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Bioassays and virtual screening to identify potent natural antifouling compounds from the brown macroalga *Dictyota dichotoma*

by

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Abstract

Macroalgae are one of the potential natural sources for the isolation of novel eco-friendly antifouling compounds. In this study, the antifouling activity of an extract of the brown macroalga *Dictyota dichotoma* collected from the Red Sea was tested against bacteria isolated from the marine biofilm and larval forms of the barnacle. A maximum inhibition of barnacle larval settlement of 89.36% was observed in 25 µg ml^{-1} extract concentration at 24 h treatment. The secondary metabolite composition of the extract was analyzed by GC-MS and compounds were used as ligands for molecular docking with barnacle cement protein. The toxicity profile of secondary metabolites present in the extract was predicted through in silico analysis. The results indicate that the crude extract of the alga inhibited the biofilm formation by the bacteria and significantly reduced the settlement of the barnacle larvae. GC-MS analysis of the extract revealed the presence of five metabolites, including two fatty acids. All metabolites showed higher binding affinity with barnacle cement than the reference compound, copper. Among the secondary metabolites detected in the algal extract, the cholestane derivative exhibited maximum binding affinity (–14.2 kcal mol^{-1}) with barnacle cement. The metabolites also showed positive for crustacean and fish toxicity in toxicity prediction using an in silico method.

Key words: seaweed, biofouling, biofilm, antibiofilm, bioactive compounds, natural products, molecular docking, Red Sea

1. Introduction

Marine macroalgae have strong chemical defense against herbivores and colonizing organisms (Hay & Fenical 1988; Hay 1997; Pereira & Vasconcelos 2014). Protection by producing metabolites, generally known as secondary metabolites, is a well-known chemical defense in most algal species (Hay & Fenical 1988, Paul 1992; Paul et al. 2001; Da Gama et al. 2014; Pereira et al. 2023). Metabolites produced by macroalgal species are rich and diverse and include polyphenols, acetogenins, terpenoids and halogenated compounds (Blunt et al. 2018; Salehi et al. 2019; Cotas et al. 2020; Ghallab et al. 2022). Secondary metabolites produced by marine macroalgae are primarily used in pharmacology to screen lead compounds for potential antibacterial, anti-inflammatory and anti-cancer agents (Cho et al. 2022; Nigam et al. 2022). Many compounds extracted from marine macroalgae are also tested for their antifouling activity to control the biofouling growth (Dahms & Dobretsov 2017; Saha et al. 2018; Plouguerne et al. 2020).

Biofouling is a costly issue for maritime and aquaculture sectors, which frequently involves expensive cleaning or antifouling measures (Satheesh et al. 2016). Many antifouling measures are currently in use to control the biofouling growth, including coatings containing toxic biocides (Omae 2003; Yebra et al. 2004; Takahashi 2009; Paz-Villarraga et al. 2022). Due to environmental concerns about the use of chemical compounds to control biofouling, many natural products are proposed as environmentally friendly antifouling compounds (Gu et al. 2020; Tian et al. 2021). Natural antifouling compounds have been described from various marine organisms, ranging from microbes to higher organisms (Satheesh et al. 2016; Liu et al. 2020). Macroalgae (seaweeds) are one of the important groups of organisms largely screened for natural antifouling compounds (Dahms & Dobretsov 2017; Qi & Ma 2017).

Although many natural products or extracts from marine algae have been tested for their antifouling effects, much less is known about how these natural products affect fouling organisms. Fouling organisms, mainly barnacles and bivalves, which dominate on submerged structures in seawater (Vinagre et al. 2020), produce specific adhesive materials for their attachment. The adhesive produced by barnacles is referred to as 'barnacle cement', as quickly glues materials together in water (Liang et al. 2019). Previous studies indicated that protein was the major component of the adhesive produced by cyprid larvae and adult barnacles (Kamino et al. 1996; Gohad et al. 2014; So et al. 2016). A compound that interferes with the production or biochemical composition of adhesive materials may provide better protection against the attachment of these fouling organisms (Qian et al. 2013). Therefore, this study analyzed the efficacy of natural products present in the extract of *Dictyota dichotoma* to understand the interaction between the compounds and barnacle cement protein using the *in silico* method. Further, the antibiofilm and antifouling effects of the extract obtained from the alga were tested using two bacterial strains isolated from the biofilm and barnacle larvae. Brown algae of the *Dictyota* genus are common in coastal ecosystems and are considered a rich source of many secondary metabolites (Chen et al. 2018). Some isolated compounds or extracts from *Dictyota* species showed antifouling activity against micro- and macrofouling organisms (Barbosa et al. 2007; Viano et al. 2009; Murugan et al. 2012; Othmani et al. 2014; Siless et al. 2018; Gadhi et al. 2018). Therefore, *D. dichotoma* was selected for this study to test the antifouling activity.

