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Research Article

Antibiofilm and Antifouling Properties of *Sargassum plagiophyllum* (C. Agardh, 1824) Extract

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Abstract
Biofouling poses significant challenges to the maritime industry. While many commercial antifouling agents are effective, their toxic effects on marine ecosystems have raised environmental concerns. To address this issue, this study aimed was to evaluate antifouling properties of *Sargassum plagiophyllum* extracts through a series of assays, including crystal violet antibiofilm activity, antibacterial screening, cytotoxicity evaluation, aquarium testing, and *in situ* field experiments. The methanolic crude extract of *S. plagiophyllum* demonstrated potent antibiofilm activity with an IC_{50} value of 0.014 mg/mL, while the aqueous fraction exhibited a higher IC_{50} of 0.690 mg/mL. Cytotoxicity assay using the brine shrimp lethality assay revealed that *S. plagiophyllum* crude extract indicated non-toxic action across various concentrations. *S. plagiophyllum* does not exhibit antibacterial activity against *Pseudomonas aeruginosa* but disrupts bacterial quorum sensing, as evidenced by the formation of a colorless opaque zone in *Chromobacterium violaceum* assay. Aquarium-based antifouling tests further demonstrated that a 5% formulation of *S. plagiophyllum* extract significantly reduced bacterial biofilm formation. Panels treated with the 5% *S. plagiophyllum* formulation exhibited a marked reduction in bacterial adhesion compared with the negative control. These results were comparable to those obtained with commercial antifouling paints: specific commercial antifouling formulations reference 1 (RF1) and reference 2 (RF2), which showed bacterial counts of 0.92×10^8 CFU/mL ($p < 0.001$) and 0.95×10^8 CFU/mL ($p < 0.001$), respectively. The active fractions identified several compounds, which likely contribute to the observed antifouling properties. *In situ* testing conducted in Redang Island and Kuala Kemaman, Malaysia, confirmed the efficacy of the 5% *S. plagiophyllum* formulation in reducing marine fouling over a three-month period, outperforming both RF1 and RF2. These findings highlight the potential of *S. plagiophyllum* as a sustainable and effective alternative for eco-friendly antifouling coatings on the environmental implications of using *S. plagiophyllum*.

Keywords: biofilm, brown algae, marine biofouling, seaweed

1. INTRODUCTION

The biofouling process occurs when an undesirable quantity of marine creatures, including macro- and micro-foulers (algae, bacteria and invertebrates), accumulate on submerged surface. Microorganisms secrete organic compounds that form a conditioning layer, facilitating the attachment of secondary colonizers such as seaweed spores and protozoa. This microfouling stage prepares the surface for macrofouling, which involves the settlement of larger invertebrates like barnacles, mussels, and tunicates. Biofouling represents a critical challenge in the maritime industry, with well-documented impacts on operational efficiency and infrastructure. It has been associated with increased drag resistance on ship hulls, leading to higher fuel consumption and operational costs. Additionally, biofouling can cause occlusions in fuel and hydraulic systems due to valve and sieve blockages, as well as clogging of nets and meshes in marine aquaculture systems, thereby compromising system performance and productivity [1]. The maritime industry invests substantial financial resources in the maintenance and removal of fouling organisms from offshore facilities and marine equipment. The presence of marine life in the pipeline has been shown to negatively impact the pumps' functionality. While biofouling on ship hulls significantly increase operational cost of fuel accounts for 60% of a ship's operating expenses; however after 6 months without antifouling protection, the fuel consumption increase by additional 40% due to the drag caused by heavy fouling [2]. In this context, antifouling refers to any strategy or coating that prevent marine organisms adhesion to artificial surfaces [3]. Consequently, the application of antifouling coatings to ship hulls is considered one of the most effective methods to mitigate the adverse effects of biofouling.

Antifouling paints have traditionally been formulated by incorporating biocides, with toxic

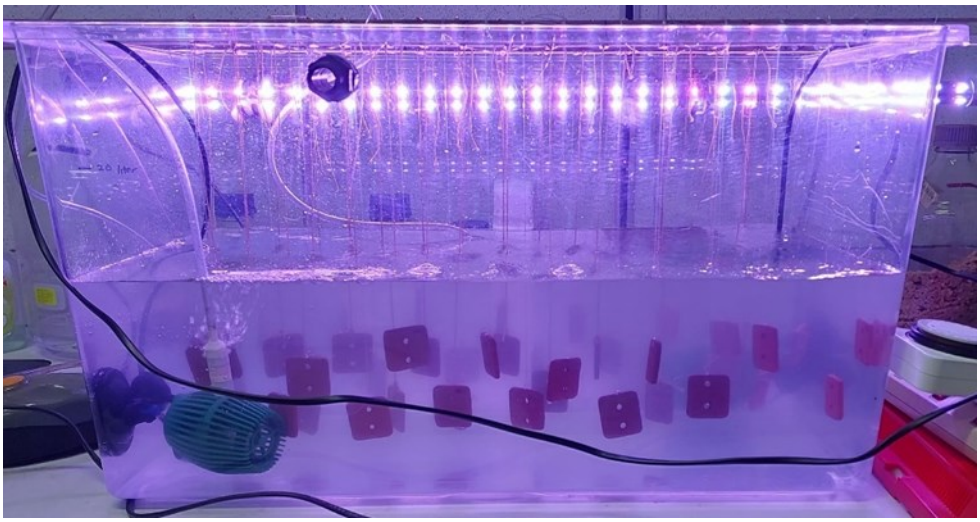

Figure 1. Aquarium test of antifouling coating panels that immersed at artificial seawater.

metals such as copper or organotin compounds being among the most effective agents for mitigating biofouling [4]. However, many of these biocides, despite their efficacy in preventing biofilm formation, are highly toxic and pose significant risks to non-target marine species such as fish, crustaceans, and marine mammals [5]. Tributyltin (TBT), a widely used biocide in antifouling coatings, is a notable example. Its application has led to severe ecological consequences due to its detrimental effects on marine life. As a result, the International Maritime Organization's Marine Environment Protection Committee implemented a global ban on TBT-based antifouling systems, effective January 1, 2008, in response to growing environmental concerns [6]. In light of these challenges, there has been increasing interest in developing more ecologically sustainable alternatives to conventional biocidal antifouling technologies. One promising approach involves the use of natural resources to formulate environmentally friendly antifouling coatings. By leveraging bioactive compounds derived from nature, researchers aim to address biofouling issues while minimizing adverse impacts on marine ecosystems.

Marine organisms is naturally susceptible to biofouling, which can cause a variety of physiological problems, that eventual led to the death of the host [7]. Marine species have evolved several defensive measures, including as the synthesis of antifouling chemicals to thwart such colonization. Seaweed have been thoroughly investigated as a possible source of antifouling agents among marine creatures due to their natural production of bioactive secondary metabolites. Seaweed produce secondary metabolites that act as defenses against organisms like bacteria, fungi, algae, and invertebrates trying to grow on them. Extracts from seaweeds can disrupt bacterial communication systems, specifically *N*-acyl homoserine lactone (AHL)-based quorum sensing, which controls biofilm formation. These compounds contained in seaweeds are effective at preventing the growth and attachment of bacteria such as *Vibrio*, *Pseudomonas*, and *Staphylococcus* The two main types of antifouling compounds identified in marine macroalgae are terpenoids and phenolic compound [8].

Numerous studies have explored the incorporation of crude extracts derived from marine organisms, which contain bioactive compounds with the potential to prevent fouling. Extensive studies has demonstrated that a variety of marine natural products exhibit antifouling [9], antibacterial [10], antibiofilm [11], and antiprotozoan [12] properties. Furthermore, the bioactive compounds derived from seaweeds, soft corals, sponges, and other marine species demonstrate potent antifouling effects, which could mitigate biofouling in marine ecosystems and industrial settings [13][14]. In an effort to address biofouling challenges, we focused on seaweed from Aceh, Indonesia with demonstrated antifouling properties. Brown algae, particularly those belonging to *Sargassum* genus have been

extensively studied for their biological applications. These organisms are known to produce a range of bioactive compounds, including sulphated polysaccharides, meroterpenoids, carotenoids, polyphenols, and phlorotannins [15]-[18]. These compounds exhibit diverse biological activities, such as anti-quorum sensing, anti-larval [19], anti-algal [20], anti-diatom [21], anti-bacterial [22], and antifouling [19][23][24]. Such findings underscore the potential of Sargassum species as a source of natural antifouling agents for mitigating biofouling issues.

