=4956.37$; $df=4,10$; $P<0.001$*) content of the EPS was noted in the bacterial cultures treated with the macroalgal extracts (Fig. 3). In all the extract treatments, the reduction in carbohydrate was significant from the control (Table 2). For protein content, the reduction was significant except 10 sec. surface extract treatment (Table 2). The maximum decrease in carbohydrate and protein concentration was observed in the bacterial culture treated with 30 sec. surface extract.

3.4 Anti-Larval Settlement Activity of the Macroalgal Extracts

The barnacle larval settlement assay results showed the anti-settlement activity of the macroalgal extracts. In the control plates, 82.66% of larval settlement was observed after 48 h under laboratory conditions. However, the settlement percentage was reduced on the plates which contained the larvae treated with macroalgal extracts (Fig. 4). The settlement percentage was very low for the larval groups treated with 30 sec. surface extract and whole thallus extract. In this assay also, the 30 sec. surface extract exhibited a higher settlement inhibition rate than other extracts. Two-way ANOVA results revealed a significant variation in larval settlement percentage between the extract treatments (Table 3). Further, post-hoc Tukey test indicated significant variation in the settlement rate between control and extract treated groups (Table 2).

Table 1. Post-hoc Tukey test results for the pair-wise comparison of bacterial growth inhibiting activity and biofilm inhibitory activities of the extracts ($P<0.05$=significant).

Bacterial growth inhibiting activity			Biofilm inhibitory activity		
Extract	Extract	P	Extract	Extract	P
10 s	20 s	0.041	10 s	20 s	0.999
	30 s	<0.001		30 s	<0.001
	W	0.020		W	0.999
20 s	30 s	<0.001		Control	<0.001
	W	0.950	20 s	30 s	<0.001
30 s	W	<0.001		W	0.999
				Control	<0.001
			30 s	W	<0.001
				Control	<0.001
			W	Control	<0.001

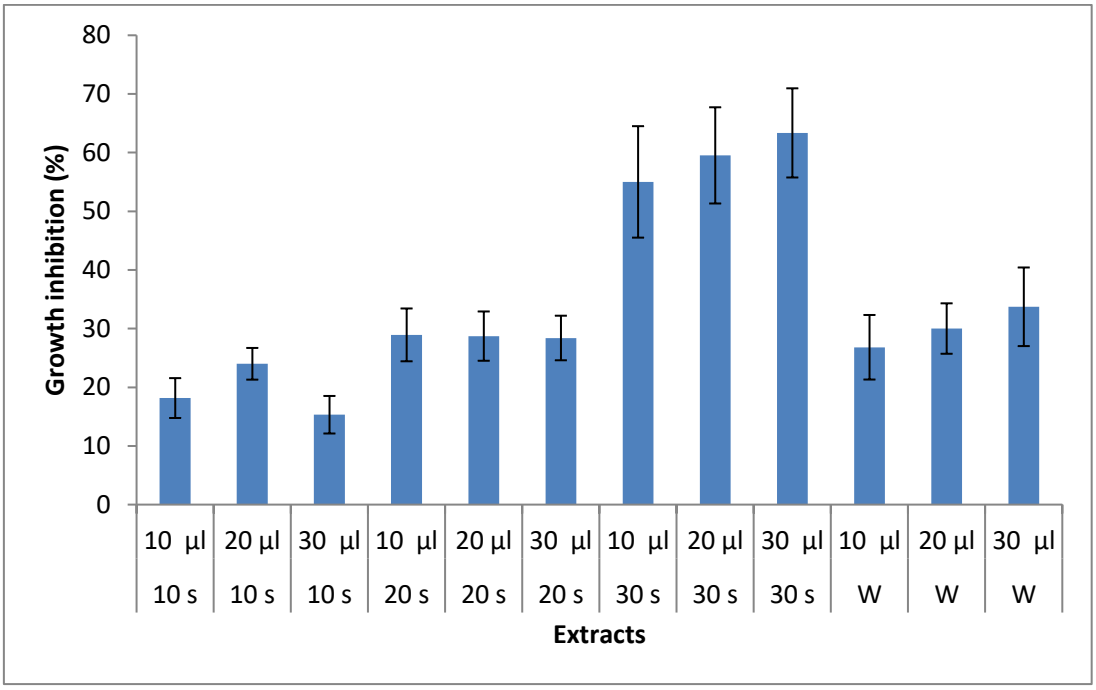

Fig. 1. Bacterial growth inhibiting activity of the extracts of *D. dichotoma*. The bacterial growth inhibitory activity was measured using spectrophotometer method. Error bars indicate the SD of mean (n=3). 10 s, 20 s, 30 s are the surface extracts prepared by different dipping duration. W=whole thallus extract.

