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Sterols compositions, antibacterial, and antifouling properties from two Malaysian seaweeds: *Dictyota dichotoma* and *Sargassum granuliferum*

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ABSTRACT

Seaweeds or macroalgae are the primary producers of an oceanic food source, widely distributed across the globe and known for their excellent defensive properties against numerous biotic and abiotic factors. These defensive traits come from their secondary metabolites that act as protective barriers against pathogens and harmful organisms where among these compounds some have been found possessing the antifouling characteristic. In this study, *Dictyota dichotoma* and *Sargassum granuliferum* collected from Pulau Nunuyan Laut, Sabah, Malaysia, were studied to determine its sterol composition, and isolation was carried out to isolate their pure sterol compounds. Two assays consist of disk diffusion method for antibacterial activity and crystal violet assay were carried out to study its antifouling activity. Campesterol, stigmasterol, and β-sitosterol were the dominant sterol compound detected in both samples and six pure sterols were isolated (compounds **1**–**6**). Results from the antibacterial and antifouling analysis showed better inhibition for Gram-negative compared to Gram-positive bacteria. Fucosterol (**4**) and epicoprostanol (**5**) gave the best antibacterial activity with two bacteria inhibited compared to other compound. Meanwhile, coprostanol (**1**), campesterol (**2**), stigmasterol (**3**), epicoprostanol (**5**), and 5β-cholestan-3-one (**6**) showed strong antifouling activity towards the selected bacterial strains with IC_{50} values ranges from 266.3 to 425.8 µg/ml.

INTRODUCTION

The extensive coastline with rocky shores and sandy bays, as well as numerous coral fringe islands found along the coastlines of Peninsular Malaysia, Sarawak, and Sabah, provides niche habitat for a variety of seaweed species in Malaysia water. In recent years, the use of natural antifouling based materials has been greatly explored and studies have been reporting on their capability against antifouling activities (Almeida and Vasconcelos, 2015; Guanther *et al*., 2007; Qian *et al*., 2015). Present technologies in inhibiting settlement and growth of marine fouling organisms mostly rely on antifouling paint formulated with inorganic compounds, such as Irgarol 1051, tributyl-tin (TBT), and other brands that are available in the market (Fisher *et al*., 1999; Grinwis *et al*., 1998). The most effective component in antifouling paints is TBT which is detrimental and not degraded in the natural ecosystem (Soliman *et al*., 2017). These inorganic chemicals action mode of either being toxic or sub-lethal and have been proved affecting the marine ecosystem (Bhadury and Wright, 2004; Soliman *et al*., 2017). In addition to that, the effects of biofouling also give the implication to the aquaculture industries where they implicate the cultured organisms for space and food deteriorating farming infrastructure as well as affecting the natural habitat on neighboring areas (Gopikrishnan *et al*., 2015). Thus, it is important in developing a green antifoulant alternative for

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combatting foulants at the same time safe to the environments. (De Nys and Steinberg, 2002; Fusetani, 2004; Guenther and De Nys, 2007). The green antifoulant may consist of isolated compounds from natural resources such as marine organism (Clare, 1996). Researchers studying on ascidians, bryozoans, cnidarians, and seaweeds had proven that these marine organisms are potentially for antimicrobial, antifungal, and antilarval activities (Clare, 1996; Fusetani, 2004; Qian *et al*., 2012;). Seaweed is a marine macroalga and they are rich sources of secondary metabolites (Bhadury and Wright, 2004) of which these metabolites functioned as antibacterial, antifungal, antiprotozoan, antiherbivores, antiepibiosis, or antifouling and so on (Clare, 1996; Da Gama *et al*., 2002; Hellio *et al*., 2001; Pawlik, 1992). *Dictyota dichotoma* and *Sargassum granuliferum* are abundantly distributed at Pulau Nunuyan Laut, Sabah, Malaysia. To the best of our knowledge, there is still no report on sterol composition for these seaweeds. Thus, we initiate this study to determine their sterol composition as well as the ability in antibacterial and antifouling.

