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ANTIFOULING PROPERTIES OF GREEN SEAWEED *Halimeda opuntia* FROM THE COAST OF ACEH, INDONESIA

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Submitted: 14 April 2025/Accepted: 5 June 2025
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How to cite (APA Style 7th): Gazali, M., Ismail, N., Saidin, J., Bakar, K., & Siong, J. Y. F. (2025). Antifouling properties of green seaweed *Halimeda opuntia* from the Coast of Aceh, Indonesia. *Jurnal Pengolahan Hasil Perikanan Indonesia,* 28(6), 510-529. http://dx.doi.org/10.17844/jphpi.v28i6.63699

Abstract

Marine biofouling remains a critical challenge in the maritime sector, prompting researchers to explore sustainable, eco-friendly, and antifouling solutions derived from marine organisms. This study aimed to determine the optimal concentration of *H. opuntia* extract for effective antifouling activity. The research methods included antibiofilm, cytotoxicity, antibacterial, anti-quorum sensing assays, and in situ tests. The results revealed that the methanol extracts of *H. opuntia* exhibited significantly higher antibiofilm activity, with an IC_{50} value of 0.020 mg/mL. Cytotoxicity assays demonstrated the lowest toxicity against the L6 cell line, with an IC_{50} value of 70.79 µg/mL. Mechanistically, the *H. opuntia* methanol extract did not exhibit a bactericidal effect against *Pseudomonas aeruginosa* but blocked bacterial communication mechanisms through quorum quenching activity, as evidenced by the formation of colorless opaque zones in reporter assays. In situ trials were conducted in the waters of Redang Island and Kuala Kemaman, Malaysia. Panels coated with *H. opuntia* 5% extract demonstrated superior antifouling performance over three months, with fouling coverage rates of 11.19% and 9.10%, respectively. Further research is needed on the antifouling properties of *H. opuntia* to identify its active compounds, evaluate its long-term effectiveness, and determine whether it is cost-efficient for mass production.

Keywords: antibiofilm, bacterial, cytotoxicity, green seaweed, *Pseudomonas aeruginosa*

Sifat *Antifouling Halimeda opuntia* dari Pesisir Aceh, Indonesia

Abstrak

Biofouling merupakan tantangan yang dihadapi dalam sektor maritim dan mendorong para peneliti untuk mengeksplorasi secara berkelanjutan dengan mencari solusi berupa *antifouling* yang ramah lingkungan dan berasal dari organisme laut. Penelitian ini bertujuan menentukan konsentrasi terbaik ekstrak *H. opuntia* berdasarkan aktivitas *antifouling*. Metode penelitian ini meliputi uji antibiofilm, sitotoksisitas, antibakteri, *anti-quorum* sensing, dan uji in situ. Hasil penelitian menunjukkan bahwa ekstrak metanol *H. opuntia* memiliki aktivitas antibiofilm lebih tinggi dengan nilai IC_{50} 0,020 mg/mL. Uji sitotoksisitas menunjukkan toksisitas terendah terhadap sel L6, dengan nilai IC_{50} 70,79 µg/mL. Secara mekanistik, ekstrak metanol *H. opuntia* tidak memiliki efek bakterisidal melawan bakteri *Pseudomonas aeruginosa*, tetapi dapat menghalangi mekanisme komunikasi bakteri melalui aktivitas *quorum quenching*, yang dibuktikan dengan pembentukan zona buram tidak berwarna dalam uji tersebut. . Uji in situ yang dilakukan di perairan Pulau Redang dan Kuala Kemaman, Malaysia menunjukkan panel yang dilapisi dengan formulasi 5% *H. opuntia* menunjukkan

http://dx.doi.org/10.17844/jphpi.v28i6.63699

kinerja *antifouling* yang superior selama tiga bulan percobaan, dengan tingkat penutupan *fouling* masing-masing sebesar 11,19% dan 9,10%. Penelitian selanjutnya diperlukan berkaitan dengan sifat *antifouling* dengan melakukan identifikasi senyawa aktif, evaluasi efektivitas jangka panjang, dan menentukan efisiensi biaya untuk produksi massal.

Kata kunci: antibiofilm, alga hijau, bakteria, *Pseudomonas aeruginosa*, sitotoksitas

INTRODUCTION

Biofouling refers to the progressive accumulation of microorganisms, algae, and invertebrates on submerged surfaces, a phenomenon that is pervasive in marine environments. This process poses significant operational and ecological challenges for anthropogenic structures, such as ship hulls, pipelines, and offshore platforms, by facilitating the colonization of both biotic and abiotic substrates. The fouling community spans multiple trophic levels, starting with bacterial biofilm formation and culminating in the attachment of macrofoulers, leading to increased drag, corrosion, and biocontamination (Demirel *et al.,* 2022).

Initial biofilm formation (bacteria, diatoms) promotes the settlement of invertebrates (e.g., barnacles, tubeworms), resulting in increased hydrodynamic drag (up to 40% higher fuel costs), structural loading and corrosion. With over 400 fouling species implicated globally, mitigation strategies must address both microbial and macrofouling phases to reduce economic and environmental impacts (Richard *et al.,* 2024; Roepke *et al.,* 2022). Bacteria are often the initial organisms to colonize surfaces during biofilm formation (Avelino-Jiménez *et al.,* 2023; Caruso, 2020). Bacteria form complex biofilms that serve as a foundation for the settlement and growth of other organisms, including macrofoulers, such as macroalgae, barnacles, bryozoans, and sponges. These biofilms create a habitat that supports the attachment and development of various invertebrates and stationary plants and animals, indicating a mutualistic or commensal relationship within the biofilm community (Muthukrishnan *et al.,* 2022; Zhao *et al.,* 2023). Microbial biofilms release bioactive compounds that serve as settlement cues for marine invertebrate larvae, including key habitat-forming species such as corals, barnacles, and macroalgae. Larvae detect these biofilm-derived into chemicals through chemoreceptors, enabling them to identify optimal settlement sites. By mediating larval attachment via chemical signaling, biofilms critically regulate recruitment success in marine ecosystems (Cooney *et al.,* 2024; Freckelton *et al.,* 2022; Lau & Qian, 2001). Biofouling organisms construct stratified communities via biofilm-mediated co-aggregation, where mucus secretions enhance recruitment and interspecies synergies, yielding tenacious ecosystems resistant to removal (Zhang *et al.,* 2024). Antifouling is the process of preventing fouling organisms from accumulating (Vijayan *et al.,* 2022). Biofouling involves both natural defenses used by marine organisms to prevent other organisms from attaching to them and man-made methods to protect structures such as ships, fish farming equipment, and underwater cameras from being covered in unwanted organisms (Abdulrahman *et al.,* 2022; Guo *et al.,* 2025). Until recently, most antifouling treatments relied on paints containing organotin (tributyltin) or heavy metals (copper and zinc), which act as broad-spectrum biocides, adversely affecting both target and non-target marine organisms (Beaumont & Budd, 1984; Cima & Varello, 2023).