Previous investigations into the antifouling potential of Indonesian marine macroalgae remain limited, with only three relevant studies identified to date. Hakim et al. [25] demonstrated promising antifouling activity in crude extracts of *Sargassum echinocarpum* and *S. cinereum* sourced from Gunungkidul, Yogyakarta. Similarly, Fahruddin *et al*. reported the potential of *S. duplicatum* from Barang Lompo Island, Makassar, South Sulawesi, for incorporation into marine coatings [26]. Additionally, Oktaviani et al. investigated *Turbinaria ornata* from Karapyak Beach, Pangandaran, West Java. Despite these efforts, the antifouling properties of seaweed species from the Aceh region of Indonesia remain largely unexplored, particularly those of the brown alga *Sargassum plagiophyllum* [27]. In light of this gap, the present study aims to evaluate the antifouling properties of *S. plagiophyllum* extracts against bacterial biofilm formation and to develop antifouling coatings incorporating these crude extracts as bioactive additives.

2. MATERIALS AND METHODS

2.1. Identification and Preparation

Samples of *Sargassum plagiophyllum* were collected at the coast of Lhok Bubon, Samatiga Subdistrict, West Aceh, Indonesia with coordinate point 4°11′42.55″ N 98°1′34.52″ E. This plant specimen underwent taxonomic identification following established morphological criteria. Diagnostic features of the thallus, including the presence and morphology of vesicles, holdfast, leaf types, branching patterns (primary and secondary), stem structure, and receptacles, were meticulously examined. Taxonomic assignments were cross-referenced with the AlgaeBase.org database and relevant scientific literature to ensure accuracy. Representative voucher specimens were preserved and deposited in the South China Sea Repository and Reference Centre (RRC) at the Institute of Oceanography and Environment (INOS), Universiti Malaysia Terengganu (UMT), with unique voucher numbers assigned by the RRC for traceability with specimen reference number UMTP 3355.

2.2. Chemical and Instruments

Chemicals used in this research were industrial methanol, ethyl acetate (Merck), chloroform (Merck), *n*-hexane (Merck), dimethyl sulfoxide (DMSO, Merck), crystal violet (CV, Sigma Aldrich), *Pseudomonas aeruginosa* (ATCC 27853), *Chromobacterium violaceum*, nutrient agar (Oxoid), and nutrient broth (Merck). All solvents used were of industrial grade, commercial antifouling paint references 1 and 2 provided by local petroleum company (Bandar Baru Bangi, Selangor, Malaysia), commercial thinners containing a mixture of xylene

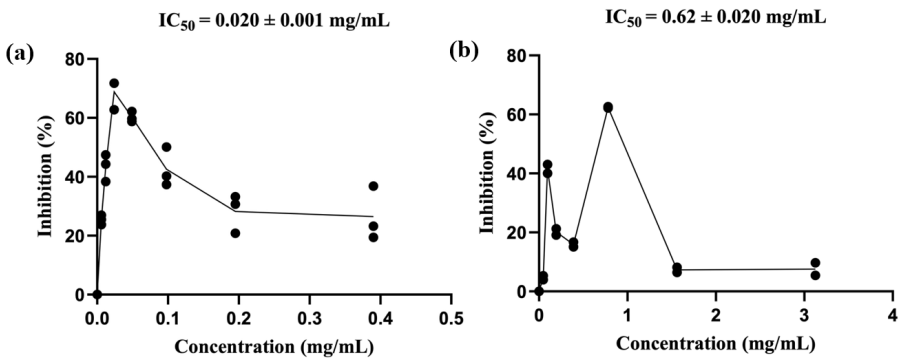

Figure 2. Inhibition of *Pseudomonas aeruginosa* biofilm formation by *Sargassum plagiophyllum* (a) MCE and (b) aqueous fraction after incubation for 24 h.

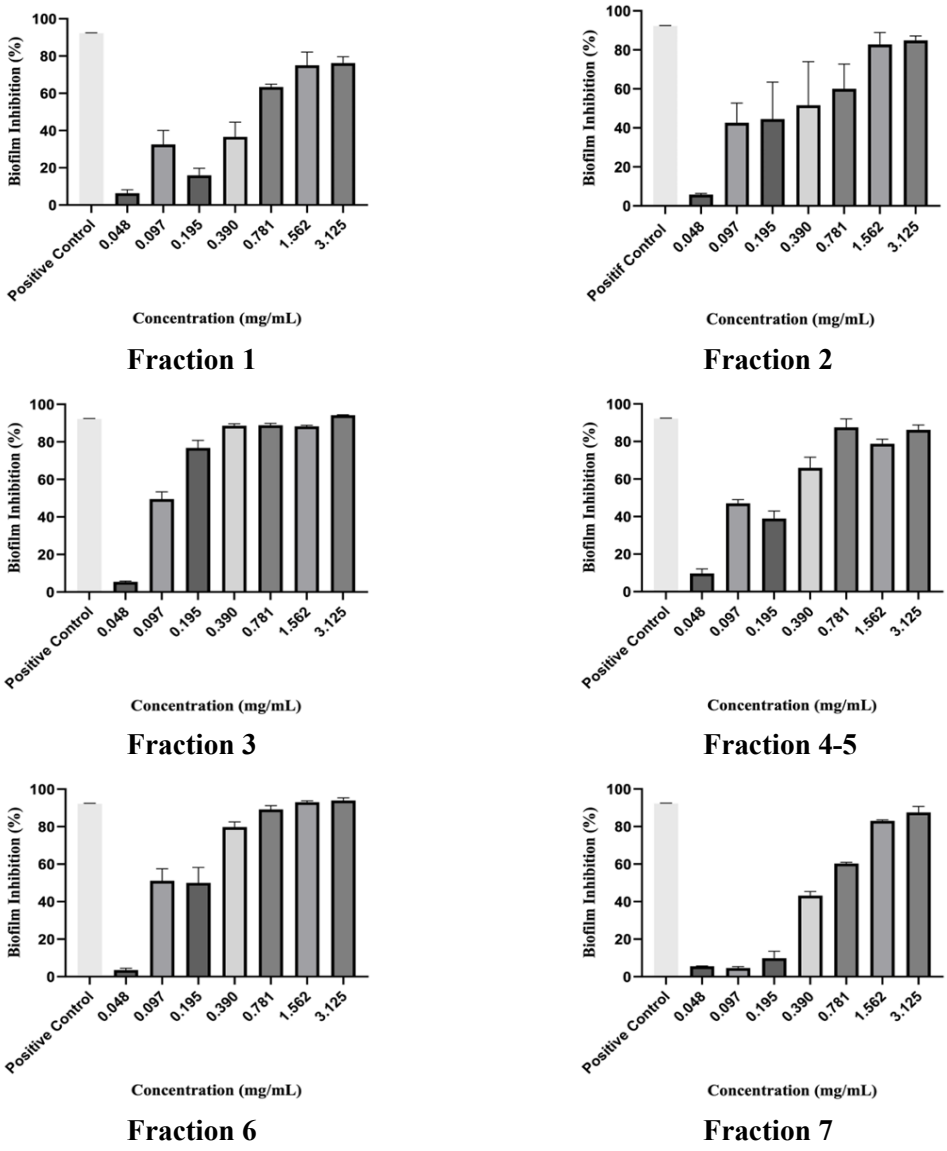

Figure 3. Inhibition of *P. aeruginosa* biofilm formation by *S. plagiophyllum* fractions after incubation for 24 h with sodium hypochlorite as positive control.

and ethylbenzene. Instruments used in this research includes rotary evaporator Buchi R300, column chromatography, preparative thin-layer chromatography, UV-vis spectroscopy (UV-800 Shimadzu), gas chromatograph-mass spectrometer (GC-MS) QP2010 ULTRA (SHIMADZU), and ELISA reader (SpectraMax® iD3 Multi-Mode Microplate Reader).