MATERIALS AND METHODS

Seaweeds collection and preparation

Fresh *D. dichotoma* and *S. granuliferum* were collected via scuba diving (6–9 m) at Pulau Nunuyan Laut, Sabah, Malaysia (05° 56.030′N; 118° 06.609′E) on June 2009 Voucher specimens (MARC08P0054, MARC08P0073) were deposited at Institute of Oceanography and Environment (INOS), Universiti Malaysia Terengganu. Collected seaweeds were cleaned from debris and air-dried. Dried samples of *D. dichotoma* and *S. granuliferum* were cut into small pieces and ground into powder using a metal grinder.

Extraction and isolation of sterols from S. granuliferum and D. dichotoma

Dried powder of *D. dichotoma* (2 kg) and *S. granuliferum* (2.3 kg) was soaked separately with 2.5 L of methanol and shake using orbital shaker at the speed of 120 rpm for a period of 3 days. Soaked samples were then filtered to remove solid particles from the solvent extracts. These procedures were repeated thrice and the collected extracts were evaporated using a rotary evaporator. Samples were then partitioned with petroleum ether, chloroform, ethyl acetate, and butanol, with a similar process as mentioned above. Isolation and characterization of pure compounds from the extracts of *D. dichotoma* and *S. granuliferum* were done using column chromatography and preparative thin-layer chromatography techniques. Table 1 shows the different solvent extracts for *D. Dichotoma* and *S. granuliferum*. Butanol extract of *D. dichotoma* (DDB) was not tested in this study due to the low yield of crude extract.

Table 1. Different solvent extracts for *D. dichotoma* and *S. granuliferum*.

Types of solvent	Seaweed samples	
	D. dichotoma	*S. granuliferum*
Methanol	DDM	SGM
Petroleum ether	DDP	SGP
Chloroform	DDC	SGC
Ethyl acetate	DDE	SGE
Butanol	DDB	SGB

Sterol analysis

Sterols were fractionated from each crude extracts using liquid–solid chromatography technique with silica and alumina as its stationary phase. 5α-androstanol was spiked into the crudes and sterol compounds were eluted using Dichloromethane: MeOH (1:9 v/v). The sterol fractions collected were then pre-concentrated using rotary evaporator to a volume of approximately 3 mL and the final volume were adjusted to 1 ml using nitrogen blowdown. Sterol compounds were silylated using N,O-Bis(trimethylsilyl) trifluoroacetamide-trimethylchlorosilane (99:1) and identification was carried out using a Shimadzu QP2010 GCMS equipped with 5% phenyl methyl siloxane (DB-5) column with helium as the carrier gas. The oven temperature was programmed at 70°C (2 minutes) ramp to 180°C (4°C/minute), increase to 300°C (5°C/minute) and isothermal for 15 minutes. Injector and detector temperatures were maintained at 300°C. Sterol compounds detected were identified and verified using major mass ion (M^+), retention times compared to those of external sterol standards (e.g., campesterol, cholesterol, *β*-sitosterol, stigmasterol) and/or mass spectra from published literature (Hayee-Memon *et al*., 1991; Kaloustian *et al*., 2008; Kala *et al*., 2015; Rohloff, 2015) and GCMS library (NIST 2014 and WILEY 229).

Antibacterial assay

Antibacterial activity of pure sterols isolated was tested using paper disk diffusion technique against five bacteria, namely, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, which were obtained from Microbiology Laboratory of Institute of Marine Biotechnology, Universiti Malaysia Terengganu. Gentamycin and Penicillin-G (10 µg), while Chloramphenicol (30 µg) were used as positive controls while all pure sterol tested were 1 mg/ml. All bacteria were cultured in appropriate broths at 30°C for overnight and concentration were matched to the density of a 0.5 McFarland standard. Agar cultures and bacterial test were prepared as described by Razmavar *et al*. (2014). All determination was carried out in triplicates.