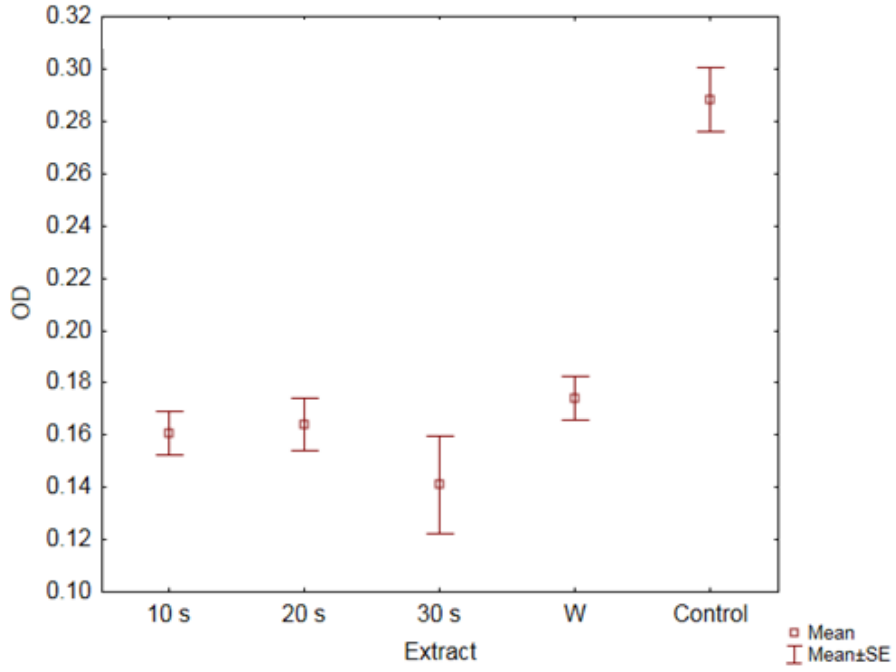

Fig. 2. Biofilm inhibiting activity of the extracts of *D. dichotoma*. The biofilm inhibitory activity was measured using microtitre plate method. Error bars indicate the SE of mean (n=3). 10 s, 20 s, 30 s are the surface extracts prepared by different dipping duration. W=whole thallus extract.