2.3. Methods

2.3.1. Extraction, Fractionation and Isolation

The powder of *S. plagiophyllum* was successively extracted used maceration method was performed following with modification [28]-[32]. The dried samples of *S. plagiophyllum* were immersed in methanol 99.9% (1:10). The extraction process lasted 24 h at room temperature. Subsequently, the samples were filtered with Whatman filter paper No. 42. The sample filtrate was evaporated with a vacuum rotary evaporator (BUCHI R300, Flawil, Switzerland) set 40 °C under pressure. The methanol crude extract (MCE) underwent sequential liquid-liquid extraction refer to Rawa et al. [33] with minor modification using a series of solvents with increasing polarity: *n*-hexane, chloroform, and ethyl acetate [32]. This partitioning technique involved dissolving the MCE in an aqueous solution and successively extracting it with each solvent in a separating funnel until no

further solute transfer was observed. The remaining aqueous phase after these extractions was designated as the residual aqueous fraction (RAF). Each fraction was evaporated with a vacuum rotary evaporator set 40 °C under pressure. Chromatographic separation techniques exploit differences in physicochemical properties (charge, size, and shape) of compounds within a mixture. These methods rely on the interaction between a stationary phase, such as Sephadex LH-20 or silica gel with a calcium sulfate binder, and a mobile phase consisting of extraction solvents [33][34]. Isolation and characterization of compounds from the extracts of brown algae *S. plagiophylum* fractions were done using size exclusion chromatography (SEC), column chromatography and thin-layer chromatography (TLC) techniques.

2.3.2. GC-MS Analysis of Bioactive Compounds

The chemical composition of fraction 6 of *Sargassum plagiophylum* was elucidated using GC-MS m. Samples were prepared by dissolving them in methanol prior to injection into the GC-MS. The chromatographic separation was performed on a fused silica capillary column (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness). The mass spectrometer was operated in electron ionization (EI) mode with an ionization energy of 70 eV. Helium (99.999%) served as the carrier gas at a constant flow rate of 1 mL/min. The interface and injector temperatures were maintained at 220 and 290 °C, respectively. The oven temperature was programmed with a gradient: initial temperature of 50 °C, ramped to 150 °C at a rate of 3 °C/min, held at 150 °C for 10 min, and subsequently increased to 300 °C at a rate of 10 °C/min. Diluted samples (1:100, v/v in methanol) with an injection volume of 1 µL were introduced into the GC-MS system using a split injection mode with a split ratio of 120:1. The relative abundance of individual chemical constituents within each fraction was determined by normalizing the peak areas from the total ion chromatogram.

2.3.3. Antibiofilm and CV Assay

P. aeruginosa was recovered from -80 °C in the freezer after being obtained from the Institute of Climate Adaptation and Marine Biotechnology at Universiti Malaysia Terengganu. A three-fold dilution was utilized to inject 100 µL of *P. aeruginosa* overnight culture into each well of a 96-well plate that contained 100 µL of nutritional broth and various quantities of seaweed crude extract or fractions, ranging from 3.125 to 0.048 mg/mL. In the last row of the plate, nutrient broth was utilized as the negative control, and 0.025% sodium hypochlorite was employed as the positive control [35]. The CV assay, which was slightly adjusted while the plate was drying. Following incubation, the contents of each well were pipetted out and

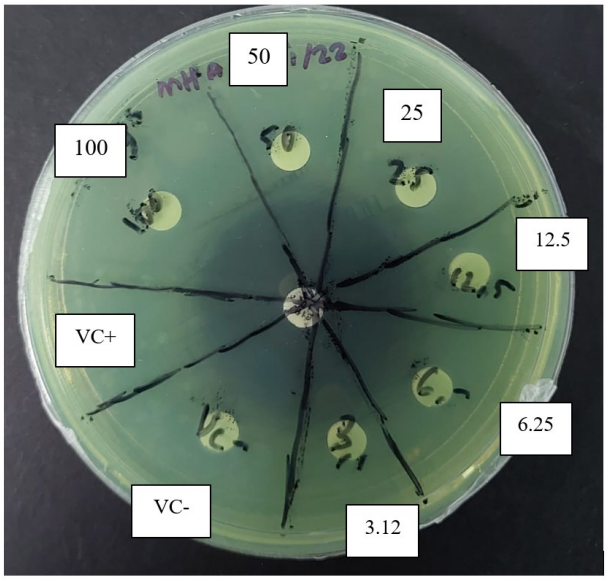

Figure 4. Antibacterial activities using disc diffusion method of *Sargassum plagiophylum* crude extract. VC– negative control; VC+ ciprofloxacin as positive control.

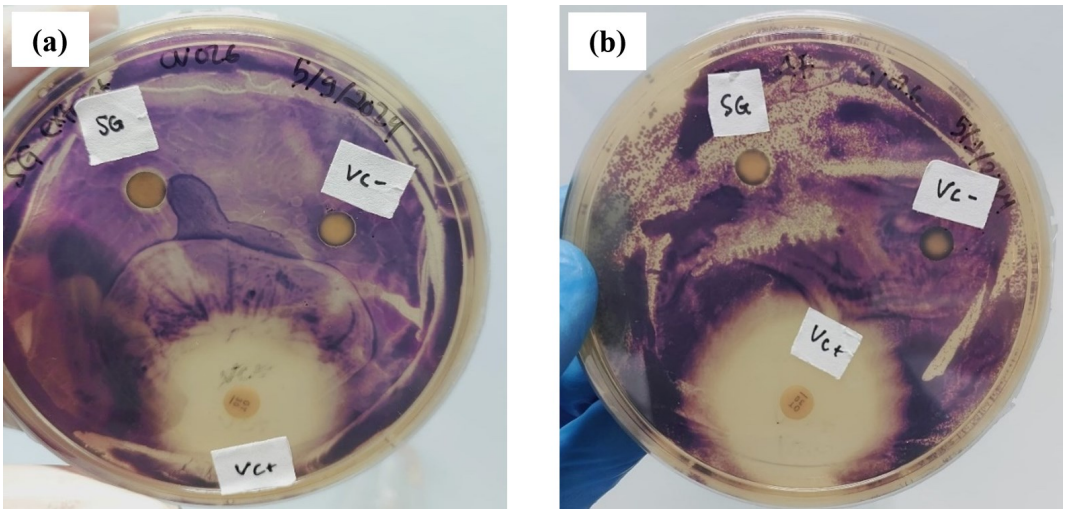

Figure 5. Anti-quorum sensing (anti-QS) activity by *Sargassum plagiophyllum* crude extract (a) and aqueous fraction (b) against *Chromobacterium violaceum* bacteria using agar disc diffusion. SG(a)- *S. plagiophyllum* crude extract; SG(b)- *S. plagiophyllum* aqueous fraction; VC- - blank; Vc+- oxytetracycline as positive control.

dried in a dryer set to 60-70 °C for 30 min. After administering 200 µL of a 0.1% (w/v) CV solution to each well, 5 min were permitted. The microtiter 96-wells were then carefully rinsed with tap water until they were clear, and the CV staining solution was discarded. The 96-well plate underwent a second 30-min drying cycle at 60–70 °C. A microtiter plate reader (SpectraMax® iD3 Multi-Mode Microplate Reader) was used to record the absorbance for CV-stained plates at 595 nm and standardised to 0.2 [36].

2.3.4. Antibacterial Activity

An antibacterial assay is performed on the positive fractions to determine the mechanism that prevents biofilm development. The disc diffusion method (Kirby-Bauer method) was used to investigate the antibacterial activity of crude extracts from selected seaweed. Various dilutions of the extracts (4 mg/disc to 0.008 mg/disc) were impregnated into sterile commercial blank discs with a diameter of 6.0 mm. The discs were stored at -5 °C before being used. Using a turbidometer, broth cultures were adjusted overnight to yield approximately 10^8 CFU/mL. Discs impregnated with extract (20 µL) were placed on agar plates and incubated at 37 °C for a day. The positive control was 30 µL of ciprofloxacin discs, while the negative control was 20 µL of DMSO [37].

2.3.5. Anti-quorum Sensing Activity

To determine whether *S. plagiophyllum* species could prevent bacteria from communicating with each other, we used a simple method called disk diffusion [38]. We grew a type of bacteria called *Chromobacterium violaceum* on a special type of agar in petri dishes. Then, we placed paper disks soaked in *Agave americana* extract or oxytetracycline (a known antibiotic) onto the agar. After a few days, we measured the size of the clear areas around the disks, which showed us how well the extract or antibiotic stopped the bacteria from communicating.