Anti-fouling assay

The crystal violet biofilm assay method was performed on the pre-sterilized 96-well flat-bottom polystyrene microtiter plates in triplicate as mentioned by Stepnovic *et al*. (2000) with some modifications. In this assay, *V. alginolyticus*, *V. mimicus*, *V. parahaemolyticus*, *P. aeruginosa*, and *B. subtilis* were chosen for their ability to form a biofilm. Different compounds isolated from *D. dichotoma* and *S. granuliferum* were added at the specified concentrations. Negative control well was also included for bacterial culture without compounds addition. Briefly, 20 µl of bacteria suspension having 0.5 OD_{595} was inoculated in 230 µl Luria Bertani broth on a microtiter plate and incubated at 37°C for 24 hours period. Once incubated, the microtiter plate was washed twice with sterile water and 200 µl of absolute methanol was added and allowed to contact for 15 minutes. Crystal violet in water solution (1% v/v) was added to the plate and then removed and washed with distilled water and dried. Finally, 33% (v/v) glacial acetic acid was added and agitated in ELISA for 15 minutes. The agitated solution was then transferred to a new sterile 96-well

plate. Optical density (OD) was measured using ELISA reader at 595 nm and the percentage of bacteria inhibition concentration was calculated as follows:

$$\% \text{ Inhibition concentration} = [(\text{OD growth control} - \text{OD sample}) / \text{OD growth control}] \times 100$$

RESULTS AND DISCUSSION

Pure sterol compounds isolated from D. dichotoma and S. granuliferum

In this section, six pure sterol compounds were obtained from the isolation of the partition extracts from *D. dichotoma* and *S. granuliferum*. Isolation and purification of the main compounds were carried out on silica gel and Sephadex LH20 column chromatography to obtain six pure compounds. Structural elucidation of these compounds was based on the data obtained from ^{1}H-NMR and ^{13}C-NMR studies. Separated compounds from *S. granuliferum* and *D. dichotoma* were identified as coprostanol (**1**), campesterol (**2**), stigmasterol (**3**), fucosterol (**4**), epicoprostanol (**5**), and 5β-cholestan-3-one (**6**) compared to the spectral data reported in the literature (Fig. 1). ^{13}C-NMR data of sterols isolated from *S. granuliferum* and *D. dichotoma* are summarized in Table 2.

Sterol composition in D. dichotoma and S. granuliferum

Results of overall sterol compounds identified in the crude extracts of *D. dichotoma* and *S. granuliferum* using GCMS are presented in Table 3. Table 4 shows the percentage of sterol compounds detected in different crude extracts of *D. dichotoma* and *S. granuliferum*. *S. granuliferum* sample exhibited more sterol with 13 compounds compared to *D. dichotoma* which detected 11 compounds. In *D. dichotoma* sample, 24-nor-22, 23-methylenecholest-5-en-3β-ol, 25-hydroxy-24-methylcholesterol, and saringosterol were not detected in all extracts while *S. granuliferum* sample did not exhibit the presence of fucosterol in all the extracts. It was observed that both *D. dichotoma* and *S. granuliferum* samples were mainly dominated by campesterol, stigmasterol, and β-sitosterol. These phytosterols are found abundant in plant and they function in maintaining the structure and physiology of cell membranes. They function by the limiting movement of fatty acyl chains which decrease membrane permeability and regulate its fluidity (Piironen *et al*., 2000), and Schuler *et al*. 1991 found sitosterol to be effective in limiting water permeability in the soybean. Previous studies on brown algal reported that fucosterol [stigmasta-5, (*E*)-24(28)-dien-3β-ol] (Easa *et al*., 1995; Newburger *et al*., 1979; Smith *et al*., 1973) and saringosterol [24-ethylcholesta-5, 28-diene-313, 24-diol] (Amico *et al*., 1980; Ikekawa *et al*., 1968) were the dominant compound found. In this study, fucosterol was found in all *D. dichotoma* crude extracts but not as dominant sterol while saringosterol was only found in methanol, petroleum ether, and ethyl acetate crude extract of *S. granuliferum* sample. The absence of fucosterol in the studied sample extracts is due to degradation into saringosterol epimers where fucosterol is highly reactive and easily oxidize to saringosterol (Bouzidi *et al*., 2014), whereas the absence of saringosterol in *D. dichotoma* extracts was due to that saringosterol peak in the respective samples exhibits weak molecular mass fragmentation in the GCMS total ion chromatogram; thus, difficult for conformation with NIST 2014 and WILEY 229 library and is not reported in this study. Furthermore,