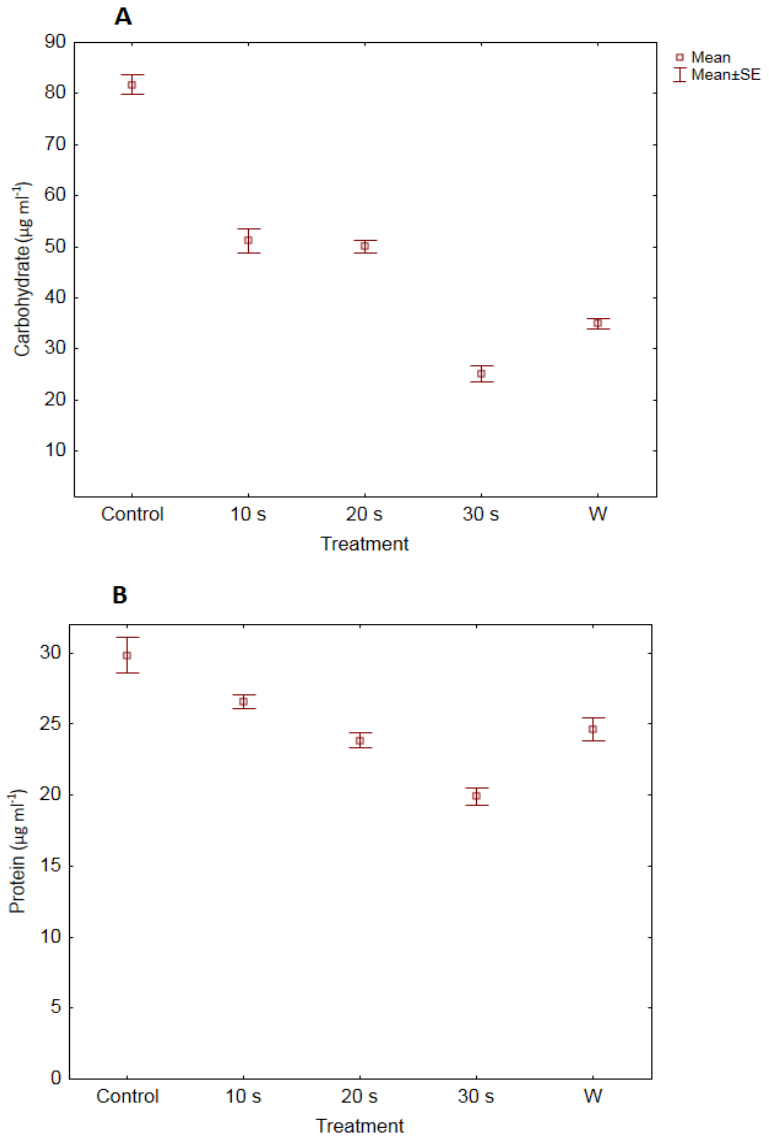

Fig. 3. Effects of macroalgal extracts on extracellular polymeric substance production in biofilm-forming bacterium *V. harveyi*. A). Changes in carbohydrate concentration after algal extract treatment. B). Changes in protein concentration after algal extract treatment. Error bars indicate the SE of mean (n=3). 10 s, 20 s, 30 s are the surface extracts prepared by different dipping duration. W=whole thallus extract.

Table 2. Post-hoc Tukey test results for the effects of macroalgal extracts on carbohydrate content of the extracellular polymeric substances produced by biofilm-forming bacterium and barnacle larval settlement (P<0.05=significant).

		Carbohydrate	Protein	Barnacle larval settlement
Extract	Extract	P	P	P
Control	10 s	<0.001	0.093	<0.001
	20 s	<0.001	0.002	<0.001
	30 s	<0.001	<0.001	<0.001
	W	<0.001	0.006	<0.001
10 s	20 s	0.987	0.177	<0.001
	30 s	<0.001	0.001	<0.001
	W	<0.001	0.458	<0.001
20 s	30 s	<0.001	0.035	<0.001
	W	<0.001	0.945	0.037
30 s	W	0.012	0.011	0.005