2.3.6. Brine Shrimp Lethality Assay (BSLA)

The BSLA has been widely utilized as a preliminary toxicity screening method for evaluating the effects of various concentrations of crude plant extracts refer to Meyer with minor modification [39]. Juvenile brine shrimp (*Artemia franciscana*) were obtained from a commercial hatchery. Individuals were collected using a Pasteur pipette and transferred into 5-mL vials containing sterile seawater solutions of crude extract at concentrations of 10, 5, 2.5, and 1.25 mg/mL. Each treatment was replicated three times. Ten *A. franciscana* nauplii were exposed to each concentration per replicate. DMSO served as the negative control. After 24 h of exposure, mortality was assessed by counting live and dead individuals;

shrimps were considered dead if no internal or external movement was observed for 30 s. The percentage mortality for each concentration was calculated, and the median lethal concentration (LC_{50}) values were determined by plotting percent mortality against the logarithm of extract concentration, followed by probit regression analysis.

2.3.7. Aquarium Study of Biofilm Formation

All the panels were suspended in an aquarium tank with 141 L of fresh seawater, which was kept at ambient temperature and set to simulate waves as well as artificial light penetration (Figure 1). The seawater was supplemented with 1.41 L of sterile artificial seawater [40] to give the necessary nutrients. After 24 h, the panels were removed from their aquarium tank. The biofilm attached to the panels (control and antifouling paint) was scraped off with a cotton swab and suspended in 1 mL of sterile synthetic seawater. This suspension was serially diluted and then disseminated over Zobell marine agar (ZMA) plates. Bacterial colonies were grown on agar plates in an incubator set to 37 °C. Twenty-four hours later, the developing colonies were measured using a microbial colony counter [41]. All panels were made in triplicate.

2.3.8. Preparation of Panels with Seaweed Extracts

The painted steel panels used in this project were from a local oil and gas firm. Bandar Baru Bangi, Selangor, Malaysia. The panels were chopped into squares of 2.5 cm by 0.3 cm. The paint on the panels was removed using hand tools and a sanding machine in line with the SSPC-SP2 standard. Before beginning the coating process, the steel panels were polished with rough-grade abrasive paper mounted to an orbital sander to eliminate rust. The panels were then cleaned with methanol according to the SSPC-SP1 standard to eliminate any visible oil, grease, dust, or other soluble pollutants [42][43]. This study's negative and positive controls were blank paint with no antifouling agent and commercial antifouling paint, known as reference paint 1 (RF1) and reference 2 (RF2), obtained from industry (supplied by a local oil and gas business). Blank paint is a paint mixture containing the following chemicals: colophony, xylene, zinc oxide, ethylbenzene, hydrocarbons (C9, aromatic), 1-methoxy-2-propanol, and fatty acids. The composition of RF1 and RF2 was largely comparable to that of the blank paint, with the exception of the absence of fatty acids and certain other additives. Additionally, the reference paints contained the active antifouling agents copper(I) oxide and zineb.

2.3.9. Marine Field Antifouling Study

The coated steel Panels were immersed vertically in saltwater at Kuala Kemaman (depth 24 m) and Redang Island (depth 14 m), Malaysia for three months and secured with buoys and anchors. The panels were placed two metres below the ocean's surface and immersed for three months at each location. They were secured to a stainless-steel frame. The panels were recovered through monthly

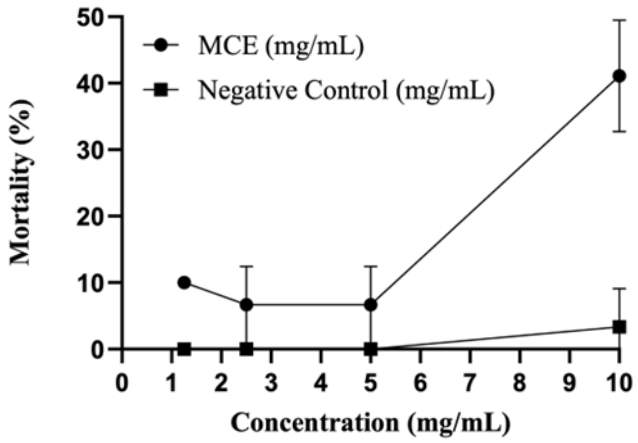

Figure 6. Cytotoxicity activity of MCE of *S. plagiophyllum* against *artemia franciscana* with DMSO as negative control.

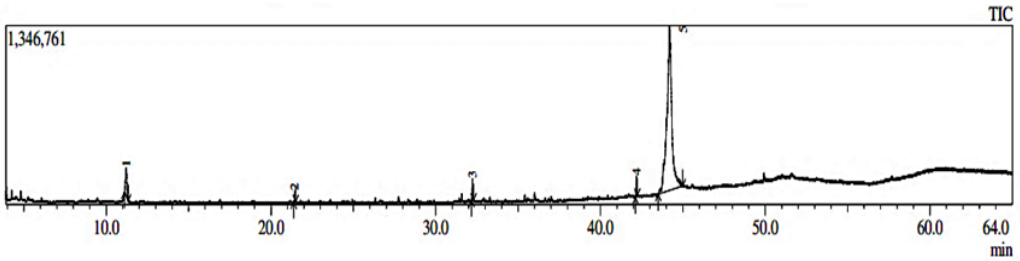

Figure 7. Chromatogram analysis GC of *Sargassum plagiophyllum* fraction 6.

diving and then returned to the lab for further evaluation. The physical properties of adjacent seawater were measured with a portable multimeter. Each month, pictures were collected to measure the antifouling performance of polymer coats [44][45].

2.3.10. Statistical Analysis

The proportion of biofilm inhibition (%) was calculated using Nadri *et al*. formula [46]. A plot was constructed by plotting the percentage of biofilm inhibition versus the crude extract concentration. By means of linear interpolation, the crude extracts' IC_{50} was discovered. The standard deviation of the mean (SEM) was used to display the data. GraphPad Prism (GraphPad Software, San Diego, CA, USA) version 10.2.3 was used to statistically analyse the information. The groups were compared using a one-way ANOVA and Tukey's post hoc test. Statistical significance was considered as a *p*-value less than 0.05.

3. RESULTS AND DISCUSSIONS

3.1. Antibiofilm Results

The research findings indicate that the MCE *S. plagiophylum* is the most effective among the ten seaweeds in inhibiting biofilm formation by *P. aeruginosa* bacteria. Its MCE demonstrated strong antibiofilm activity, with an IC_{50} value of 0.020±0.001 mg/mL. This means that even at very low concentrations, *S. plagiophylum* can effectively prevent bacteria from forming biofilms (Figure 2 (a)). This suggests that *S. plagiophylum* has the potential to be a valuable natural antifouling agent. Based on liquid-liquid extraction which aqueous fraction of *S. plagiophyllum* depicted antibiofilm activity activity against *P. aeruginosa* bacteria, with an IC_{50} value of 0.62 ± 0.020 mg/mL shows excellent antibiofilm according to the experimental results (Figure 2(b)). It has been proposed that the antibiofilm action of aqueous fraction of *S. plagiophyllum* may be impacted by the polarity-based chemical separation.

Based on the experimental research exhibited the higher antibiofilm efficacy of the MCE of *S. plagiophyllum* at lower concentrations (0.006–0.390 mg/mL), compared to the aqueous fraction of *S. plagiophyllum* (0.048–3.125 mg/mL), can be attributed to the differences in solvent polarity and the selective extraction of distinct bioactive compounds, which vary in their potency and mode of action. Methanol is a polar organic solvent known for its high efficiency in extracting a diverse array of secondary metabolites from plant and algal tissues. These include bioactive compounds such as phlorotannins [47], flavonoids [48], terpenoids [49], and fatty acids that many of which have been associated with antimicrobial and antibiofilm activities. Due to its polarity, methanol effectively dissolves these compounds, making it a preferred solvent for isolating natural products with potential antifouling applications. Nevertheless, aqueous being a highly polar solvent, is capable of extracting certain bioactive compounds; however, it is generally less effective in dissolving lipophilic or moderately polar molecules that are often associated with potent antimicrobial and antibiofilm activity. Aqueous fraction typically contain polysaccharides, proteins, amino acids, and highly polar phenolic compounds or glycosides. While these constituents may exhibit some biological activity, they are generally less potent compared to the secondary metabolites extracted using semi-polar solvents like methanol [47].