2. Materials and methods

2.1. Collection and extraction of algal samples

The macroalgal species *Dictyota dichotoma* (Hudson) J.V. Lamaouroux 1809 was used for the extraction. Macroalgal samples were collected from the Obhur Creek (21°42'33.52"N; 039°05'45.71"E) on the Jeddah coast of Saudi Arabia. The debris and epifauna associated with the alga were removed by gently rinsing the samples in filtered seawater (Millipore, 0.45 µm). After rinsing, *D. dichotoma* samples were kept at room temperature (28°C) and dried in the shade. The dried algal samples were ground into powder using an electric grinder. The powdered algal sample was extracted using methanol as a solvent (100 g of algae with 300 ml of methanol). The container with macroalgal powder and methanol was covered with aluminum foil, and the extraction process was carried out in a shaker for 72 h. The resulting extract was collected and centrifuged for 15 min at 3000 rpm at 4°C. After centrifugation, the extract was filtered through a filter paper (Whatman filter paper). The filtered extract was concentrated using a rotary vacuum evaporator. The crude extract was collected and kept in glass vials at 4°C. Prior to bioassays, the crude extract was reconstituted in methanol at the desired concentration.

2.2. Biofilm-forming bacteria for antibiofilm assays

Two bacterial strains – *Pseudoalteromonas shioyasakiensis* (NCBI GenBank accession number: KY224086) and *Planomicrobium* sp. (NCBI GenBank accession number: KY224087) – were used for

antibiofilm assays. These bacteria were previously isolated from the biofilm developed on aquaculture cage nets submerged in the Obhur Creek (Balqadi et al. 2018). As reported earlier, these two bacteria are active biofilm formers under laboratory conditions (Siddik & Satheesh 2019).

2.3. Antibiofilm assay

The antibiofilm activity of the extract was tested using the microtiter plate method according to the available protocol (Coffey & Anderson 2014). Biofilm-forming bacteria were cultured in marine broth (Difco, 2216) at 28°C in an incubator for 24 h. Overnight grown bacterial culture (cell density adjusted to 0.4 OD at 600 nm) was collected (500 µl in each well) into microtiter plate wells. The algal extract was added to the wells in three different concentrations (5, 10, and 25 µg ml^{-1}). Replicate wells (n = 6) were maintained for each concentration and the control. Wells with 25 µl ml^{-1} methanol added were considered controls. The experiment was conducted for three incubation durations (24, 48 and 72 h). The plates were placed at 28°C in an incubator for respective incubation periods. Plates were then rinsed and stained with crystal violet to measure optical density (OD) according to the described procedure (Satheesh & Ba-Akdah 2022). OD values were used to calculate the percentage of biofilm inhibition using the following formula:

$$PF = T \times 100/C$$

$$PI = 100 - PF$$

where *PF* = percentage of biofilm formation, *T* = OD of treatment wells, *C* = OD of control wells, *PI* = percentage of biofilm inhibition.

2.4. Culturing of barnacle larvae for settlement and toxicity studies

The barnacle *Amphibalanus amphitrite* (= *Balanus amphitrite*) was used as a model organism for a laboratory settlement assay. Samples of adult barnacles were collected from the Obhur Creek, the Jeddah coast, Saudi Arabia and brought to the laboratory. In the laboratory, the barnacles were kept in small tanks (10 L) under a 16:8 (light:dark) light cycle. Adult barnacles were fed with *Artemia* nauplii. The procedure for maintaining adult barnacles and rearing larvae in the laboratory has been described previously (Satheesh and Ba-Akdah 2022). In brief, nauplii released by adult barnacles were collected and transferred into larvae rearing jars. Nauplii were fed with the microalga *Chaetoceros calcitrans*. The jars were kept inside a temperature-controlled chamber at 28°C under 16:8 light-dark cycles. Stage III nauplii were sorted under a stereomicroscope for toxicity studies. Nauplii were cultured to the cypris stage for a settlement assay.

2.5. Toxicity assay using nauplius larvae

This experiment was conducted to understand the toxicity of algal extract against barnacle larvae. Stage III nauplii collected from the larvae rearing tank were used for the toxicity test. Twenty-five nauplii were placed in each well of 6-well plates containing filtered seawater. The extract was added to the wells at three different concentrations, as in the antibiofilm assay (5, 10, and 25 µg ml^{-1}). The plates were kept inside a temperature-controlled chamber at 28°C under dark conditions. Wells into which 25 µl ml^{-1} of methanol was added were considered controls. Control and treatment (in each concentration) wells were maintained in replicates (n = 6). The number of dead nauplii in each well was checked periodically for 96 h. The 96 h LC_{50} value was calculated from mortality data using the online tool Quest Graph (https://www.aatbio.com/tools/lc50-calculator).