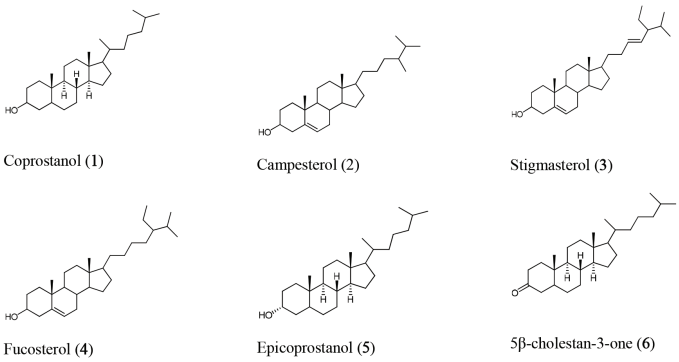

Figure 1. Chemical structures of isolated sterols from *S. granuliferum* and *D. dichotoma*.

Table 2. ^{13}C NMR (125 MHz, $CDCl_3$ dc) chemical shifts of compounds.

Carbon number	1	2	3	4	5	6
1	35.5	36.5	37.3	37.3	35.4	37.2
2	31.6	31.7	31.7	31.7	30.6	37.1
3	71.4	71.8	71.8	71.8	71.9	213.5
4	38.3	42.3	42.3	42.3	36.2	42.4
5	40.1	140.8	140.8	140.8	42.2	40.9
6	28.8	121.7	121.7	121.7	28.3	26.7
7	32.1	33.9	31.9	31.9	26.5	28.3
8	35.8	36.1	31.9	31.9	35.8	35.6
9	54.4	50.2	50.2	50.1	40.5	44.4
10	35.5	37.3	36.5	36.5	34.6	34.9
11	21.3	26.3	21.1	21.1	20.9	21.2
12	42.6	39.8	39.7	39.8	40.2	39.5
13	44.9	42.3	42.2	42.4	42.7	42.7
14	56.5	56.8	56.9	56.8	56.6	56.5
15	23.8	24.3	24.4	24.3	24.3	25.8
16	28.2	29.2	28.9	55.8	27.2	24.2
17	56.3	56.1	55.9	11.9	56.4	56.4
18	12.1	11.9	12.1	19.4	12.0	18.7
19	12.3	19.8	18.9	36.4	22.6	12.1
20	36.2	33.9	40.5	18.8	36.5	37.8
21	18.7	21.2	21.2	35.2	18.7	22.7
22	37.0	45.9	138.3	25.7	35.9	36.2
23	24.2	26.1	129.3	147.0	23.9	23.8
24	28.0	19.3	51.2	34.8	39.5	40.1
25	39.5	29.7	31.9	22.2	28.0	28.0
26	22.8	21.1	21.2	22.1	22.8	22.6
27	22.6	18.8	19.4	115.6	23.4	22.8
28		11.8	25.4	13.2		

the high concentration of campesterol, stigmasterol and β-sitosterol and fucosterol in this study was similar to Kanias *et al*. (1992) who studied *Padina pavonia* from the Aegean Sea. The studied area Pulau Nunuyan, Sabah, is located in the Sulu Sea having a warm tropical climate with an average seawater temperature of 28°C. This warmer temperature might be the reason for higher campesterol, stigmasterol, and β-sitosterol compared to fucosterol. The differences in sterol

Table 3. GCMS data of sterols from *D. dichotoma* and *S. granuliferum*.

Compound name	Experimental major mass Ion (M^+)	GCMS major mass ion (M^+)
Coprostanol	370, 355, 257, 215	370, 355, 257, 215
Epicoprostanol	370, 355, 257, 215	370, 355, 257, 215
24-Nor-22,23-methylenecholest-5-en-3β-ol	456, 366, 3255, 129	
Cholesterol	386, 368, 328, 129	386, 368, 329, 129
Cholestanol	388, 373, 215	388, 373, 355, 215
24-Methyl-22-dehydrocholesterol	470, 380, 215, 129	
25-Hydroxy-24-methylcholesterol	470, 386, 256, 129	
Brassicasterol	470, 380, 255, 129	470, 380, 255, 129
Campesterol	472, 382, 255, 129	472, 382, 255, 129
Stigmasterol	484, 394, 255, 129	484, 394, 255, 129
Fucosterol	412, 314, 281, 129	412, 314, 281, 129
β-Sitosterol	486, 396, 357, 129	486, 396, 357, 129
Saringosterol	416, 396, 357	416, 396, 357

Table 4. Sterol compound detected in different crude extracts of *D. dichotoma* and *S. granuliferum*.