Biofouling, characterized by the accretion of marine organisms on submerged substrates, continues to present a substantial impediment to the maritime sector, resulting in both economic detriment and ecological perturbation. Conventional antifouling coatings, which are based on the release of cytotoxic biocides, including arsenic, copper, and tributyltin (TBT), have demonstrated efficacy in mitigating biofouling. However, these formulations introduce significant environmental hazards by leaching toxic compounds into marine ecosystems, leading to food web contamination and deleterious effects on non-target biota. Despite the global prohibition of TBT in 2008, copper-

based coatings remain prevalent, despite their documented adverse effects on marine organisms. Consequently, the development of ecologically sustainable and nontoxic alternatives is critical to address this persistent challenge. Even in very small amounts, these toxic organometallic and heavy metal compounds pose a significant threat to the environment (Satasiya *et al.,* 2025), and their use is banned due to environmental harm. Natural products are being explored as possible replacements for tributyltin, one of the most effective antifouling agents (Roepke *et al.,* 2022).

Seaweed naturally protect themselves from microbial growth, making them a potential source of environmentally friendly antifouling substances. Crude extracts from *Sargassum muticum* and *Ceramium botryocarpum* have demonstrated inhibitory effects on the growth of diatom species, including *Fragilaria pinnata* and *Cylindrotheca closterium* (Dahms & Dobretsov, 2017; Tunkal *et al.,* 2022). Nevertheless, the investigation of seaweed-derived antifouling compounds remains limited, notably in geographically less-studied areas such as the Aceh region of Indonesia.

A survey of the extant scientific literature reveals a notable scarcity of investigations into the antifouling capabilities of Indonesian seaweed. Although a study by Oktaviani *et al.* (2019) explored the antibacterial characteristics of the green alga *Chaetomorpha antennina*, harvested from Karapyak Beach in Pangandaran, West Java, the antifouling properties of seaweed species indigenous to the Aceh region of Indonesia remain unexamined. Notably, the current body of knowledge lacks research focusing on the antifouling potential of the specific green algal species *Halimeda opuntia* within this geographically distinct area. This significant gap in our understanding underscores the critical need for comprehensive research to elucidate the bioactive compounds present in Aceh seaweed. Such investigations hold the promise of identifying sustainable and ecologically viable alternatives to the currently employed toxic antifouling agents, thereby contributing to the development of environmentally responsible marine coatings and materials.

Aceh, Indonesia, has a nearly even distribution of Chlorophyta, Rhodophyta, and Phaeophyta seaweeds (Diansyah *et al.*, 2018). *Halimeda* sp. is a common and ecologically important green seaweed found along the coast of Lhok Bubon, West Aceh (Gazali, 2018). Erniati *et al.*, (2022) found that *Halimeda* sp. is present on the West coast of Simeulue Island, Aceh, making up about 9.22% of the seaweed found there. The green seaweed *Halimeda* sp., particularly *H. opuntia*, is used less than brown and red seaweeds, particularly for bioactive substances in the maritime industry. *H. opuntia* is a green seaweed that exhibits antibacterial and antioxidant activities and is rich in calcium carbonate (Daveza *et al.*, 2025). Nufus *et al.* (2017) reported that *H. opuntia* contains the minerals Ca (124.39%), Na (21.16%), Mg (2.63%), K (2.29%), and Fe (0.13%).

Recent studies on the biological activities of *Halimeda* sp. from Aceh waters, such as *H.* macroloba as an antioxidant agent (Gazali *et al*., 2019b), *H. opuntia* possesses antioxidant activity (Gazali *et al.*, 2019a), *H. tuna* has in vitro α-amylase and α-glucosidase inhibitory activity (Gazali *et al.*, 2023), *H. tuna* has cytotoxic activity against lung cancer cells (Husni *et al.*, 2024), *H. tuna* has antioxidant and anti-arthritic activities (Gazali *et al.*, 2024), and *H. tuna* has anticancer activity against cervical cancer cells (Gazali *et al.*, 2024). However, there is no evidence on the biological impacts of *H. opuntia* from Aceh waters, focusing on their antifouling paint properties. Thus, the aim of this study was to determine the best concentration of *H. opuntia* extract formulation that is most effective in antifouling activity.

MATERIALS AND METHOD

Sample Collection

Green seaweed *H. opuntia* samples were collected from the shore of Lhok Bubon in the Samatiga Subdistrict, West Aceh, Aceh Province, Indonesia (Figure 1). The samples were rinsed with fresh water to remove any clinging sand and grime. Fresh seaweed was then allowed to dry at room temperature. Wet

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and dry samples were delivered to the South China Sea Repository and Reference Center (RRC) at the Institute of Oceanography and Environment (INOS), Universiti Malaysia Terengganu (UMT), Malaysia (voucher number UMTP 3357).

Extraction and Liquid-Liquid Partition

The green seaweed *H. opuntia* was extracted using a modified version of the method described by Azizi *et al.* (2019). Dried seaweed samples were mixed with methanol at a ratio of 1:10 (%w/v) and extracted for 24 h at room temperature. The mixture was then filtered, and the filtrate was evaporated to dryness using a rotary evaporator. *H. opuntia* methanol crude extract was subjected to liquid-liquid partition using a modified method described by Vidyapeeth *et al.* (2017) subjected the methanol crude extract (MCE) to sequential liquid-liquid partitioning to isolate compounds based on their varying solubilities. The MCE, initially dissolved in an aqueous solution, was subjected to extraction using n-hexane, chloroform, and ethyl acetate. Each extraction involved vigorously mixing the MCE solution with an equal volume of the respective solvent in a separatory funnel, allowing phase separation, and collecting the organic layer for analysis. This process was repeated until the solvent layer showed no further color change, indicating the complete liquid-liquid extraction of the compounds.

Cytotoxicity Assay

The cytotoxicity of various materials against L6 rat muscle cells was evaluated based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sul- fophenyl)-2H-tetrazolium (MTS) assay, adapted from Andriani *et al. (*2019). L6 cells were cultured in a humidified incubator at 37°C with 5% CO_2 until they reached 80% confluence. Subsequently, 100 µL aliquots of the cell suspension were seeded into a 96-well plate and incubated for 24 h. The culture medium was then replaced with 100 µL of crude extract diluted to various concentrations. A positive control was included at the highest concentration used for the sample. The plate was incubated for an additional 72 h, and cell viability was assessed using the MTT assay. The percentage of cell viability was estimated using the following formula:

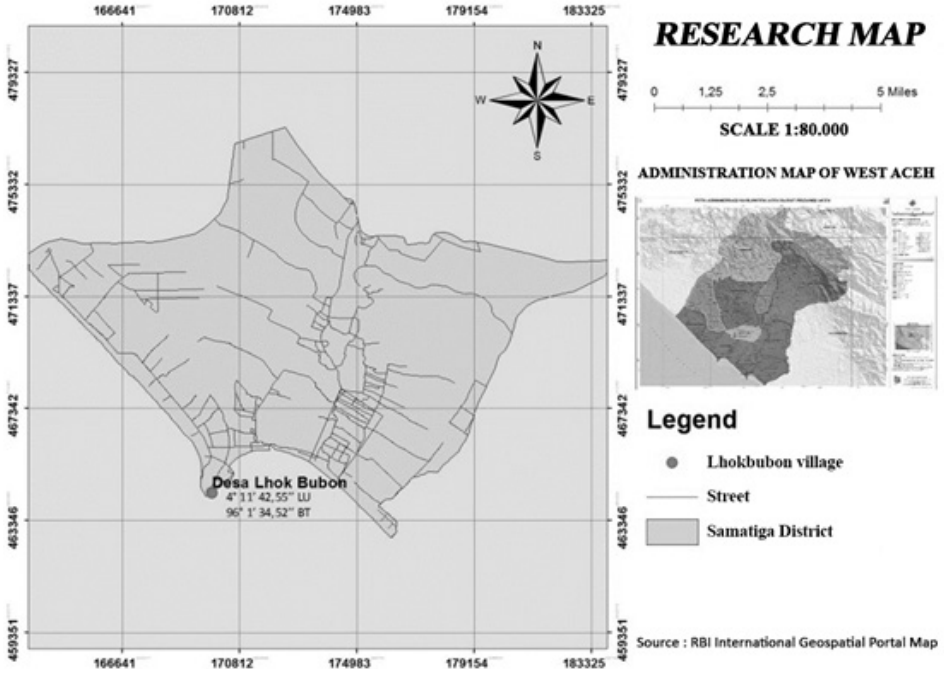

Figure 1 Sampling site
Gambar 1 Lokasi pengambilan sampel

$$IC_{50} = \frac{\text{sample}}{\text{blank}} \times 100$$

The IC_{50} value was directly interpolated from the graph. According to (Andriani *et al.,* 2019), if the IC_{50} value is higher than 30 µg/mL, the material is considered non-toxic.

Antibacterial Assay

The antibacterial activity of the crude extract from *H. opuntia* against *Pseudomonas aeruginosa* (ATCC 27853) was evaluated using the disc diffusion method at concentrations of 100, 50, 25, 12.5, 6.25, and 3.1 ppm. The extract was impregnated onto sterile discs and placed on agar plates inoculated with bacteria. The zones of inhibition surrounding the discs were measured after incubation to determine the antibacterial activity of the extracts. Ciprofloxacin was used as a positive control and dimethyl sulfoxide (DMSO) as a negative control (Indraningrat *et al.*, 2024).

Anti-Quorum Sensing Activity

The anti-quorum-sensing activity of the *H. opuntia* crude extract was evaluated using the disk diffusion method. *Chromobacterium violaceum*, a bacterium that produces violet pigments, was used as a reporter strain. The extract was impregnated onto sterile paper discs and placed on agar plates inoculated with bacteria. The zones of growth inhibition around the discs were observed to determine the anti-quorum-sensing activity of the crude extract. Oxytetracycline and LB broth were used as positive and negative controls, respectively (Tang *et al.,* 2020).

Identification of Bioactive Compound by GC-MS

The chemical composition of the aqueous fraction of *H. opuntia* was characterized via gas chromatography-mass spectrometry (GC-MS) using a GCMS-QP2010 ULTRA system (Shimadzu). Samples were prepared in methanol and introduced via split injection (1 µL, 120:1 split ratio) into a fused silica capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness). The GC-MS system employed electron ionization (EI) at 70 eV, with ultra-high-purity helium (99.999%) as the carrier gas (constant flow rate: 1 mL/min). The injector and mass-transfer line temperatures were maintained at 290°C and 220°C, respectively. The oven temperature gradient initiated at 50°C, ramped at 3 °C/min to 150°C (held for 10 min), then increased at 10°C/min to 300°C. Sample dilutions (1:100, v/v, in methanol) were analyzed, and the relative constituent abundances were quantified via peak area normalization, expressed as percentages of individual peaks relative to the total integrated chromatographic area.

Preparation of Panels Incorporated with Seaweed Extracts

The painted steel panels used in this study were obtained from a local oil and gas company (Bandar Baru Bangi, Selangor, Malaysia). The panels were cut into squares measuring 2.5 cm × 0.3 cm. The paint on the panels was removed using hand tool cleaning and a sanding machine in accordance with the Society for Protective Coatings-Surface Preparation Standard No. 2 (SSPC-SP2 standard). Before proceeding with the coating method, the steel panels were polished with rough-grade abrasive paper attached to an orbital sander to remove the rust. The panels were then cleaned with methanol according to the SSPC-SP1 standard to remove any detectable oil, grease, dust, or other soluble contaminants (Noor Idora *et al.*, 2015; Rahman *et al.*, 2023).

To prepare the final antifouling paint formulations, crude extracts of *Halimeda opuntia* (HO) were incorporated into a blank paint matrix at a concentration of 5% (w/w), resulting in the HO5% treatment. The mixtures were homogenized using a drilling machine to ensure the uniform dispersion of the bioactive components. For testing, three replicate panels (n = 3) were coated using an air spray compressor (Model ZL-550W2-50L, Uma, Cheras, Selangor, Malaysia), with a drying period of two days between coats to allow proper film formation. For this study, we used blank paint (negative control), which contained no antifouling agent, and two types of commercial antifouling paints (positive controls), designated as Reference 1 (RF1) and (RF2), supplied by a local Oil and

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Gas company (Bandar Baru Bangi, Selangor, Malaysia). Blank paint is a mixture of paint consisting of some chemicals, including: colophony (CAS number: 8050-09-7), xylene (CAS number: 1330-20-7), zinc oxide (CAS number: 1314-13-2), ethylbenzene (CAS number: 100-41-4), hydrocarbons (C9, aromatic, CAS number: 64742-95-6), 1-methoxy-2-propanol (CAS number: 107-98-2), and fatty acids (CAS number: 91001-64-8). Some of the components included in the reference paints were similar to those of the blank paint, but without fatty acids and other added ingredients. In addition, the other ingredients contained in the reference paints were dicopper oxide (CAS number: 1317-39-1), and zineb (CAS number: 12122-67-7).

The Antifouling Effectiveness of Painted Panels in a Field Trial

The coated steel panels were immersed in seawater at Kuala Kemaman and Redang Island, Malaysia. The panels were deployed in Kuala Kemaman on April 24, 2024, and on Redang Island on April 25, 2024. The panels, which were linked to a stainless-steel frame, were submerged for three months at both sites before being dropped two meters from the ocean's bottom. The sea depth in Kuala Kemaman was 27 m, whereas that in Redang Island was 17 m. Monthly diving was used to retrieve the panels, which were then returned to the laboratory for further processing. A handheld multimeter (Aquaread Asia, Singapore) was used to measure the physical properties of the surrounding saltwater.

Statistical Analysis

Data were analyzed using statistical software to determine whether there were significant differences between the groups. One-way analysis of variance (ANOVA) was employed, followed by Tukey's test to identify specific differences between groups. The results are presented as mean values with standard errors of the mean. Statistical significance was set at p less than 0.05. This analysis allowed for a rigorous comparison of the data and identification of any significant variations among the groups.

RESULT AND DISCUSSION

Antibiofilm Activities

Marine environments, with their vast biodiversity, serve as a source of inspiration for the development of natural antimicrobial agents. Despite the high bacterial density in seawater (approximately 1 million cells/mL), including *Pseudomonas* species, and the propensity for bacterial biofilm formation, sessile marine organisms, such as algae, effectively counteract these threats. This indicates an inherent capacity to produce protective metabolites (Shannon & Abu-Ghannam, 2016). Recent investigations have demonstrated that compounds and extracts derived from green, brown, and red seaweeds exhibit potent antibacterial, antibiofilm, and antifouling properties (Bhowmick *et al*., 2020; Dahms & Dobretsov, 2017). This result showed that the green seaweed *H. opuntia* methanol extract exhibited the maximum inhibitory activity with a value of 62.16% at a concentration of 0.024 mg/mL against *P. aeruginosa* bacteria (Figure 2a), whereas *H. opuntia* aqueous fraction possessed antibiofilm activity with a maximum percentage of inhibitory action with a value of 58.44% at a concentration of 1.562 mg/mL (Figure 2b). These results are comparable to those of a previous study. Nik Mohd Sukrri *et al*. (2024) reported that Malaysian green seaweed, *Ulva lactuca* methanol crude extract possesses the highest inhibition at 63% at a concentration of 0.031 mg/mL.