2.6. Barnacle cyprid settlement assay

The cyprid settlement and mortality assay was conducted in 6-well plates with 10 cyprids in each well. Similarly to the larval toxicity assay, three concentrations of the extract (5, 10 and 25 µg ml^{-1}) were used for this experiment. Wells with 25 µl ml of methanol added were used as controls. The treatment and controls were maintained in replicates (n = 6). The plates were kept inside a temperature-controlled chamber at 28°C. The cyprid larval settlement on the wells was checked after 24, 48 and 72 h by placing the plate under a microscope (stereomicroscope, Leica S6E). The larval settlement values were used to calculate the percentage of settlement inhibition (the percentage of larvae not settled) using the following formula:

$$Percentage\ of\ settlement\ (PS) = T \times 100/C.$$

$$Percentage\ of\ settlement\ inhibition = 100 - PS$$

where *T* = larvae settled in treatment wells, *C* = larvae settled in control wells, *PS* = percentage of settlement

2.7. Analysis of secondary metabolite composition of the algal extract

The composition of secondary metabolites of the *D. dichotoma* extract was analyzed using GC-MS. The procedure described by El-Din & El-Ahwany (2016) for macroalgal metabolite analysis was used in this study. In brief, a GC/MS system (Agilent GC-MS 5975 Inert XL MSD, United States) equipped with a J&W 122-5532G DB-5ms 30 x 0.25 mm 0.25 µm column and a mass detector was used for the analysis. The compounds were identified from the spectral library (NIST 11 – Mass Spectral Library) based on the comparison spectrum peaks.

2.8. *In silico* analysis: docking of cement protein and algal compounds

Secondary metabolites detected from the extract of *D. dichotoma* through GC-MS analysis were virtually screened for their activity against barnacle cement protein. The structure of cement protein of *Megabalanus rosa* (PDB code: 6LEK) was downloaded from the RCSB protein data bank (RCSB PDB). The structure of the compounds identified in the extract was obtained from Pubchem. The 2D chemical structures were downloaded as SDF files and converted to PDB format using OpenBabel (open source chemistry toolbox). Copper (Cu) was used as a reference compound due to its common use in antifouling coatings. The protein–ligand interaction was analyzed by cavity-detection guided blind docking using the CB-DOCK2 server (Liu et al. 2022) available online at https://cadd.labshare.cn/cb-dock2/php/index.php. The binding affinity between barnacle cement protein and ligands (secondary metabolites) was calculated using PyRx software. The ligand that showed the best binding affinity was further analyzed for its interaction with amino acid residues of the barnacle cement protein using BIOVIA discovery studio software.

2.9. Prediction of toxic properties of algal secondary metabolites

Toxic properties of the compounds detected in the extract were predicted by the *in silico* method using admetSAR (version 2) available online at http://lmmd.ecust.edu.cn/admetsar2 (Yang et al. 2019). Properties such as water solubility, biodegradability, crustacean toxicity and fish toxicity were predicted using admetSAR.

2.10. Statistical analysis

The variation of larval mortality between the control and macroalgal extract-treated groups was analyzed using one-way ANOVA (analysis of variance). Two-way ANOVA was used to analyze the effects of extract on larval settlement and biofilm formation. The concentrations of the extract and observation time were used as factors in two-way ANOVA. Tukey's post-hoc test was used to analyze pairwise interactions. The data were checked for homogeneity using Levene's test. For all statistical tests, $p < 0.05$ was considered significant. Statistical analysis was conducted using Statistica (ver. 13. 5.0.17).

3. Results

3.1. Antibiofilm assay

The extract of *D. dichotoma* inhibited the biofilm formation of the two bacterial strains used in this study (Figs 1 & 2). The higher concentration of algal extract showed an increase in biofilm inhibition. Further, of the three incubation periods tested, the higher percentage of biofilm inhibition was observed at 24 h in both *P. shioyasakiensis* and *Planomicrobium* sp. Two-way ANOVA results indicate a significant variation in biofilm development between the control and treatments (Table 1). The biofilm inhibition also varied significantly ($p < 0.05$) depending on the experiment duration (Table 2).