In addition, the MCE typically contains a higher concentration of bioactive compounds per unit mass compared to the aqueous fraction. This higher concentration of active molecules in the MCE explains its effectiveness at much lower concentrations (0.390–0.006 mg/mL), as a smaller

amount of the extract is sufficient to deliver a biologically effective dose. In contrast, the aqueous fraction requires significantly higher concentrations (3.125–0.048 mg/mL) to achieve comparable antibiofilm activity, reflecting a lower relative abundance of the key active constituents within that fraction.

The specific composition of these compounds can vary significantly depending on the macroalgal species, its growth environment, and the extraction method employed. While research specifically focusing on *P. aeruginosa* and its aqueous extract may be limited, the antibacterial and antibiofilm properties of marine macroalgae in general have been extensively studied. Alreshidi et al. documented a substantial anti-biofilm effect of a methanol extract of *Sargassum* sp. against *Staphylococcus epidermidis* [50]. Specifically, they observed an 82.35% reduction in biofilm formation when the extract was applied at a concentration of 12.5 mg/mL. Through detailed chemical characterization, the researchers identified several prominent compounds within the extract, including 6,10,14-trimethyl-2-pentadecanone, methyl hexadecenoate, hexadecanoic acid, and mono(2-ethylhexyl) phthalate. Aqueous fraction of *S. plagiophyllum* were subjected to SEC, yielding 6 fractions including fractions 1, 2, 3, 4–5, 6, and 7. These fractions were evaluated for their inhibitory potential against *P. aeruginosa*, a bacterium implicated in marine biofilm formation.

Subsequently experimental using SEC with Sephadex LH20, the researcher in this study chose the aqueous fraction for subsequent testing, yielding six fractions, the results showed that 1, 2, 3, 4–5, 6 and 7 of *S. plagiophyllum* fractions has antibiofilm activity against *P. aeruginosa* bacteria with an inhibition percentage of 76.18%, 84.90%, 94.25%, 87.55%, 93.93%, and 87.56% for 3.125, 3.125, 3.125, 0.781, 3.125, and 3.125 mg/mL, respectively (Figure 3). Based on the statistical analysis, the results indicate that fraction 6 from the SEC experiment exhibited one of the highest antibiofilm activity against *P. aeruginosa* bacteria. on the other hand, we focused on small size molecules that the last drop out in glass column chromatography namely fraction 6 and this fraction have high yield for analytical advance.

In vitro assays assessing antibiofilm activity demonstrated that extracts derived from the brown alga *S. plagiophyllum* exhibited significant inhibition of biofilm formation across tested microbial species. Notably, effective inhibition was observed at exceptionally low concentrations, suggesting potent antibiofilm properties. Based on the experimental results, it is indicated that among these four fractions, the aqueous fraction of *S. plagiophylum* exhibits strong antibiofilm activity against *P. aeruginosa*. It is suggested that the separation of compounds based on polarity may

Table 1. The result of compound identification of *S. plagiophyllum* fraction 6 from GC-MS.

Peak	RT	%Area	Component Name	Molecule Formula	MW	SI
1	11.213	4.60	Undecane	$C_{11}H_{24}$	156	95
			Tridecane	$C_{13}H_{28}$	184	92
			Dodecane	$C_{12}H_{26}$	204	92
2	21.445	0.74	1-Chloroundecane	$C_{11}H_{23}Cl$	190	91
			Dodecane	$C_{12}H_{26}$	204	91
			n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	91
3	32.229	1.53	Dihexadecanoate	$C_{38}H_{68}O_8$	652	89
			Pentadecanoic acid	$C_{15}H_{30}O_2$	242	88
4	42.181	1.52	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390	92
			3,3'-Thiobis-propanoic acid	$C_{30}H_{58}O_4S$	514	82
5	44.191	91.61	ditetradecyl ester	$C_{32}H_{62}O_4$	570	77
			Diethylmalonic acid	$C_{23}H_{44}O_4$	384	64

Note: RT: Retention Time; MW: Molecule weight; SI: Similarity index

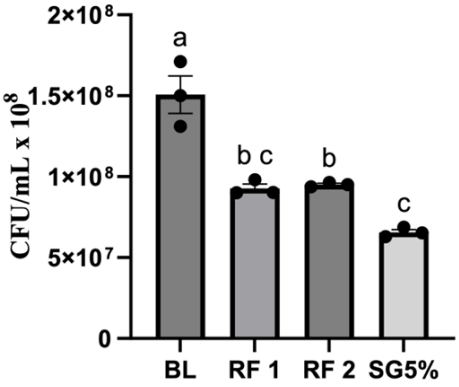

Figure 8. Bacterial count for the tested panels in an aquarium after 24 h of incubation whereas BL: blank, RF 1: reference 1, RF 2: reference 2, and SG 5%: *S. plagiophyllum* 5%.

influence the antibiofilm activity of the brown algae *S. plagiophylum.* In this study, the aqueous fraction was selected for subsequent separation using SEC, which resulted in 7 fractions. The research findings indicate that fraction 1 of *S. plagiophylum* exhibits antibiofilm activity against *P. aeruginosa* bacteria, with an IC_{50} value of 0.51 mg/mL. Among them, fraction 6 of *S. plagiophyllum* exhibited a unique TLC pattern, suggesting a distinct chemical composition compared to other fractions obtained from the extract that possesses a remarkable ability to inhibit biofilm formation by *P. aeruginosa*. This indicates that it is a highly effective antifouling agent, even at low concentrations. Fraction 6 was selected for subsequent analysis due to its potent antibiofilm activity against *P. aeruginosa*, indicating the presence of highly effective inhibitory compounds. Furthermore, chromatographic data, specifically retention times from SEC, suggested distinct chemical characteristics for fractions 6, potentially reflecting novel bioactive compounds or mechanisms. Targeted investigation of fraction 6 aims to elucidate the specific molecular entities responsible for its antibiofilm efficacy. This knowledge is crucial for identifying target interactions and exploring the potential of these compounds as antifouling agents. Further purification of fraction 6 via column chromatography, followed by structural elucidation using mass spectrometry and nuclear magnetic resonance spectroscopy, will enable the identification and characterization of individual active compounds. These structural insights can then facilitate the synthesis of these compounds or the development of structural analogs with enhanced properties. Overall, SEC is a valuable tool for studying complex mixtures like plant extracts. By separating components based on their size and molecular weight, SEC enables researchers to identify and isolate compounds of interest and investigate their biological activities. In the case of *S. plagiophyllum* extract, SEC was used to investigate the potential antifouling properties of specific components within the extract, providing valuable insights into the mechanisms underlying its antifouling activity.

3.2. Antibacterial Activity of S. plagiophyllum

The MCE of *S. plagiophyllum* did not exhibit any antibacterial effect against *P. aeruginosa*, according to the disc-diffusion result (Figure 4). However, the extract exhibited significant inhibitory effects on biofilm formation by *P. aeruginosa*. This antibiofilm activity suggests a potential quorum quenching mechanism, whereby the extract interferes with bacterial cell-cell communication. Furthermore, the extract was effective in disrupting pre-formed biofilms and preventing the development of new biofilms, indicating its potential for combating biofilm-associated infections. These findings align with a previous study by Schwartz *et al*., which reported low antibacterial activity of a *Sargassum* sp. crude extract from Omani waters against *Escherichia coli* and *Pseudomonas putida* [19].

Our study revealed that the MCE of *S. plagiophyllum* sourced from West Aceh waters, Indonesia, did not exhibit antibacterial activity against *P. aeruginosa*, a finding that contrasts with previous reports on other Sargassum species from

different geographical locations. For instance, Schwartz *et al*. demonstrated effective antibacterial activity of *S. muticum* extract from Oshima, Japan, against *E. coli* and *P. putida* [19]. Furthermore, Plouguerné et al. investigated the antibacterial activity of polar and nonpolar extracts of *S. vulgare* against five marine biofilm-forming bacteria, revealing varying levels of inhibition across different extract fractions [51]. Notably, the methanol fraction of *S. vulgare* exhibited the most potent antibacterial effect, with a minimum inhibitory concentration of 0.2 µg/mL against *Vibrio aestuarianus* and *Pseudoalteromonas elyakovii*. These contrasting results underscore the potential for species-specific and location-dependent variations in the antibacterial properties of *Sargassum* extracts.