Gadhi *et al.* (2018) found that a methanol extract from *Halimeda* sp. effectively inhibited the growth and biofilm formation of *Vibrio harveyi*. The extract was particularly effective at a concentration of 0.032 mg/mL, significantly reducing both bacterial growth and the production of extracellular polymeric substances (EPS), which are crucial components of biofilms. Interestingly, the aqueous fraction of *H. opuntia* also showed excellent antibiofilm activity against *P. aeruginosa,* with an IC_{50} value of 1.20 mg/mL, which exhibited a maximum inhibitory action of 58.4% of inhibition percentage against *P. aeruginosa* biofilm activity. It has been proposed that the antibiofilm activity of

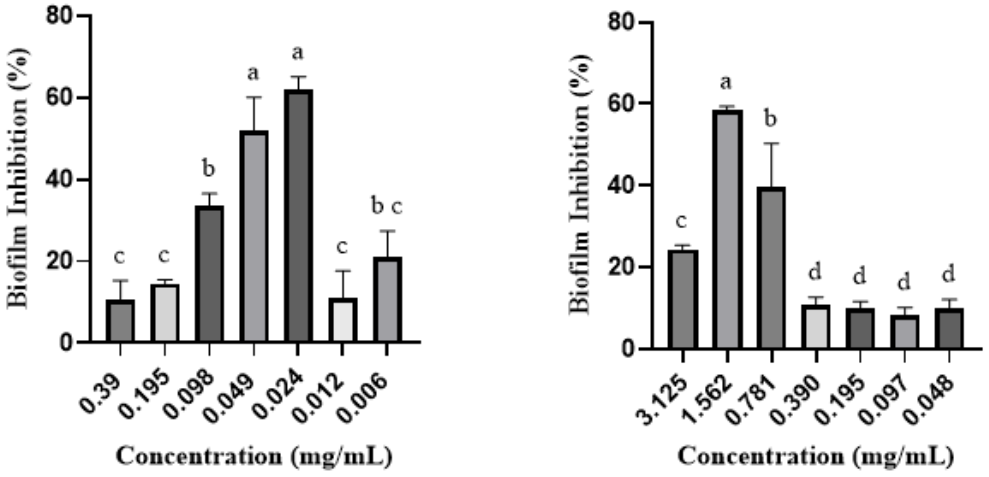

Figure 2 Inhibition of *P. aeruginosa* biofilm formation by *H. opuntia* methanol crude extract (A) and aqueous fraction (B) after incubation for 24 hours in the 96-well plate

Gambar 2 Penghambatan pembentukan biofilm *Pseudomonas aeruginosa* oleh ekstrak kasar metanol *H. opuntia* (A) dan fraksi *aqueous* (B) setelah inkubasi 24 jam dalam 96 *well plate*

the green seaweed *H. opuntia* may be affected by polarity-based chemical separation. These findings imply that the methanol extract and aqueous fraction *of H. opuntia* demonstrate a remarkable ability to inhibit biofilm formation and may have further value as antifouling agents.

Rahman *et al*. (2023) investigated the *Sonneratia lanceolata* and *Diadema setosum* methanol crude extract showed the comparable highest inhibitory activity in biofilm formation with value of almost 100% at even low concentration of 0.6125 mg/mL. Farizan *et al*. (2024) also reported that *Melaleuca cajuputi* methanol crude extract has potential antibiofilm activity, with an optimum of up to 61% at a low concentration of 0.031 mg/mL. Based on a previous study with other plants tested, the green seaweed *H. opuntia* methanol crude extract has comparable antibiofilm activity against *P. aeruginosa*, indicating a promising candidate for natural antifouling agents to mitigate biofouling, which has economic and ecological aspects in maritime industries.

H. opuntia aqueous fractions possess potential antibiofilm activity that can inhibit *P. aeruginosa* biofilm formation. Numerous water-soluble chemicals are abundant in the aqueous fractions of seaweed. The precise composition can change considerably depending on the macroalgal species, growing environment, and extraction technique. Although *P. aeruginosa* and its aqueous portion may receive little attention, the antibacterial qualities of macroalgae and their ability to prevent biofilm development have been extensively studied.

Antibacterial Activity

The methanol crude extract of the green seaweed *Halimeda opuntia* crude extract did not exhibit antibacterial activity against *P. aeruginosa* (ATCC 27853) in the concentration range of 100 to 3.125 ppm (Figure 3). Neither the bacteria nor the pre-existing *P. aeruginosa* biofilm can be eliminated by *H. opuntia* crude extract. This indicates that the *H. opuntia* crude extract has a quorum quenching mechanism. Furthermore, the crude extract of *H. opuntia* exhibited antibiofilm properties against *P. aeruginosa*, an aquatic bacterial pathogen, and considerably reduced its biofilm production. According to reports, this

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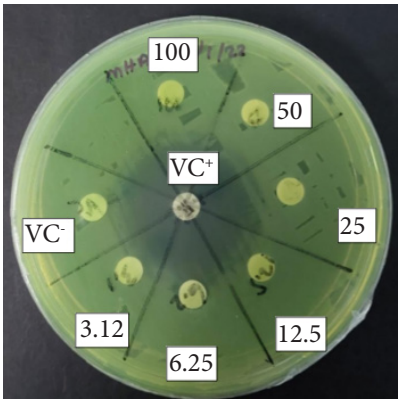

Figure 3 Antibacterial activities using disc diffusion method of *H. opuntia* crude extract; VC-negative control; positive control-ciprofloxacin

Gambar 3 Aktivitas antibakteri menggunakan metode difusi disk pada ekstrak kasar *H. opuntia*, VC-kontrol negative; kontrol positif- ciprofloxacin

extract was successful in stopping the initial attachment, breaking up the structure of the biofilm, and preventing the formation of new biofilms.

Jae-Suk Choi *et al.* (2010) have reported that *Ulva pertusa* (green seaweed) extracts was evaluated against *Gardnerella vaginalis*, revealing antibacterial effects with maximum inhibition (6.5 mm zone of inhibition at 5 mg/disk). This previous study contradicts the findings of the present study, which may be attributed to the differences in the bioactive compound composition of both green seaweed species. Interestingly, the green seaweed *H. opuntia* crude extract has promising antifouling activity, and this crude extract has a quorum-quenching mechanism compared to other green seaweed species. This result indicates that the green seaweed *H. opuntia* crude extract possesses the potential to disrupt or block bacterial communication involving N-acyl homoserine lactones (AHLs) as signal molecules. Kumar *et al.* (2017) have reported that the first quorum sensing (QS) inhibitory compound was identified from the red seaweed *Delisea pulchra*, which produces halogenated furanones structurally similar to bacterial acyl-homoserine lactone (AHL) signaling molecules. Following this discovery, further studies have reported QS-inhibitory and biofilm-suppressing activities in several other macroalgal species, indicating a broader potential of seaweeds as natural sources of anti-quorum-sensing agents. Additionally, the brown alga *Laminaria digitata* produces hypobromous acid, a compound that can disrupt bacterial QS signaling pathways and modulate QS-regulated gene expression (Borchardt *et al.,* 2001). Additionally, Bacterial communities associated with the green seaweed *Ulva* sp. and *Colpomenia sinuosa* have demonstrated quorum sensing (QS) inhibitory activity and the ability to prevent biofouling (Kanagasabhapathy *et al.,* 2009). However, the precise origins of these QS inhibitors remain undetermined. Although they may be produced by algae or epiphytic bacteria, the specific mechanisms through which algal metabolites interfere with QS are not yet fully understood, highlighting the need for further research (Goecke *et al*., 2010).