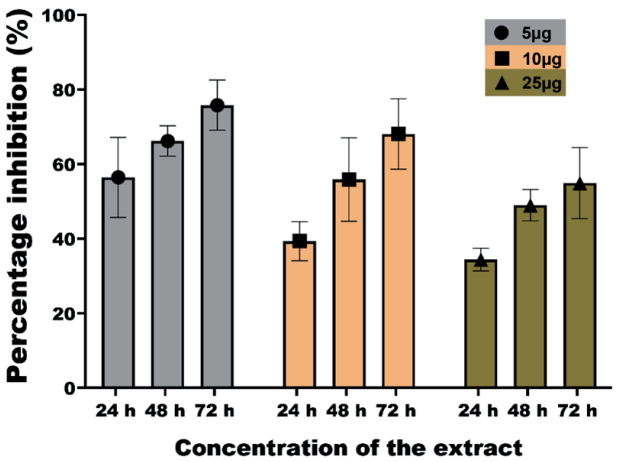

Figure 1

Antibiofilm activity of *D. dichotoma* extract against the biofilm-forming bacterium *P. shioyasakiensis*. Values are mean ± SD, $n = 6$.

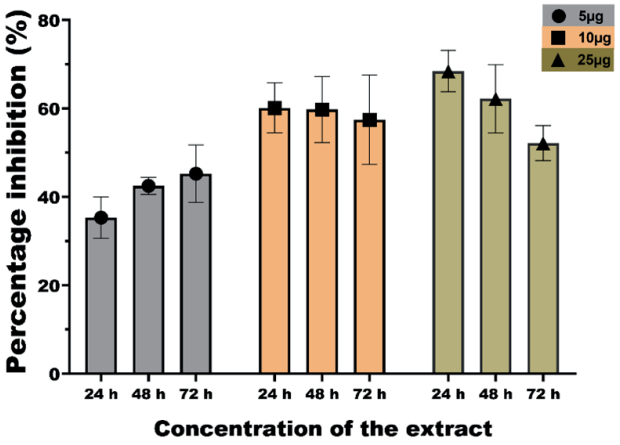

Figure 2

Antibiofilm activity of *D. dichotoma* extract against the biofilm-forming bacterium *Planomicrobium* sp. Values are mean ± SD, $n = 6$.

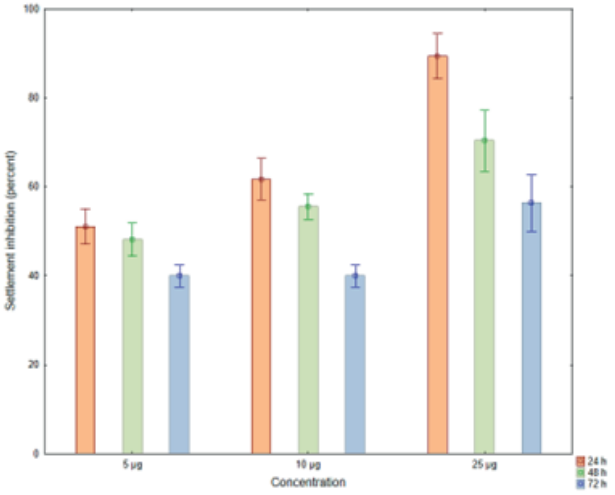

Figure 3

Inhibitory effect of *D. dichotoma* macroalgae extract on barnacle larval settlement. Values are mean ± SE, n = 6.

Table 1

Approximate probabilities for the variables used in Tukey's post hoc test.

Treatments		Antibiofilm activity		Barnacle larval toxicity	Barnacle larval settlement
		P. shioyasakiensis	*Planomicrobium* sp.		
Control	5 µg	< 0.001	< 0.001	0.677	< 0.001
	10 µg	< 0.001	< 0.001	0.508	< 0.001
	25 µg	< 0.001	< 0.001	0.026	< 0.001
5 µg	10 µg	0.687	< 0.001	0.992	0.388
	25 µg	0.065	< 0.001	0.231	< 0.001
10 µg	25 µg	0.488	0.974	0.354	< 0.001

$p < 0.05$ is considered significant

Table 2

Two-way ANOVA results for antibiofilm and barnacle larval settlement assays.

Effect	df	Antibiofilm activity				Barnacle larval settlement	
		P. shioyasakiensis		*Planomicrobium* sp.			
		F	*P*	*F*	*P*	*F*	*P*
Treatment	3	104.64	< 001	168.37	< 001	139.24	< 001
Time	2	20.75	< 001	19.55	< 001	32.02	< 001
Treatment*Time	6	0.41	0.866	1.83	0.108	1.39	0.229
Error	60						

$p < 0.05$ is considered significant

3.2. Cyprid settlement assay

The settlement of the cyprid larva was reduced considerably after treatment with the algal extract (Fig. 3). The maximum reduction of 89.36% was observed at 25 µg ml^{-1} concentration of extract after 24 h. The percentage of settlement inhibition decreases with increasing duration of the experiment (Fig. 3). ANOVA results indicate significant differences in the larval settlement between the treatment groups depending on the concentration and duration of the experiment (Table 2). Further, settlement in the extract-treatment groups differed significantly from the control wells (Table 1).