3.3. Anti-quorum Sensing Activity of S. plagiophyllum

The MCE and aqueous fraction of *S. plagiophyllum* exhibited limited quorum quenching activity, as evidenced by a 0.2 mm inhibition zone in the assay (Figure 5). These findings indicate that both the crude extract and the aqueous fraction possess a mechanism of action consistent with quorum quenching.

3.4. Cytotoxicity Activity of Methanolic Crude Extract S. plagiophylum

The mortality of *Artemia franciscana* following 24-h exposure to varying concentrations of MCE, with DMSO serving as the negative control. A concentration-dependent increase in mortality was observed. However, the results indicate that MCE did not induce 50% mortality in *A. franciscana* even at the highest tested concentration. growth observed (Figure 6). The *A. salina* BSLA is widely used as a rapid and reliable method for assessing the cytotoxic effects of bioactive compounds, providing a preliminary evaluation of toxicity among different plant-derived extracts [52].

Exposure of *A. franciscana* to the MCE of *S. plagiophyllum* at concentrations ranging from 1.25 to 10 mg/mL did not result in significant mortality, indicating low or no toxicity. No clear dose-dependent effect was observed within the tested range. This study corroborated by Premarathna *et al*. have reported that the crude extracts of the green seaweeds *Caulerpa racemosa* and Caulerpa *sertularioides* as well as the brown seaweed *Padina antillarum* did not induce mortality in *A. salina* across the tested concentration range [53]. These findings suggest low or no cytotoxicity under the experimental conditions. Nevertheless, the BSLA proved to be a reliable and effective tool for evaluating the toxicological profile of seaweed-derived extracts, supporting its use in future studies on marine natural products. Therefore, this MCE can be considered as promising candidates for the development of antifouling agents derived from marine biota, particularly due to their bioactive potential and low toxicity to non-target organisms. Although no significant lethality was observed within the tested concentrations, the overall trend in the data suggests that with higher doses, potential cytotoxic effects might become apparent. This indicates the importance of further investigations using extended concentration ranges or alternative extraction methods to isolate and identify bioactive compounds. The results also highlight the need to explore other biological activities of these seaweed species, such as anti-quorum sensing or antibiofilm properties, which may be relevant for eco-friendly antifouling applications.

3.5. GC-MS Analysis of Sargassum plagiophyllum

The GC-MS analysis of the fraction 6 of *S. plagiophylum* from SEC technique demonstrating the most potent antibiofilm activity led to the identification of several compounds. The chromatogram of the *S. plagiophyllum* fraction 6 is presented in Figure 7. The highest activity exhibited by fraction 6 of SEC technique prompted us to do the further chemical investigation by GC-MS analysis. A high-resolution mass spectrometer equipped with a data system in combination with GC was used for the chemical analysis of fractions of the plant. The spectra of the unknown constituents were compared with known constituents deposited in the NIST library. Chemical constituents of fraction 6 of *S. plagiophyllum* species analyzed by GC-MS were found to contain a mixture of fatty acids with volatile compounds. The identified compounds constitute 100 % of the total fractions. Five peaks were observed in the case of *S. plagiophyllum* fraction 6 from size exclusion chromatography

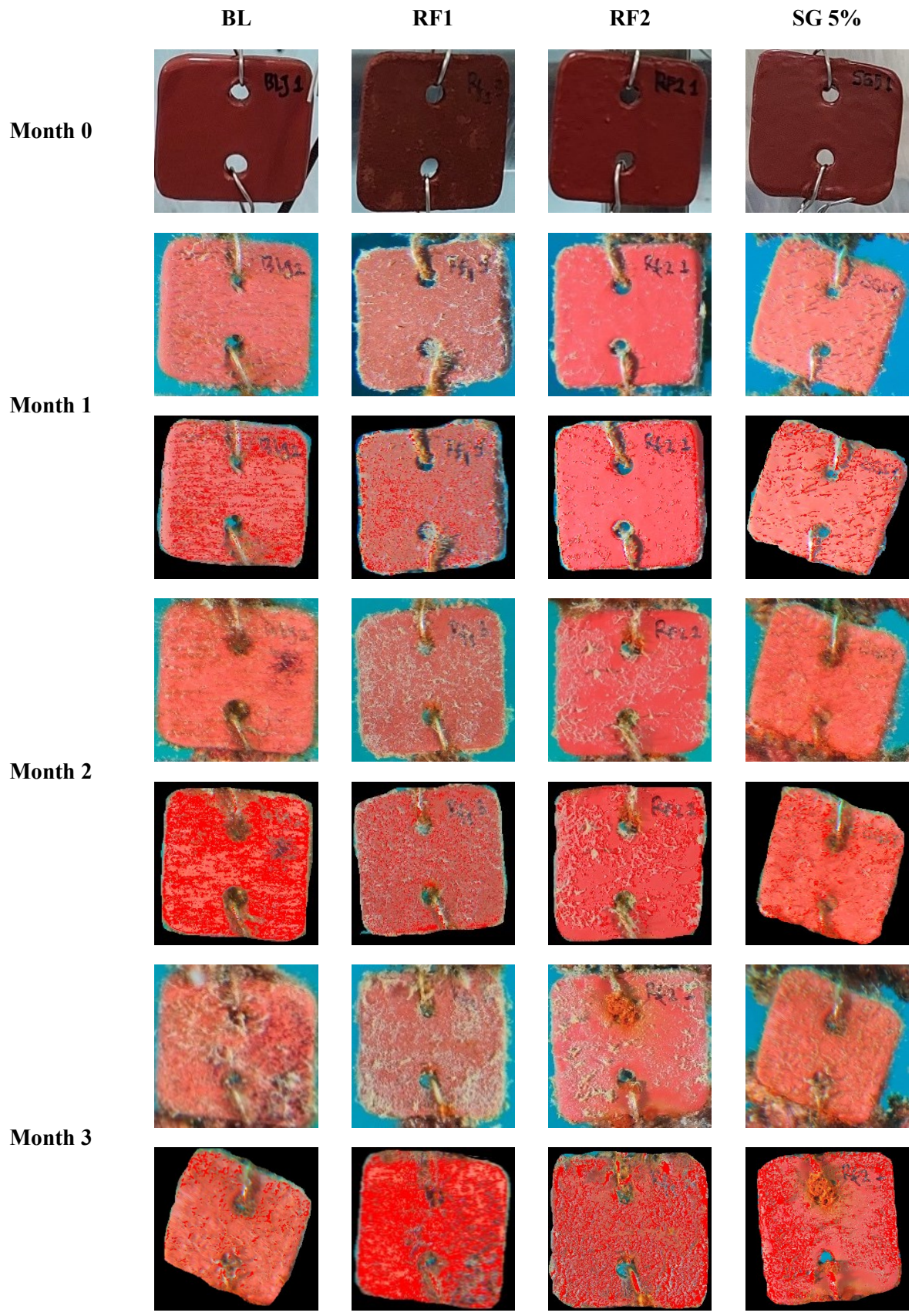

Figure 9. Antifouling Panels after immersed in Redang Island, Malaysia with 3 months period whereas BL: blank, RF 1: reference 1; RF 2: reference 2; SG5%: *S. plagiophyllum* 5%. Yellow circle is barnacles' attachment.

stage. The retention time, molecular weight and the bioactivity of the compounds corresponding to the five peaks are presented in Table 1. Major compounds identified were different types of alkanes (4.60 %), alkyl halides (0.74 %), fatty acids (1.53 %), phthalates (1.52 %), and thioesters (91.61 %). Among the 5 peaks, a few compounds including *n*-hexadecanoic acid, pentadecanoic acid, 3,3'-thiobis-propanoic acid, and ditetradecyl ester were the prominent antibiofilm compounds. Previously, the *S. plagiophyllum* fraction 6 have already been evaluated in antibiofilm activities.