In addition to its intrinsic and acquired resistance, the exceptional capacity of this bacterium to build biofilms adds to its strength by acting as a protective barrier against host defenses and anti-Pseudomonas antibiotics (Talebi *et al.,* 2019). Other assays are necessary for the initial screening of bioactive substances.

Anti-Quorum Sensing

A colorless, opaque circle (Figure 4) was formed around the extract-loaded disk (concentration 10 mg/mL). The negative control (LB broth-loaded disk) showed no inhibition, but oxytetracycline, the positive control, demonstrated extremely apparent,

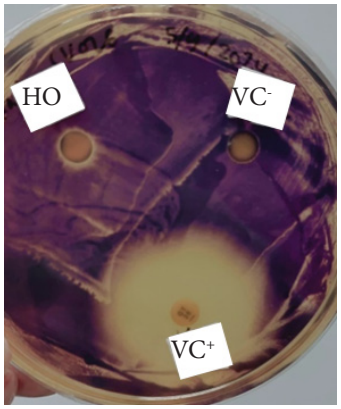

Figure 4 Anti-quorum sensing (anti-QS) activity by *H. opuntia* (HO) extract against *Chromobacterium violaceum* bacteria using agar disc diffusion. Ho-*H. opuntia* crude extract; VC-blank; Vc+-positive control, Oxytetracycline

Gambar 4 Aktivitas anti-quorum sensing (anti-QS) oleh ekstrak kasar *H. opuntia* (HO) melawan bakteri *Chromobacterium violaceum* menggunakan difusi disk agar. Ho- ekstrak kasar *H. opuntia*; VC-Blanko; Vc+ control positif Oxytetracycline

visible inhibition. The presence of the tested extract resulted in the loss of purple pigmentation in *C. violaceum,* indicating QS inhibition.

It has been widely documented that quorum sensing signalling molecules play a role in biofilm formation, which is the cause of various forms of marine biofouling. To control the propagation of biofilms and potentially be a valuable tool in the treatment of bacterial infections in the future, quorum sensing systems can be utilized to expand the application of quorum sensing inhibitors as an alternative strategy for the inhibition of specific phenotype expression. This blocks cell-to-cell communication.

Melander *et al.* (2020) provided a thorough analysis of natural antibiofilm compounds derived from marine sources. Halogenated furanones, produced by some algae species, such as the red marine algae *Delisea pulchra,* are the first class of quorum sensing inhibitors that competitively prevent N-acyl homoserine lactones (AHL) from binding to intracellular receptors, which in turn prevents bacterial colonization and the formation of biofilms. In another similar study, 30 different types of marine macroalgae were evaluated for their ability to interfere with bacterial communication. This was performed using a specific type of bacteria, *C. violaceum*. Of these 30, *Asparagopsis taxiformis* was particularly effective. It not only inhibits bacterial growth but also disrupts their communication processes (quorum quenching activity) (Jha *et al.,* 2013). This has occurred as a result of blocking the important quorum-sensing pathway that passes through bacterial cells, including the AI-2 signalling network in both Gram-positive and Gram-negative species and the AHSL regulation system in gram-negative bacteria (Borges & Simões, 2019).

Cytotoxicity activity

Halimeda opuntia crude extract exhibited cytotoxic activity with an IC_{50} value of 70.79 µg/mL (Figure 5). This indicates that the *H. opuntia* crude extract has low cytotoxicity against L6 cells. Therefore, *H. opuntia* crude extracts are environmentally friendly to marine organisms because they are not toxic. There are no previous studies that reported macroalgae cytotoxicity with L6 cell line for antifouling standard

To be deemed effective, a natural antifouling remedy must be safe for the ecosystem. The main objective is to prevent marine creatures from adhering to submerged surfaces while avoiding damage to marine ecology. It should not endanger fish, invertebrates, or other marine life. The L6 cell line drastically increased in concentration from 12.5-50 µg/mL against *H. opuntia*

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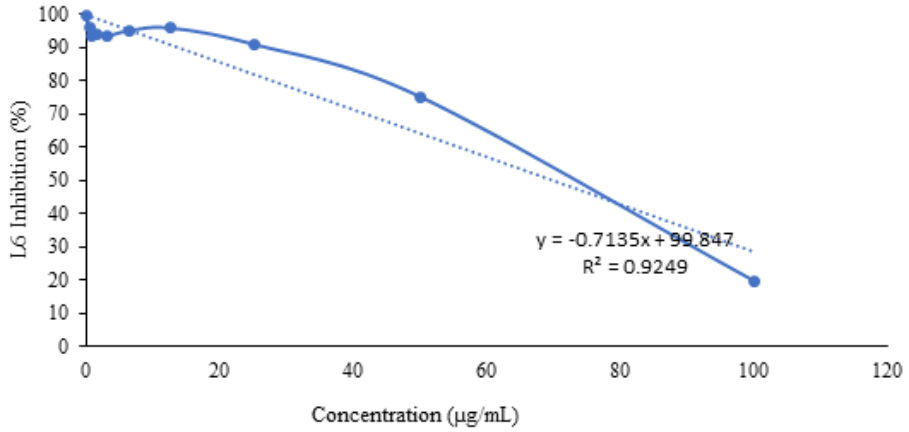

Figure 5 Cytotoxicity of *H. opuntia* with L6 cell line (normal cell)
Gambar 5 Sitotoksisitas *H. opuntia* dengan sel L6 (sel normal)

extracts for 3 days of experimental research (Figure 5). This indicates that *H. opuntia* extract has low cytotoxic activity. *H. opuntia* extracts might not produce compound with cytotoxic properties against L6 cells. L6 cells grew well for 3 days with the addition of *H. opuntia* extracts, which was almost similar to the negative control, where L6 cells were not treated with the extract. L6 cells grew in the wells, indicating many spots under an inverted microscope, as illustrated in Figure 6. Moreover, Vincristine sulfate was used as a positive control in this experimental study. Vincristine sulfate was used as a positive