3.3. Toxicity of the extract against barnacle nauplii

The percentage of mortality of A. *amphitrite nauplii* was significant between the control group and the larvae treated with 25 µg ml^{-1} of the *D. dichotoma* extract ($F = 3.28$; $df = 3.20$; $p < 0.05$). Although an increase in the percentage of mortality was observed

in the other two concentrations of the extract (Fig. 4), the differences were not significant (Table 1). The 96-hour LC_{50} value for crude extract against the barnacle nauplii was 83.75 µg ml^{-1}.

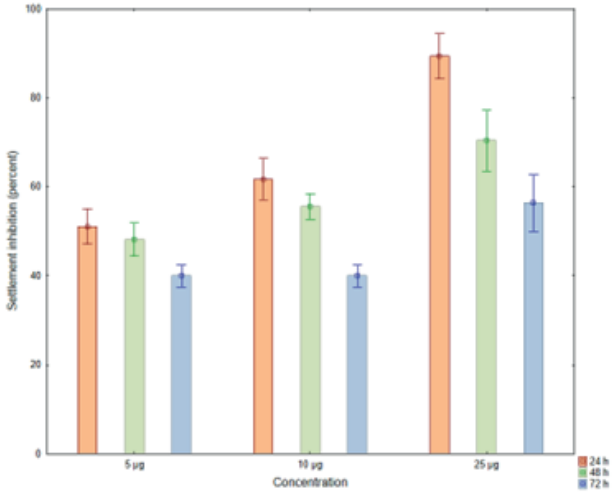

Figure 4

Toxicity of crude extract of *D. dichotoma* against barnacle nauplii. Values are mean ± SE, n = 6.

3.4. Secondary metabolite composition of the algal extract

The GC-MS analysis of the macroalgal extract yielded five compounds (Fig. 5, Table 3). The compounds identified from the extract based on the similarity match of the GC-MS spectrum peak were: 3-Trifluoroacetoxypentadecane, 9,9-Dimethoxybicyclo [3.3.1]nona-2,4-dione, hexadecanoic acid, cholestane, 4,5-epoxy and oleic acid. All these compounds have been previously reported to have bioactivity, including antibiofilm activities (Table 3).

3.5. Molecular docking analysis

The *in silico* analysis based on protein-ligand docking results indicate a higher binding affinity of the cholestane derivative (–14.2 kcal mol^{-1}) against the barnacle cement protein (Table 4). Hexadecanoic acid showed the lowest binding affinity of –5.7 kcal mol^{-1} among the compounds detected in the extract. Further, all compounds showed higher binding affinity than the reference compound copper (–4 kcal mol^{-1}). Figure 6 shows the binding pattern of ligands (algal compounds) with the barnacle cement protein based on cavity-detected blind docking. The interaction between the cholestane derivative and amino acid residues of barnacle cement is depicted in Figure 7. The interaction of cholestane with barnacle cement showed the presence of hydrogen and alkyl bonds.

3.6. Toxic properties of secondary metabolites

The *in silico* prediction of the toxic properties of the secondary metabolites detected in the extract of *D. dichotoma* is presented in Table 5. Cholestane, 4,5-epoxy is the least soluble among the secondary

Table 3

Secondary metabolite composition of *D. dichotoma* extract.

Compound name	Molecular formula	Molecular weight	Biological activity
3-Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$	324	Antioxidant (Hussein et al. 2016)
9,9-Dimethoxybicyclo [3.3.1]nona-2,4-dione	$C_{11}H_{16}O_4$	212	Cytotoxic (Dassamiour et al. 2022)
Hexadecanoic acid	$C_{17}H_{34}O_2$	270	Antibiofilm (Prasath et al. 2020)
Cholestane, 4,5-epoxy	$C_{27}H_{46}O$	386	Antifungal (cholestane derivatives; Alarif et al. 2011.)
Oleic acid	$C_{18}H_{34}O_2$	282	Antibiofilm (Pan et al. 2019; Khadke et al. 2021)

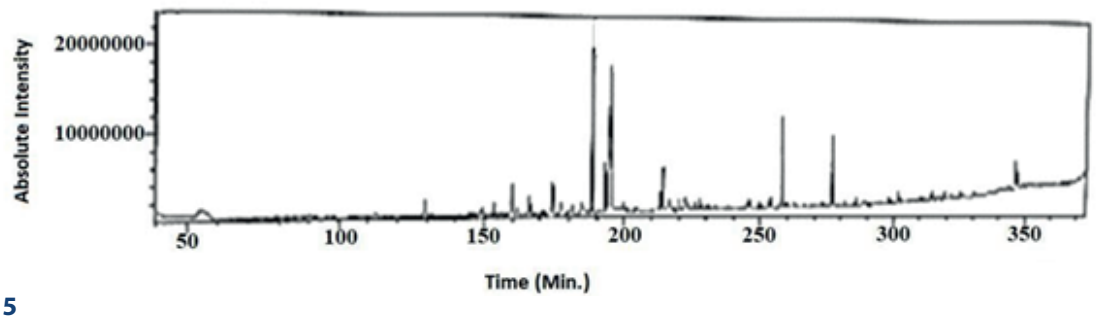

Figure 5

GC-MS spectrum of the *D. dichotoma* macroalgae extract.