Compound Name	Percentage of sterol in different solvent partition crude extract								
	Dictyota dichotama				*S. granuliferum*				
	DDM	DDP	DDC	DDE	SGM	SGP	SGC	SGE	SGB
Coprostanol	0.19	-	0.38	0.12	0.02	0.02	-	0.03	0.01
Epicoprostanol	0.50	-	-	-	0.21	0.17	7.60	-	-
24-Nor-22,23-methylenecholest-5-en-3β-ol	-	-	-	-	1.78	3.92	-	5.17	16.6
Cholesterol	9.60	-	-	1.20	5.51	6.72	-	13.2	11.8
Cholestanol	28.8	1.91	5.40	-	0.51	1.13	-	1.79	-
24-Methyl-22-dehydrocholesterol	5.23	8.79	6.31	5.84	5.16	7.29	-	6.39	20.1
25-Hydroxy-24-methylcholesterol	-	-	-	-	3.10	-	-	8.40	16.7
Brassicasterol	3.61	8.04	4.33	4.87	5.04	7.15	-	5.43	20.1
Campesterol	18.2	30.5	20.4	17.7	14.7	29.6	-	3.99	8.32
Stigmasterol	25.1	22.3	30.9	8.07	32.8	32.0	31.9	4.10	6.39
Fucosterol	3.37	24.3	6.81	30.0	-	-	-	-	-
β-Sitosterol	5.32	4.18	25.4	32.2	29.2	5.60	60.5	34.9	-
Saringosterol	-	-	-	-	2.03	6.49	-	16.6	-

SGM = Methanol extract of *S. granuliferum*, SGP = Petroleum etherl extract of *S. granuliferum*, SGC = Chloroform extract of *S. granuliferum*, SGE = Ethyl acetate extract of *S. granuliferum*, SGB = Buthanol extract of *S. granuliferum*, DDM = Methanol extract of *Dictytota dichotoma*, DDP = Petroleum ether extract of *Dictytota dichotoma*, DDC = Chloroform extract of *Dictytota dichotoma*, DDE = Ethyl acetate extract of *Dictytota dichotoma*, - Not detected

composition and its abundances could be due to differences in the life cycle of the algae and their ecological conditions (Ikekawa *et al*., 1968; Kanias *et al*., 1992; Kamernarska *et al*., 2002; 2003; Petkov *et al*., 1992). In addition, coprostanol (5β-cholestan-3β-ol) and epicoprostanol (5β-cholestan-3α-ol) were also found in *D. dichotoma* and *S. granuliferum* crude extract. It is interesting to note that these two compounds were normally associated with sewage discharge; thus, the presence of them in the sample studied should be further studied to ascertain their origin.

Antibacterial activity

Antibacterial activities of all pure sterol compounds were tested against adhesive Gram-positive bacteria (*B. subtilis*) and Gram-negative bacteria (*V. alginolyticus*, *V. parahaemolyticus*, *V. mimicus*, and *P. aeruginosa*). In this study, the bacteria selected were mostly from the Gram-negative strains due to Gram-negative bacteria with different characteristics were the major strain in the marine environment and accounted for 90% of marine bacteria population (Iyyapparaj *et al*., 2012). The high density of this bacteria is due to their cell wall is highly adapted for the high salinity environment (Das *et al*., 2006). Results of antibacterial activities obtained for all pure sterol compounds tested were shown in Table 5. In general, all pure sterol compounds of both seaweeds showed better inhibition against Gram-negative bacteria compared to Gram-positive bacteria. Compound **5** gave the best inhibition with two Gram-positive bacteria inhibited while other compounds only inhibited one Gram-positive bacteria. The zones of growth inhibition for Gram-negative bacteria were almost similar for all compounds with inhibition range of 7.2–9.6 mm. Meanwhile, compound **4** is the only compound that gave

Table 5. Antibacterial activity of sterol pure compounds against (a) *V. alginolyticus* (b) *V. mimicus* (c) *Vibrio parahaemolyticus* (d) *P. aeruginosa* (e) *B. subtilis*