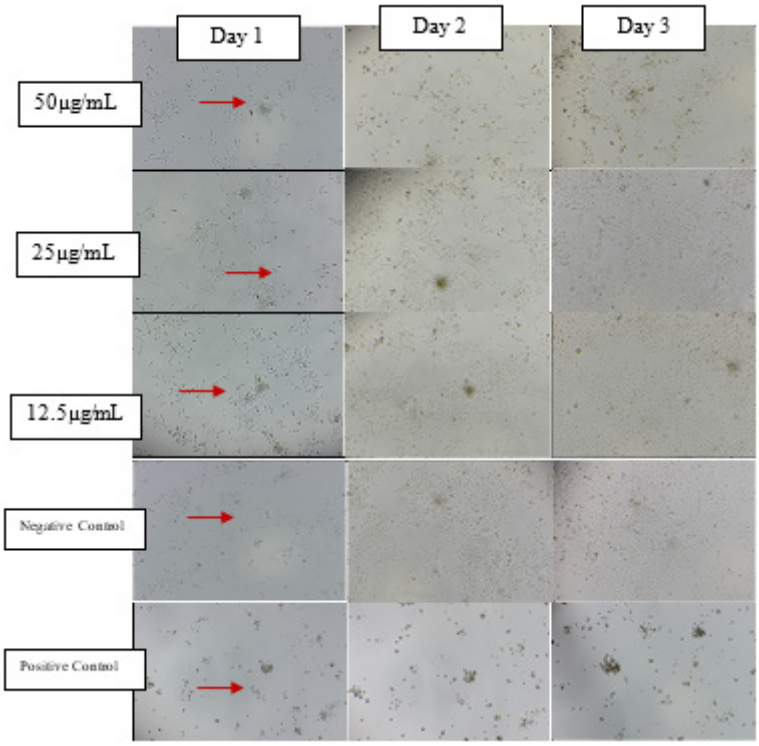

Figure 6 Morphology of L6 cell line (normal cell) that give *H. opuntia* extract with different concentration, negative control and positive control- Vincristine sulphate. A few spots indicating L6 cell grow well
Gambar 6 Morfologi sel L6 (sel normal) yang diberikan pada ekstrak *H. opuntia* dengan konsentrasi yang berbeda, kontrol negatif dan kontrol positif- Vincristine sulfate. Bintik-bintik menunjukkan sel L6 tumbuh dengan baik

control, as the cells were expected to be inhibited, thereby indicating that the assay was conducted properly. Another study by Andriani *et al.* (2019) reported that *Pandanus tectorius* fruits exhibited no cytotoxic action against RAW or the L-6 cell line (rat muscle cell).

In this study, *H. opuntia* extract has been scientifically proven to be beneficial for antifouling paint due to its low toxicity against L6 cells. The L6 cell line is a rat skeletal myoblast cell line. This means that it is a collection of cells produced from rat muscle tissue that can develop and differentiate into adult muscle fibers under specific laboratory conditions.

Identification of Bioactive Compound by GC-MS

Compound identification was performed on an aqueous fraction sample of *H. opuntia*, which exhibited the best antibiofilm activity based on the IC_{50}. The identification of these two samples aimed to determine the potential compounds responsible for antibiofilm activity. The GC-MS peak results indicated the number of detected peaks in the samples. Retention time (RT) refers to the time taken for a component or compound to reach the column detector of the chromatograph. The % area value represents the relative abundance of a compound based on its measured peak. However, the % area value does not provide a quantitative measure of the compound content (Fitri & Proborini, 2018). The chromatogram of the aqueous fraction is shown in Figure 7, and the results of the compound identification of the aqueous fraction are presented in Table 1.

These results are consistent with those of a previous study by Prabhakaran *et al.* (2012), which showed that *Halimeda macroloba* extract possesses potential antifouling activity with a group of compounds, including alcohols, phenols, aromatics, carboxylic acids, esters, and ethers. In this study, *H. opuntia* extract also had a similar group of compounds as *H. macroloba* owing to the Halimeda genus. Interestingly, Gadhi *et al.* (2018) also reported that GC-MS analysis revealed that *Halimeda* sp. extracts contain phenolic derivatives such as phenol 2,6-bis(1,1-dimethylethyl) and phenol 3,5-bis(1,1-dimethylethyl). More importantly, the methanol extract of *Halimeda* sp. contains decanol derivatives, such as 1-dodecanol, 1-hexadecanol, and n-heptadecanol. These fatty alcohols, commonly found in plants, have been reported to have antibacterial properties. In addition, they have demonstrated antifouling capabilities (Kang *et al.,* 2016). It is important to note that some of the compounds identified in the GC-MS analysis could be environmental contaminants that have been absorbed by the algae.

Marine Field Study

Each *H. opuntia* crude extract was used at two different concentrations, each at 5% (w/v). All the coated panels were firmly secured to the stainless-steel frame at Kuala Kemaman and Redang Island, Malaysia, the two immersion locations, using iron wire, and then submerged in seawater at a depth of approximately two meters. As shown in Figure 8, the tested panels for Kuala Kemaman and Redang Island were gathered at different points in time.

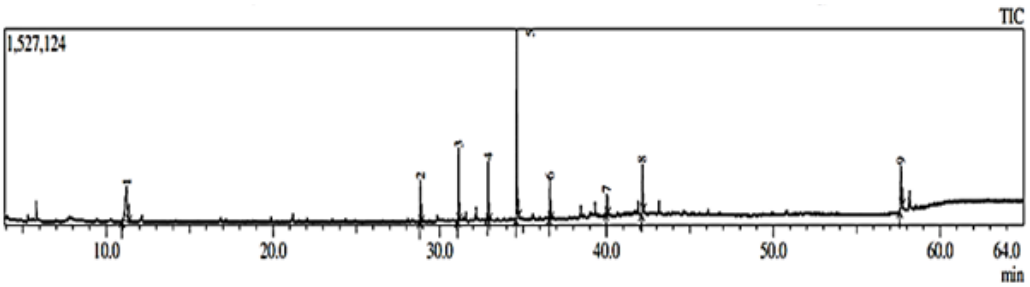

Figure 7 Chromatogram analysis GC of H. opuntia aqueous fraction
Gambar 7 Analisis kromatogram GC fraksi aqueous *H. opuntia*

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Table 1 Biological activity of compound in aqueous fraction of *H. opuntia*
Tabel 1 Aktivitas biologi senyawa pada fraksi aqueous *H.opuntia*

Peak	Retention time	% Area	Component name	Molecule formula	Molecule weight	Similarity index I
1	11.229	17.83	Undecane	$C_{11}H_{24}$	156	94
			Dodecane	$C_{12}H_{26}$	170	93
2	28.843	6.21	1-Nonadecene	$C_{19}H_{38}$	266	95
			Behenic alcohol	$C_{22}H_{46}O$	326	95
			Z-5-Nonadecene	$C_{19}H_{38}$	266	95
			n-Tetracosanol-1	$C_{24}H_{50}O$	354	94
3	31.128	11.10	7,9-di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276	86
			Olean-18-ene	$C_{30}H_{50}$	410	64
			3,5-Cyclohexadiene-1,2-dione	$C_{14}H_{20}O_2$	220	63
4	32.902	8.42	1-Nonadecene	$C_{19}H_{38}$	266	95
			1-Heptacosanol	$C_{27}H_{56}O$	396	95
			9-Tricosene	$C_{23}H_{46}$	322	95
			n-Tetracosanol-1	$C_{24}H_{50}O$	354	95
5	34.621	29.24	1-Octadecanol	$C_{18}H_{38}O$	270	96
			n-Nonadecanol-1	$C_{19}H_{40}O$	284	95
			Behenic alcohol	$C_{22}H_{46}O$	326	95
			1-Hexadecanol	$C_{16}H_{34}O$	242	95
6	36.606	5.76	1-Heptacosanol	$C_{27}H_{56}O$	396	95
			n-Tetracosanol-1	$C_{24}H_{50}O$	354	94
			Nonadecyl heptafluorobutyrate	$C_{23}H_{39}F_7O_2$	480	94
			1-Heneicosyl formate	$C_{22}H_{44}O_2$	340	94
7	40.018	2.66	1-Nonadecene	$C_{19}H_{38}$	266	93
			1-Heptacosanol	$C_{27}H_{56}O$	396	93
			Nonadecyl trifluoroacetate	$C_{21}H_{39}F_3O_2$	380	92
			1-Eicosene	$C_{23}H_{46}$	322	92
8	42.149	7.43	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390	94
9	57.652	11.34	1,4-Epoxynaphthalene-1(2H)-methanol	$C_{23}H_{36}O_2$	344	63
			Propanoic acid	$C_5H_9AgO_2$	208	60
			Mercury di-n-butyl	$C_8H_{18}Hg$	316	58
			Diboroxane	$C_{22}H_{28}B_2O_5$	394	56
			2-tert-Butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl) phenol	$C_{40}H_{58}O_3$	586	55