Table 4

Binding affinity of secondary metabolites against barnacle cement protein. Pubchem CID shows the structure of the compounds used for the *in silico* analysis.

Compound name	Pubchem CID	Binding affinity (kcal mol^{-1})
Copper (reference compound)	23978	–4.0
3-Trifluoroacetoxypentadecane	534406	–7.0
9,9-Dimethoxybicyclo [3.3.1]nona-2,4-dione	537288	–8.2
Hexadecanoic acid	985	–5.7
Cholestane, 4,5-epoxy	537014	–14.2
Oleic acid	445639	–6.4

Table 5

Predicted toxicity profile of secondary metabolites of *D. dichotoma*. Toxic properties were predicted through *in silico* analysis using admetSAR.

Algal metabolites	Water solubility (logS)	Biodegradation	Crustacean toxicity	Fish toxicity
3-Trifluoroacetoxypentadecane	–3.316	non-degradable	+	+
9,9-Dimethoxybicyclo [3.3.1]nona-2,4-dione	–3.388	non-degradable	+	+
Hexadecanoic acid	–3.502	biodegradable	+	+
Cholestane, 4,5-epoxy	–4.585	non-degradable	+	+
Oleic acid	–4.04	biodegradable	+	+

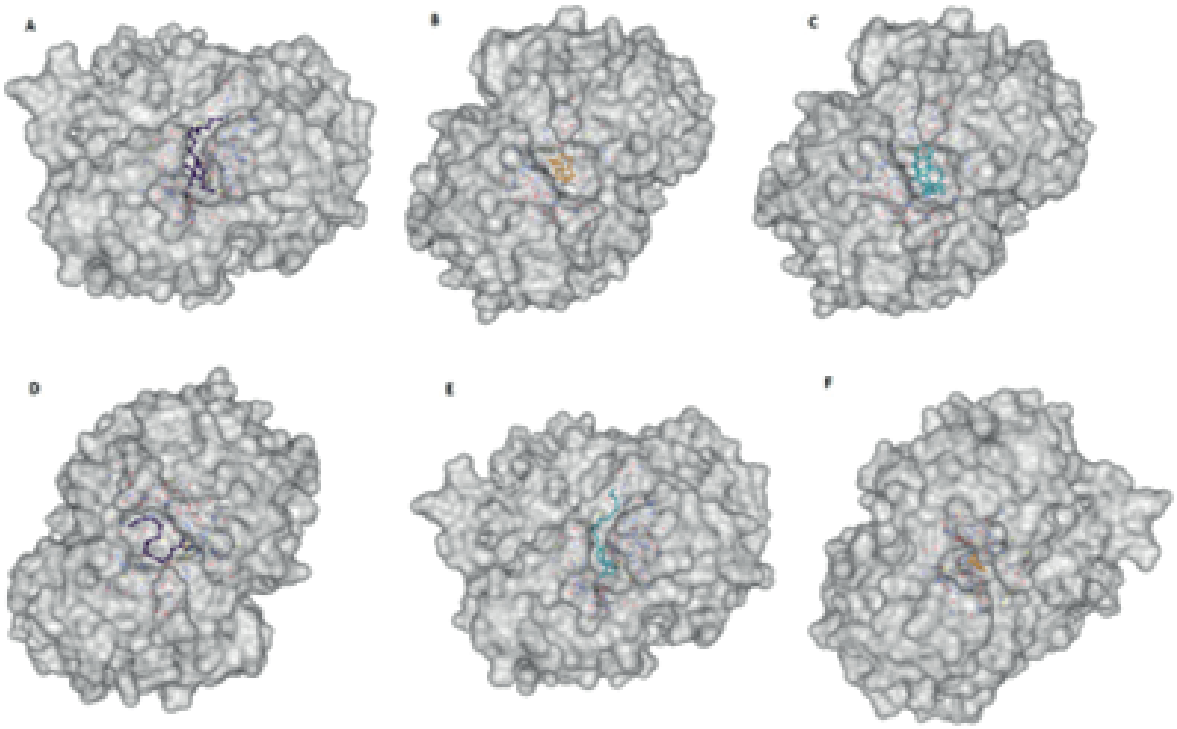

Figure 6

Binding pattern of ligands (secondary metabolites of *D. dichotoma*) with barnacle cement protein. A). 3-Trifluoroacetoxypentadecane, B). 9,9-Dimethoxybicyclo [3.3.1]nona-2,4-dione, C). Cholestane, 4,5-epoxy, D). Hexadecanoic acid, E). Oleic acid, F). Copper (reference compound).