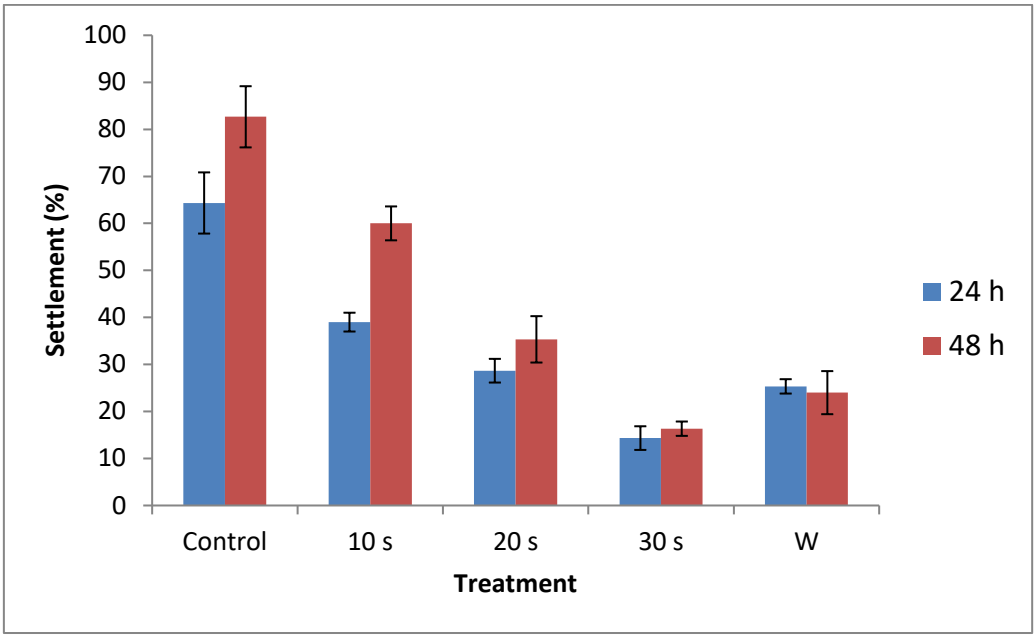

Fig. 4. Anti-larval settlement activity of the algal extracts against barnacle larva. Error bars indicate the SE of mean (n=3). 10 s, 20 s, 30 s are the surface extracts prepared by different dipping duration. W=whole thallus extract.

Table 3. Two-way ANOVA results for the anti-larval settlement of *D. dichotoma* extracts. Extract type and experiment duration were used as factors for the ANOVA (P<0.05=significant).

Effects	SS	Degree Of Freedom	MS	F	p
Extract	12690.33	4	3172.58	193.058	<0.001
Time	653.33	1	653.33	39.757	<0.001
Extract*Time	587.67	4	146.92	8.940	<0.001
Error	328.67	20	16.43		

4. Discussion

The results of this study showed the inhibitory activity of extracts obtained from *D. dichotoma* against biofilm-forming bacteria and barnacle larva. This is in accordance with the previous studies which reported the antifouling activities of *D. dichotoma* against different fouling organisms (Siless *et al*. 2018; Bakar *et al*. 2019; Gadhi *et al*. 2019). Further, in a previous study, Cho *et al*. (2001) indicated strong inhibitory activity of macroalgal extracts against invertebrates in laboratory experiments. Many other investigations also showed the production of antifouling compounds by marine macroalgae for deterring the settlement of invertebrates (for a review see: Dahms and Dobretsov 2017).

The extracts were tested against a biofilm-forming bacterium and barnacle larvae to assess the antifouling activity against the micro- and macrofouling organisms. In addition, the effect of the extracts on EPS production in biofilm-forming bacterium was also assessed in this study. EPS are produced by the microorganisms after attachment on substrates and believed to play a key role in biofilm formation (Flemming *et al*. 2007). Hence, the compounds which reduce or inhibit the production of EPS and other signal molecules are considered as good fouling control agents (Satheesh *et al*. 2016). In this study, the macroalgal extracts affected the production of EPS in *V. harveyi* which was evidenced by the reduction in carbohydrate and protein content after treatment. Microbial EPS mainly contains carbohydrates and a small amount of protein along with other macromolecules (Flemming *et al*. 2007). Hence, measurement of these macromolecules

will indicate the quantity of EPS production under different experimental conditions.

Most of the previous studies showed the results of antifouling activities from the extracts of whole thalli of marine macroalgae (e.g. Hellio *et al*. 2002; Aguila-Ramirez *et al*. 2012; Salama *et al*. 2018). This approach will not help to understand the antifouling mechanism of macroalgae in natural conditions. In marine algae, the compounds produced for defense purposes are mainly non-polar and located in the surface layers of the algae (Murugan *et al*. 2012). Further, Paradas *et al*. (2016) reported that the fatty acid derivatives that are responsible for the antifouling activity of the red alga *Laurencia translucida* are produced in the corticoid cells and stored in the surface layers. These compounds are playing multiple ecological functions in macroalgae against the colonizing epibiotic bacteria and herbivores (da Gama *et al*. 2014). As fouling organisms come into contact with the surface of the macroalgae, the metabolites located in the surface may act as a primary defense tool. Hence, surface extraction of the macroalgal samples instead of whole extracts may give more ecological relevance (Nylund *et al*. 2007), especially for testing against the bacteria.