Figure 8 shows the appearance of the antifouling coating panel after 3 months including blank, reference 1 (RF 1), reference 2 (RF 2), and *H. opuntia* 5% (HO 5%). There are barnacle communities in the blank panel that are attached to the edge of the panels with many filamentous algae. Moreover, filamentous algae were more attached to commercial antifouling coatings, reference 1 (RF1) and reference 2 (RF2), than to the antifouling of *H. opuntia* extract over three months.

The results showed that the blank panel without a sample had an increasing trend of filamentous algae as microfoulers attached to the panels over 3 months periods from the 1st month to the 3rd month (25.46%, 29.96%, and 56%) respectively. Furthermore, references 1 (RF 1) and 2 (RF 2), which are commercial antifouling paints, also indicated an increase in filamentous algae on the panel for 3 months. Uniquely, antifouling with *H. opuntia* (HO5%) extract formulation showed a natural antifouling panel in the 1st month, with an *H. opuntia* panel of 14.27% and a significant increase in the 2nd month with a macrofouler percentage of 15.2%. However, in the 3rd month, the macrofouler percentage was low at 11.19%. This indicates that the antifouling panel of *H. opuntia* has lower macrofouler than reference 1 that was immersed in Redang Island (Figure 9). Thus, the *H. opuntia* 5% extract is an environmentally friendly antifouling paint comparable to the commercial antifouling paint reference 1 (RF1).

Qualitatively, these panels showed different patterns of fouling coverage. There are barnacle communities attached to the

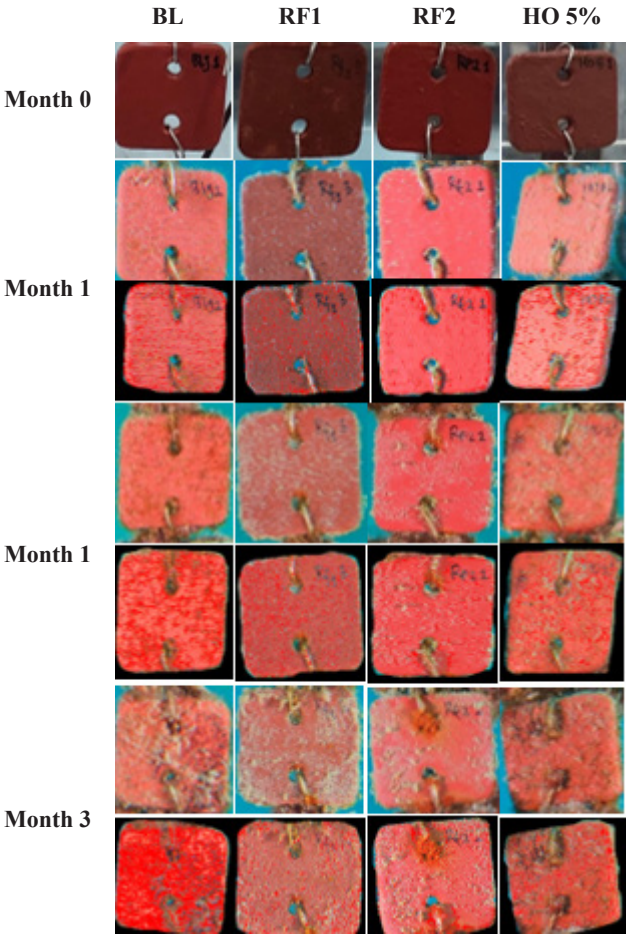

Figure 8 Antifouling coating for three months immersion period at Redang Island (BL: blank; RF 1 reference 1; RF 2: reference 2; HO5%: *H. opuntia* 5%)

Gambar 8 Lapisan *antifouling* setelah periode perendaman selama tiga bulan di Pulau Redang (BL: blangko; RF 1: referensi 1; RF 2: referensi 2; HO5%: *H. opuntia* 5%)

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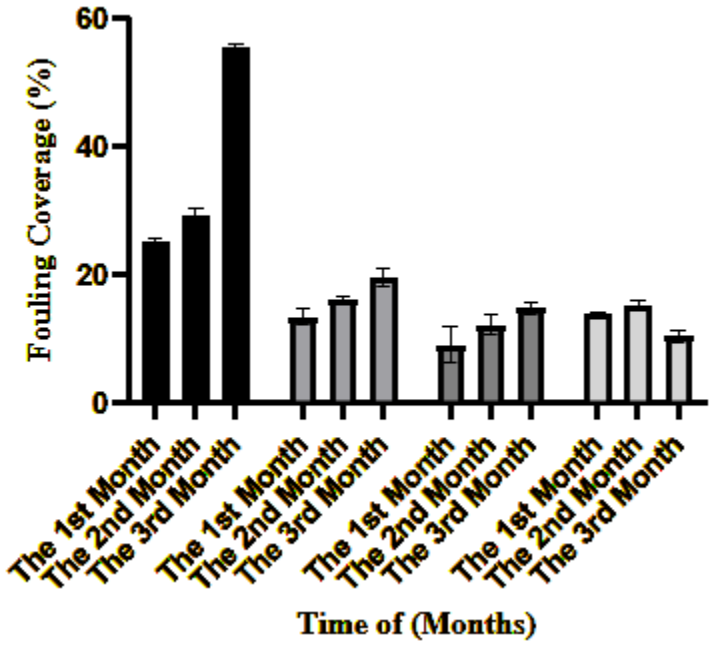

Figure 9 Percentages of the area covered by biofouling after immersion in the Redang Island (BL: (BL: blank ; RF 1: reference 1 ; RF 2: reference 2 ; HO5%: *H. opuntia* 5%).

Gambar 9 Persentase area yang ditutupi oleh *biofouling* setelah perendaman di Pulau Redang (BL: blangko ; RF 1: referensi 1 ; RF 2: referensi 2 ; HO5%: *H. opuntia* 5%)

blank panel with many filamentous algae. Interestingly, the antifouling of *H. opuntia* 5% recovered after three months of immersion, which resulted in lower fouling coverage than that with the commercial antifouling coating (reference 1).

Antifouling panel immersion in Kuala Kemaman included Blank, reference 1 (RF 1), reference 2 (RF 2), and *H. opuntia* (HO5%). The blank panel has percentages of the area covered by macrofouling for 3 months (the 1st month-the 3rd month) with values of 30.38%, 38.2%, and 45.5%, respectively, whereas reference 1 (RF1) has macrofouler percentages of 3.8%, 16.6%, and 14.3%, respectively. Reference 2 is a commercial antifouling paint with strong antifouling activity, with percentages of the area covered by macrofouling of 0.9% in the 1st month, 1.3% in the 2nd month, and 0.03% in the 3rd month. Interestingly, natural antifouling from *H. opuntia* (HO5%) showed different trends, with a macrofouler percentage of 10.73% in the 1st month, 30.7% in the 2nd month, and 9.10% in the 3rd month. These trends indicate that the antifouling panel made from *H. opuntia* (HO5%) has stronger antifouling activity than the commercial antifouling panel in reference 2 (Figure 10).