The antibiofilm activity of *S. plagiophyllum* fraction 6 was found to be higher than another fraction studied. Therefore, chemical composition of *S. plagiophyllum* fraction was studied further in detail by GC-MS analysis. However, GC-MS analysis of *S. plagiophyllum* fraction showed the presence of five peak with compound such as undecane, tridecane, dodecane, 1-chloroundecane, *n* -hexadecanoic acid, dihexadecanoate, pentadecanoic acid, diisooctyl phthalate, 3,3'-thiobis-propanoid acid, and ditetradecyl ester. All compounds have similarity index > 70. GC-MS analysis of a fraction isolated from *S. plagiophyllum* fraction 6 revealed a predominance of fatty acids, phthalates, and thioesters. These classes of compounds have previously been implicated in possessing antifouling properties, deterring the settlement and growth of organisms on surfaces. Supporting evidence comes from studies by Rosell and Srivastava who demonstrated the antibacterial activity of unsaturated fatty acids from the brown algae *Desmarestia ligulata* [54]. Additionally, Ganti et al. isolated fats and phthalic acid derivatives from *S. confusum* and demonstrated their antifouling activity [55]. Similarly, Plouguerné et al. identified saturated and unsaturated linear hydrocarbons (C12–C27) in anti-microfouling fractions from *S. muticum* chloroform extracts [51]. Interestingly, they also detected several major metabolites like arachidonic acid, palmitic, linolenic, palmitoleic acids, and galactoglycerolipids that were absent in the S. *plagiophyllum* fraction analyzed in this study. Another study, Padma et al. have highlighted that Dodecane, a member of the alkane hydrocarbon class, was identified in the crude extract as a promising antifouling compound [56]. This compound has demonstrated the antibacterial efficacy of dodecane when extracted from the thistle plant *Silybum marianum* using ethanol.

S. plagiophylum contained hexadecanoid acid that play important role in antifouling properties. This study supported by Sukrri et al. that reported green algae *U. lactuca* isolated hexadecanoic acid,

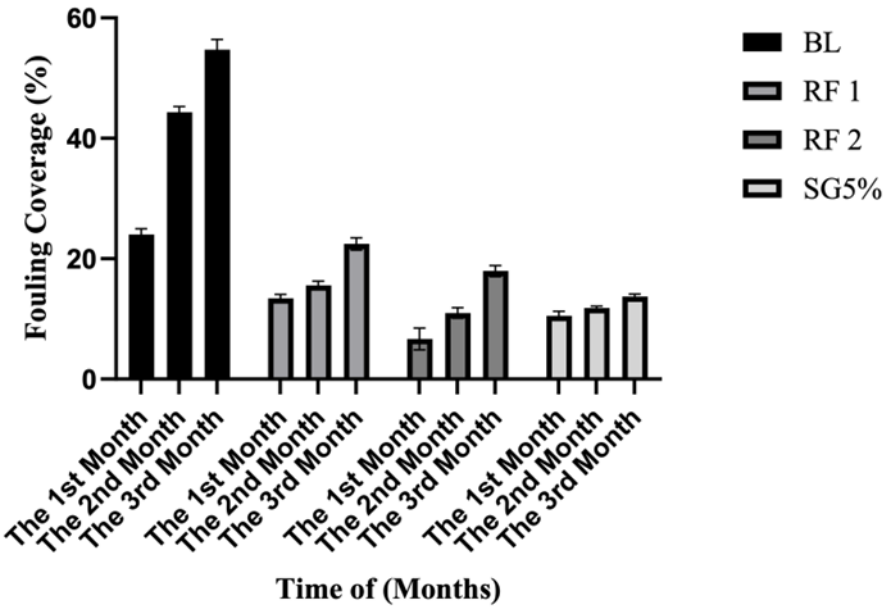

Figure 10. Percentages of the area covered by marine fouling after immersion in the Redang island, Malaysia whereas BL: blank, RF1: reference 1, RF2: reference 2, SG 5%: *S. plagiophyllum* at 5% concentration.

an antifouling compound that offer promising prospects for the development of novel, environmentally friendly antifouling paints [57]. These findings also were corroborated by research conducted by Gao et al., which demonstrated the potent antifouling properties of hexadecanoic acid produced by two bacterial strains: *Kytococcus sedentarius* and *Bacillus cereus* [58]. Additionally, a study by Bakar et al. highlighted the broad-spectrum antifouling activity of hexadecanoic acid, attributed to its ability to inhibit biofilm formation by various marine bacteria, including *Vibrio alginolyticus*, *Vibrio mimicus*, *Vibrio parahaemolyticus, P. aeruginosa,* and *Bacillus subtilis* [59]. In GC-MS analysis peak depicted that ditetradecyl ester compound is the most dominant compound in *S. plagiophyllum* fraction with %area of 91.61%. These compounds belong to the group of thioester compounds. Thioesters are organic compounds containing a C=S bond. They are similar to esters, but with the oxygen atom replaced by a sulfur atom. This compound group have antibiofilm activity in this study. Manilal et al. have reported that ditetradecyl ester compound possesses anti-inflammatory action in *Moringa stenopetala* extract but the finding of *S. plagiophyllum* compound has not yet been reported [60][61].

3.6. Biofilm Formation Test in the Aquarium

The current study used an aquarium setup to assess the biofilm formation on the coated panels. By counting the number of bacteria in the adhered biofilms, the biofilm attachment was ascertained. When compared to blank paint (negative control), it was discovered that antifouling paint containing an antifouling ingredient derived from crude extracts decreased the settlement of marine bacteria. On the agar plate spread with the bacterial suspension from the panels coated with *S. plagiophyllum* (SG 5%), the number of bacterial cells attached to the panels coated with blank paint (which served as the negative control) was found to be 1.50×10^{8} CFU/mL. In contrast, it was significantly reduced to 0.65×10^{8} CFU/mL on the panels coated with antifouling paint (SG 5%) compared to commercial antifouling paint includes RF1 and RF2 these counts were determined to be 0.92×10^{8} and 0.95×10^{8} CFU/mL, respectively (Figure 8). These results demonstrated comparable antibiofilm activity to those observed in the 96-well microtiter plate assay, effectively inhibiting biofilm formation even at low concentrations. Rawi et al. assert that incorporating low concentrations of samples into paint formulations can significantly inhibit biofilm formation by bacteria [62].

This study corroborated by Sukrri et al. study that reported that panels with incorporated paint of green algae *Ulva lactuca* methanolic crude extract successfully reduced the adherence of marine bacteria possibly as good as the commercial antifouling paint, RF1 and RF2 [57]. Additionally, Farina et al. have reported that paint formulations incorporating a 5% concentration of methanolic *Melaleuca cajuputi* crude extract exhibited superior biofilm inhibition compared to those containing 5% or 10% of the essential oil [63]. Similarly, Ramzi *et al*. also found that adding *Diadema setosum* and *Sonneratia lanceolata* crude extract to an antifouling coating greatly decreased the adhesion of biofilm bacteria in an aquarium experiment [43]. In a similar vein, the results of the present investigation demonstrated that antifouling paint combined with *S. plagiophyllum* crude extracts can prevent bacteria from settling and sticking to surfaces under laboratory settings.

3.7. In Situ Test

Two distinct concentrations of each *S. plagiophyllum* crude extract at concentration of 5% (w/v), were utilized. At Kuala Kemaman and Redang island, Malaysia the two immersion sites, all the coated panels were securely fastened to the stainless-steel frame with iron wire and submerged in seawater at a depth of approximately 24 m. The tested panels for Kuala Kemaman and Redang island, Malaysia were collected and photographed at 3-month periods. Figure 9 shows the growth of marine organisms (macrofoulers) on steel panels in the Redang Island, Malaysia. After one month immersion, there are slightly significant changes in the steel panels compared to before immersion. Biofilm formation, consisting of bacteria and plankton, was observed on all panels, with a more pronounced presence on those coated with blank paint. Following two months of immersion, barnacle settlement was evident on the blank panels, excluding the positive control and panels coated with a 5% concentration of *S. plagiophyllum*