The surface molecules are generally extracted by gently soaking the algal samples in relevant solvents (Saha and Wahl, 2013; Othmani *et al*., 2016; Gadhi *et al*., 2018). Dipping of algae in the solvents for 5-10 s is considered effective as it will not affect the algal cells (Nylund *et al*., 2007). In this study, the algal samples were soaked in the solvent for three durations such as 10, 20 and 30 seconds to understand the antifouling efficiency. Though the effects of these three soaking durations on algal thallus were not tested in this study, the extract obtained by 30 seconds soaking duration showed strong antifouling activity than the other extracts. Notably, the whole tissue extract showed less activity than 30 sec. surface extract. The variations observed in the activity may be due to the concentration of compounds in the extracts obtained by different extraction duration.

The marine algal surfaces are colonized by many microorganisms like bacteria, fungi and microalgae (Dahms and Dobretsov, 2017). The microbial communities associated with the marine organisms are distinct from the microbes associated with other substrates or those living in the water (Wahl *et al*., 2012). Numerous studies indicated the defence role of epibiotic bacterial communities associated with the macroalgae (See reviews: Hollants *et al*. 2013; Satheesh *et al*. 2016). Specifically, the antifouling activity of bacteria associated with the macroalgae was reported by Harder *et al*. (2004), Rajasree *et al*. (2012) and Dahms and Dobretsov, (2017). Hence, the observation of the higher antifouling activity of the surface extracts may be due to the presence of many microbial communities on the algal surface.

In conclusion, the results of this study proved that marine algae possess a surface-associated antifouling mechanism for defense from the colonizing organisms and herbivores. The surface-antifouling defense may be achieved mainly through the secondary metabolites. These compounds might be produced by the algae or the algal-associated bacterial communities. The extraction duration of the surface-associated metabolites strongly affected the antifouling activity. As the metabolite production and microbial association in algal communities are varied over spatial and temporal scales (Dahms and Dobretsov, 2017), further studies by including seasonal sampling regimes may provide more insights regarding the surface-associated antifouling molecules. Further, this study indicated the need for the inclusion of surface extraction methods for antifouling screening studies using macroalgae and other marine organisms.

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الخصائص المانعة للحشف من الطحالب البنية الكبيرة *Dictyota Dichotoma* التي تم جمعها من البحر الأحمر: مقارنة بين مستخلصات التالوس السطحية والكاملة

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المستخلص. تعتبر المستقلبات التي تنتجها الطحالب البحرية الكبيرة مصدرًا لمضادات التلوث الطبيعية. في هذه الدراسة، تم اختبار المستخلصات السطحية والكاملة للطحالب البحرية الكبيرة *Dictyota dichotoma* ضد السلالة البكتيرية المكونة للغشاء الحيوي *Vibrio harveyi* وbarnacle تسوية اليرقات لفهم أفضل استراتيجيات الاستخراج لفحص المقايسات الحيوية المضادة للحشف. تم تحضير المستخلصات السطحية في فترات زمنية مختلفة عن طريق غمس عينات الطحالب لمدة ١٠ و٢٠ و٣٠ ثانية في الهكسان. أشارت النتائج إلى أن المستخلصات (سواء السطحية أو الكاملة) التي تم الحصول عليها من عينات الطحالب الكبيرة لها نشاط قوي ضد الحشف ضد السلالة البكتيرية *V. harveyi* واستقرار يرقات البرنقيل. أثرت مدة استخراج المستخلصات السطحية بشدة على النشاط المضاد للحشف. في جميع المقايسات، تم الحصول على مستخلص السطح بمقدار ٣٠ ثانية. أظهر الغمس في المذيب نشاطًا مثبطًا مرتفعًا عن مستخلص الثعلب الكامل. في الختام، أشارت هذه الدراسة إلى ضرورة إدراج طرق الاستخراج السطحي لفحوصات الفرز المضادة للحشف باستخدام الطحالب البحرية كمصدر للمنتجات الطبيعية.

الكلمات المفتاحية: الحشف الحيوي، الأغشية الحيوية، يرقات البرنقيل، المنتجات الطبيعية، المركبات النشطة بيولوجيا، الأعشاب البحرية. البحر الاحمر.