Extract	Concentration (mg/ml)	Inhibition zone (mm)				
		Bacteria				
		V. alginolyticus	*V. mimicus*	*V. parahaemolyticus*	*P. aeruginosa*	*B. subtilis*
1	1	NA	7.8 ± 0.05	NA	NA	NA
2	1	9.4 ± 0.80	NA	NA	NA	NA
3	1	NA	NA	7.1 ± 0.1	NA	NA
4	1	NA	NA	9.0 ± 0.00	NA	9.6 ± 0.80
5	1	7.20 ± 0.80	8.6 ± 0.05	NA	NA	NA
6	1	NA	9.6 ± 0.05	NA	NA	NA
Control						
Penicillin	10 µg	34.2 ± 0.5	31.3 ± 0.6	33.3 ± 0.6	40.5 ± 0.5	32.8 ± 0.3
Gentamycin	10 µg	33.7 ± 0.6	31.3±0.6	35.2 ± 0.3	38.8 ± 0.8	29.7 ± 0.6
Chloramphenicol	30 µg	45.3±0.5	48.1±0.9	42.3 ± 0.6	36.2 ± 0.3	30.5 ± 0.5

Table 6. Crystal violet biofilm activity of sterol pure compounds against (a) *V. alginolyticus* (b) *V. mimicus* (c) *Vibrio parahaemolyticus* (d) *P. aeruginosa* (e) *B. subtilis*.

Sample	IC_{50} (µg/ml)				
	Bacteria				
	V. alginolyticus	*V. mimicus*	*V. parahaemolyticus*	*P. aeruginosa*	*B. subtilis*
1	NA	266.29 ± 0.09	NA	NA	NA
2	425.79 ± 0.14	NA	NA	NA	NA
3	NA	NA	560.07 ± 0.07	NA	NA
4	NA	NA	NA	NA	NA
5	269.00 ± 0.06	314.81 ± 0.07	NA	NA	NA
6	NA	143.60 ± 0.04	NA	NA	NA

NA = no activity.

inhibition for Gram-positive bacteria. The greater inhibition for Gram-negative compared to Gram-positive bacteria obtained for all sterol compounds was in opposite trend compared to other research studies on antimicrobial activities from seaweeds (Dussault *et al*., 2016; Kavita *et al*., 2014). The abundances of Gram-negative bacteria in the marine environment is well-known; therefore, results of antibacterial activity obtained in this study bring new insight for the development of antimicrobials against Gram-negative bacteria from seaweed-based natural products.

Anti-fouling activity

All pure sterol compounds isolated from *D. dichotoma* and *S. granuliferum* were tested for antifouling activity using crystal violet assay. Coprostanol (**1**), campesterol (**2**), stigmasterol (**3**), epicoprostanol (**5**), and 5β-cholestan-3-one (**6**) showed strong inhibition towards the selected bacterial strains with IC_{50} values ranges from 266.3 to 425.8 µg/mL, while fucosterol (**4**) gave no activity for all bacterial strain tested (Table 6). Results obtained showed these compounds gave better inhibition properties for Gram-negative bacteria compared to Gram-positive bacteria. Epicoprostanol (**5**) is the most active compounds which inhibit two types of bacteria *V. alginolyticus* (269.00 ± 0.06) and *V. mimicus* (314.81 ± 0.07). Microtiter plate system for quantifying biofilm formation has been investigated using many different organisms and strains (Christensen *et al*., 1985; Deighton and Balkau, 1990; Miyake *et al*., 1992; Ramage *et al*., 2001; Stepanović *et al*., 2000). Crystal violet staining method is a good technique in measuring the amount of biofilm formed but not on its activity. Thus, this technique is suitable for quantifying biofilm removal but not disinfection (Pitts *et al*., 2003).

Therefore, the results of this study present the possibility of sterol compound to be used as a potential natural antifouling substance.

CONCLUSION

Results from this study showed sterol compounds isolated from *D. dichotama* and *S. granuliferum* exhibit antibacterial and antifouling activities on Gram-negative bacteria compared to Gram-positive bacteria. This characteristic is important because Gram-negative bacteria are the dominant bacteria present in the marine ecosystem and they play an important part in bio-film processing. Therefore, the sterol compounds of these seaweeds can be utilized for developing a promising green antifouling alternative in combating marine water biofilm.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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