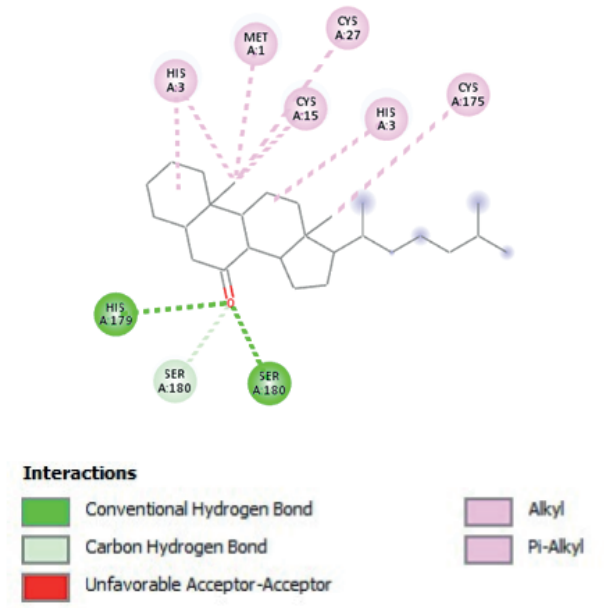

Figure 7

Interaction of ligand cholestane, 4,5-epoxy with amino acid residues of barnacle cement protein.

metabolites present in the extract. In general, all compounds are poorly soluble in water and toxic to aquatic biota such as crustaceans and fish. While hexadecanoic acid and oleic acid are predicted to be biodegradable, other compounds are not biodegradable.

4. Discussion

The results of the present study revealed that the extract of the brown macroalga *D. dichotoma* shows antibiofilm and anti-settlement effects on larvae. The extract significantly reduced the biofilm formation of two bacterial strains isolated from the microfouling community in the crystal violet microtiter plate assay. While the microtiter plate experiment using crystal violet staining can be used as a valuable tool to predict the biomass of bacterial cells attached to the plates, it will not provide detailed information on the viability or number of cells adhering to the substrate (Rima et al. 2022). Therefore, the observed reduction in biofilm biomass on microtiter plate wells may be due to the biofilm inhibitory activity or the bactericidal activity of the algal extract. Further analysis of the antibacterial activity of the extract against the biofilm-forming bacteria may provide more information on the observed antibiofilm activity.

The results also revealed a reduction in the settlement of barnacle larvae in the laboratory bioassay. This indicates that the algal extract contained antifouling metabolites. Previously, Bakar et al. (2017) studied the fatty acid composition and antifouling activity of *D. dichotoma* collected from Malaysia. Further, Siless et al. (2018) reported the antifouling activity of pachydictyol A and dictyoxide isolated from *D. dichotoma*. In general, macroalgae of the genus *Dictyota* are a prolific source of bioactive compounds, mainly terpenes (Othman et al. 2014; Chen et al. 2018). Bioactive terpene compounds isolated from *Dictyota* show antifouling activity against different fouling organisms (Barbosa et al. 2007; Viano et al. 2009; Othman et al. 2014). In addition to *D. dichotoma*, many marine algal species are known to produce antifouling compounds to inhibit the settlement of invertebrates (Dahms & Dobretsov 2017; Saha et al. 2018; Quemener et al. 2022). Most of these metabolites are present on the surface of algae and play multiple ecological roles against predators and herbivores (Murugan et al. 2012).

The GC-MS analysis of the extract obtained from *D. dichotoma* showed the presence of five bioactive metabolites. Among the detected secondary metabolites, hexadecanoic acid and oleic acid belong to fatty acids. Fatty acids isolated from marine algae show good antibacterial and antifouling activity (Bazes et al. 2009; Stabili et al. 2012; Paradas et al. 2016; Li et al. 2018). Further, hexadecanoic acid (palmitic acid) is a common fatty acid reported from many *Dictyota* species (Shameel et al. 1991). Another compound detected in the extract was the cholestane derivative (cholestane, 4,5-epoxy). The presence of cholestane derivatives in marine algae and their antibacterial activity have been previously reported (Das & Srinivas 1993; Dashtegol et al. 2021). The halogenated hydrocarbon compound, 3-Trifluoroacetoxypentadecane, detected in the algal extract was previously reported from the sea cucumber *Holothuria forskali* (Telahigue et al. 2020). Halogenated compounds isolated from marine algae show multiple biological activities, including antibacterial and antifouling activity (Blunt et al. 2009).