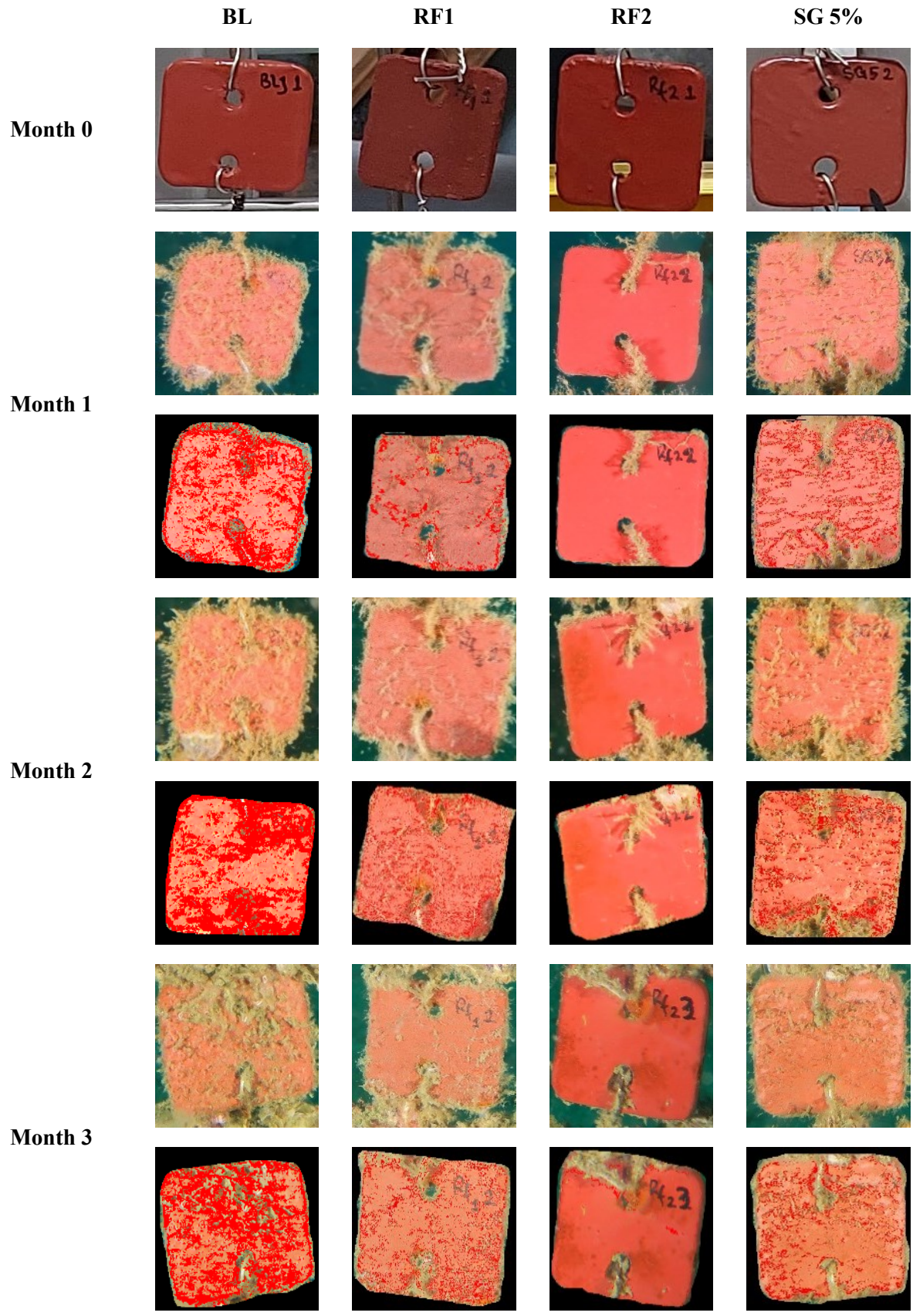

Figure 11. Antifouling panels after immersed in the Kuala Kemaman, Malaysia with 3 months period whereas BL: blank, RF 1: reference 1; RF 2: reference 2, SG5%: *S. plagiophyllum* 5%. Yellow circle is barnacles' attachment.

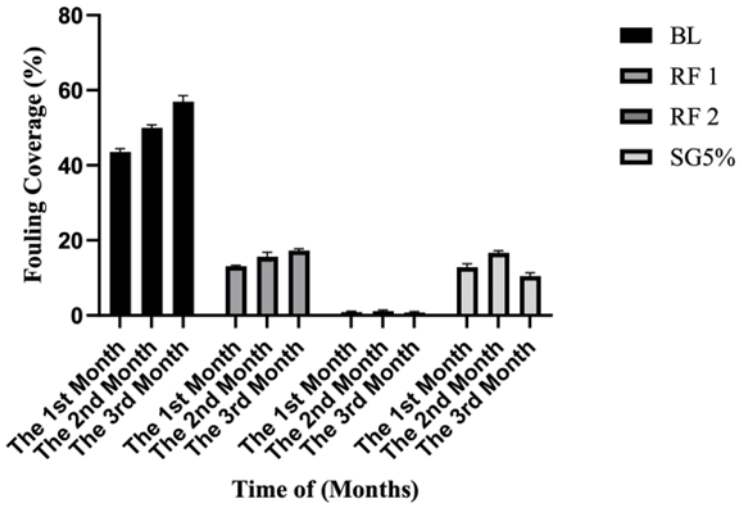

Figure 12. Percentages of the area covered by marine fouling after immersion in the Kuala Kemaman, Malaysia whereas BL: blank, RF1: reference 1, RF2: reference 2, and SG 5%: *S. plagiophyllum 5%.*

antifouling paint. Interestingly, the antifouling coating panel of *S. plagiophyllum* at 5% concentration has effectively natural antifouling paint due to the fouling coverage value of 13.71% in the 3rd month compared to commercial antifouling paint reference 1 and reference 2 have fouling coverage value of 22.46% and 17.96%, respectively (Figure 10). These results indicate that the antifouling paint with *S. plagioplyllum* at 5% concentration addition is the best environmentally friendly antifouling paint that beneficial for preventing marine biofouling.

These panels were subsequently secured to a stainless-steel frame and submerged in seawater at Kuala Kemaman, Malaysia (Figure 11). After a three-month immersion period, the panels were retrieved and photographed. A comparative analysis of the blank panels and those coated with antifouling paint revealed a significant reduction in macroalgae growth and barnacle attachment on the panels treated with the SG 5% antifouling paint. This observation suggests that the antifouling properties of the SG 5% coating effectively inhibited the settlement and growth of marine organisms, thereby mitigating biofouling. The reduced presence of macroalgae attachment on the SG 5% panels is likely due to the bioactive compounds present in the *S. plagiophyllum* extract, which may deterrent effects on marine fouling organisms. None of the antifouling paints containing crude extracts exhibited any barnacle attachment. The performance of these formulated paints was comparable to that of the commercially available antifouling paint used as a positive control, indicating their effectiveness in preventing barnacle settlement. These findings highlight the potential of natural-based antifouling coatings derived from *S. plagiophyllum* as a sustainable alternative to traditional synthetic antifoulants, which often have environmental concerns associated with their use.

Quantitatively, there are percentages of the area covered by marine fouling after immersion at Kuala Kemaman, Malaysia calculated by ImageJ software that depicted SG 5% panel have slightly reduced fouling coverage value of 10.49% in the 3rd month compared to commercial antifouling RF1 with fouling coverage of 17.30% (Figure 12). This result shown that fouling organism like macroalgae has recovered in the 3rd month immersion. Ramzi et al. have observed that there are less macroalgae fouling in crude extracts of *Diadema setosum* and *Sonneratia lanceolata* of 5% and 10% panels [43]. Farina et al. also reported that the number of microorganisms on methanolic crude extract and essential oil of *Melaleuca cajuputi*-painted steel panels was significantly reduced [63]. Soliman et al. previously created natural antifouling paint by mixing soft coral crude extracts into the base paint [64]. Hamdona et al. [66] also reported that crude extracts of marine algae demonstrated the strongest antifouling efficacy against both micro- and macro-fouling when immersed in seawater for 4.5 month [65]. These findings imply that the antifouling paint

-coated panels containing crude extracts from *S. plagiopylum* were successful in preventing biofouling formation in the maritime environment for a brief amount of time.

4. CONCLUSIONS

In this study, we concluded that *S. plagiophyllum* MCE and its aqueous fraction exhibited significant antibiofilm and antibacterial activities. *In vitro* and *in vivo* testing confirmed the ability of *S. plagiophyllum*-based coatings to reduce bacterial biofilm formation and macrofouling colonization. The GC-MS analysis identified several bioactive compounds within fraction 6 of *S. plagiophyllum* that likely contribute to these antifouling properties. These findings suggest that *S. plagiophyllum* could serve as a promising, environmentally sustainable alternative to traditional synthetic antifouling paints.

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Conflicts of Interest

The authors declare no conflict of interest.

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