The antifouling paint panels and blank panels in the ocean at Kuala Kemaman and Redang Island were compared over a three-month period. Rapid growth of filamentous algae was observed on the blank panels. Conversely, on the antifouling paint covered in the *H. opuntia* (HO 5%) panel, significantly fewer filamentous algae were observed. There are differences in the trend of fouling coverage at both sites, including Redang Island and Kuala Kemaman, with the same treatment of antifouling paint at both locations. This is indicated by different environmental factors, such as temperature, salinity, nutrient availability, type of biofouling, water movement, dynamic conditions, and anthropogenic factors. Redang Island is characterized as a relatively isolated marine protected area with a well-preserved coral reef ecosystem that is minimally influenced by mainland runoff and anthropogenic disturbances. In contrast, Kuala

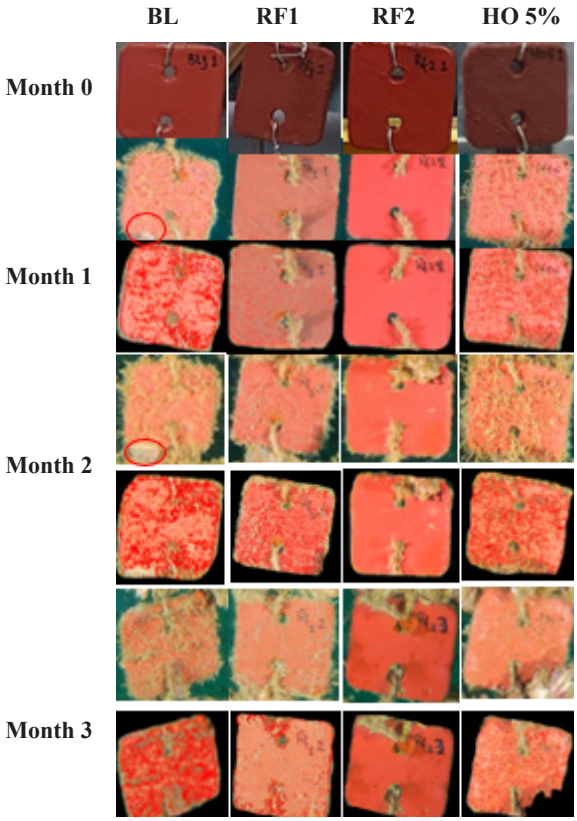

Figure 10 Antifouling coating for three months immersion period at Kuala Kemaman (BL: blank; RF1 reference 1; RF 2: reference 2; HO5%: *H. opuntia* 5%)

Gambar 10 Lapisan *antifouling* setelah periode perendaman selama tiga bulan di Kuala Kemaman (BL: blangko; RF 1: referensi 1; RF 2: referensi 2; HO5%: *H. opuntia* 5%)

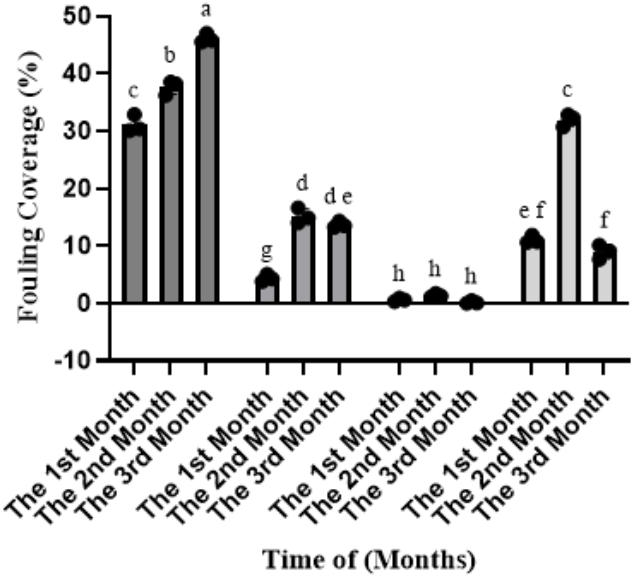

Figure 11 Percentages of the area covered by biofouling after immersion in the Kuala Kemaman (BL: blank ; RF 1: reference 1 ; RF 2: reference 2 ; HO5%: *H. opuntia* 5%).

Gambar 11 Persentase area yang ditutupi oleh *biofouling* setelah perendaman di Kuala Kemaman (BL: blangko ; RF 1: referensi 1 ; RF 2: referensi 2 ; HO5%: *H. opuntia* 5%)

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Kemaman represents a developed coastal and estuarine environment subject to significant freshwater inflow from rivers, resulting in higher turbidity, variable salinity, and elevated pollution levels. These differing environmental conditions support distinct marine ecological communities at both immersion locations, which influence biofouling attachment.

The antifouling action of the lower concentration (5 %) was more potent than that of the higher concentrations. Previous studies, Farina *et al.* (2024) have reported that *Melaleuca cajuputi* methanolic crude extract (MC5% and MC 10% respectively) compare with panels coated with essential oil (EO5% and 10% respectively) that immersed at Tok Jembal Beach and Kuala Kemaman, Terengganu for 3 months period exhibited antifouling coating paint containing 5% MCE produces the best results in combating biofilm. The antifouling coating panel of *H. opuntia* 5% showed better results than the methanolic extract *of M. cajuputi* and *essential oil* panel coating. Ramzi *et al.* (2023) also reported that antifouling paint with *Diadema setosum* crude extract paint with *Sonneratia lanceolata* crude extract paint that immersed in Kuala Kemaman and Redang Island five weeks period revealed antifouling activities in marine field study. Similarly, the antifouling panel of *H. opuntia* also exhibited the best environmentally friendly antifouling paint, as *D. setosum* and *S. lanceolata* crude extracts resulted in lower fouling coverage in all panels. *D. setosum* and *S. lanceolata* 5% showed almost similar fouling coverage in five weeks of immersion, whereas *H. opuntia* 5% showed fouling coverage only after 3 months of immersion.

CONCLUSION

H. opuntia crude extract has antibiofilm activity. *H. opuntia* extract showed no cytotoxicity against L6 cells at different concentrations. *H. opuntia* extract did not exhibit bactericidal effects against *P. aeuroginosa* and *C. violaceum* bacteria in the antibiofilm mode mechanism. The antifouling panel of *H. opuntia* 5% immersed in Redang Island and Kuala Kemaman, Malaysia showed potential antifouling properties due to the reduced macrofouler percentage in the 3rd month) which was lower than that of the commercial antifouling panels of References (RF1 and RF2). Thus, this finding has benefited the maritime industry in solving problems of marine biofouling.

ACKNOWLEDGEMENT

We thank the Institute of Climate Adaptation and Marine Biotechnology, Universiti Malaysia Terengganu, for facilitating laboratory experiments. This research was financially supported by a local Oil and Gas Company (Bandar Baru Bangi, Selangor, Malaysia) with grant number 63931 for 2019-2024 (Prof. Dr. Noraznawati Ismail as Principal Investigator).

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