Barnacles are commonly used as a model organism for screening antifoulants from various sources (Marechal & Hellio 2011). The results of this study indicate that the crude extract of *D. dichotoma* could inhibit the settlement of barnacle larvae on hard substrates. However, a study by da Gama et al. (2003) did not find antifouling activity of *Dictyota* extract in a field assay. Differences in the antifouling activity of macroalgae (even of the same species) between different studies may be due to the geographical region of algae collection, sampling seasons and metabolite composition of the extract. Therefore, a comparison of the activity of the compounds may provide more information than the extracts. Although

the algal extract inhibited the settlement of barnacle larvae in laboratory experiments, further field studies are required to confirm this activity. Since the mode of action of the compounds on target organisms is necessary for the formulation of an environmentally friendly antifouling agent, *in silico* analysis was carried out against the cement protein of barnacles. The results indicate a good binding affinity of the secondary metabolites of *D. dichotma* with the target protein. The steroid compound, cholestane, 4,5-epoxy showed a higher binding affinity indicating its antifouling defense role. Previously, Zhang et al. (2014) reported on the antifouling activity of cholestane derivatives from the gorgonian coral *Subergorgia suberosa*. Although hexadecanoic and oleic acids show low binding affinity among the compounds detected in the extract, the values are higher than those of the reference compound, copper. This indicates that the metabolites of the macroalga *D. dichotoma* may act as a more potent antifouling compound than copper.

Since the *in silico* approach is not widely used in antifouling screening studies, it is necessary to identify more potential molecular targets for inhibiting the settlement of organisms. Further, molecular docking of isolated compounds with many target proteins, ranging from bacteria and higher organisms, may provide more insight into how antifouling compounds work. Previous *in silico* studies used a G protein-coupled receptor (Inbakandan et al. 2016) and acetylcholinesterase (Arabshahi et al. 2021) as protein targets for molecular docking of antifouling compounds due to their role in the larval settlement. In this study, the barnacle cement protein was used as a target to expand our knowledge of the interaction between algal secondary metabolites and cement protein. Cypris larvae secrete protein-containing adhesives for temporary ('footprints') and permanent attachment (cyprid cement) to substrates (Khandeparker & Anil 2007; Liang et al. 2019). The adult barnacle produces cement for firm attachment to substrates (Khandeparker & Anil 2007). Therefore, compounds that interact with temporary or permanent adhesives produced by barnacle larvae, as well as adults, may prevent the settlement on surfaces. The results obtained in this study indicate a strong binding of compounds present in the algal extract with barnacle cement proteins.

The toxicity assay against a barnacle nauplius revealed that mortality was not significant at low concentrations of the extract. In addition, the settlement of the cyprid larva was very low within 24 h (higher inhibition was observed at 24 h). The decrease in the percentage inhibition of settlement (Fig. 3) with time may be due to the metabolic activity of larvae. The main function of the antifouling coating is to prevent initial attachment or deter the organisms from the surfaces. The results show that the secondary metabolites present in the extract could strongly inhibit the larval attachment during initial exposure, validating with the main feature of an ideal antifouling compound. The *in silico* toxicity prediction of the secondary metabolites showed positive against crustaceans and fish. Hexadecanoic acid and oleic acid are also predicted to be biodegradable. Natural products are considered an alternative to toxic biocides in antifouling applications due to their biodegradability (Satheesh et al. 2016; Kyei et al. 2020). Although the secondary metabolites detected in the extract of *D. dichotoma* are predicted to be toxic to fish and crustaceans, natural products are generally less toxic and environmentally friendly compared to chemical compounds used for antifouling purposes (Hellio et al. 2000). *In silico* methods for screening of toxicity of compounds will predict the toxicity based on chemical structure and available data (Bassan et al. 2021). These *in silico* prediction models will be useful for understating the toxicity of compounds. However, it is necessary to integrate experimental data with *in silico* models. Therefore, further laboratory studies are required to test the toxicity against non-target organisms, biodegradability and water solubility of the compounds present in the extract of *D. dichotoma*.

In conclusion, the present study showed that the extract of the brown macroalga *D. dichotoma* inhibited the biofilm-forming bacteria and the settlement of barnacle larvae in laboratory experiments. GC-MS analysis revealed the presence of fatty acids, halogenated hydrocarbons and the cholestane derivative in the macroalgal extract. While molecular docking analysis revealed the interaction of all the compounds present in the extract with the cement protein of barnacles, the cholestane derivative exhibited the highest binding affinity. Overall, the results of the present study confirmed that the brown alga *D. dichotoma* can be used as a natural source to extract compounds for antifouling applications. Among the compounds detected in the extract, *in silico* analysis revealed that the cholestane derivative may be one of the potential antifouling compounds for further laboratory and field studies.

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