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ANTI-BIOFOULING ACTIVITY OF SPONGE *Callyspongia pseudoreticulata* COMPONENTS EXTRACT AGAINST *Balanus amphitrite*

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ABSTRACT

Biofouling attached to floating net cages and other aquaculture containers is an obstacle in aquaculture. The presence of biofouling can reduce the quality of the environment and interfere with the health of farmed animals, resulting in large losses. This study assessed the anti-biofouling activity of the Callyspongia pseudoreticulata extract component (EMCp) against the Barnacle Balanus amphitrite. For this purpose, C. pseudoreticulata was extracted with 80% methanol using the maceration method at <40°C to obtain EMCp. B. amphitrite was cultured in an aquarium at 25 ppt. The anti-biofouling activity of EMCp was tested against B. amphitrite larvae using asbestos plates. The study used a Complete Randomized Design with three treatments, namely, A) asbestos plate smeared with EMCp + varnish, B) asbestos plate smeared with varnish, and C) asbestos plate not smeared with EMCp and varnish (control). EMCp toxicity test on the larvae used clear bottles, and the adhesion test using 24-well polystyrene plates. Toxicity observations were done after 24 hours, and the number of dead larvae was calculated. The attachment of the larvae was calculated under a microscope at 10× magnification. Identification of groups of chemical compounds using a thin-layer chromatography chromatogram detected under UV lamps and spraying of reagents. The study found that EMCp effectively inhibited the attachment and growth of B. amphitrites with the LC50 of 150 mg/L. Studies of chemical constituents identified various compounds in the extract, including alkaloids, flavonoids, and terpenoids. These results suggest that C. pseudoreticulata has potential as a natural alternative to chemical-based antifouling agents.

KEYWORDS: Anti-biofouling activity, *Callyspongis pseudoreticulata*, *Balanus amphitrite*, toxicity test

INTRODUCTION

Biological fouling or biofouling is the accumulation of aquatic animals, plants, and microorganisms on artificial surfaces immersed in water (Amara *et al*., 2018). Some examples of biofouling often grow on the surface of submerged substrates, such as bacteria and protists, and macro-organisms, such as invertebrates and algae (Kumar *et al*., 2021). However, the presence of biofouling is an obstacle to cultivation activities. Biofouling can bring huge losses to fisheries and economies worldwide (Luoma *et al*., 2022). In aquaculture, biofouling reduces water circulation and oxygen levels, harming the health and growth of fish, which can translate into significant financial losses. Maintenance costs to clean dirty equipment increase operational costs, whereas in shipping, biofouling leads to higher fuel consumption due to increased resistance, further increasing costs and emissions (Farkas *et al*., 2022; Hadžiæ *et al*., 2022). This biofouling is often attached to cultivation containers such as floating net cages. Biofouling attached to maintenance containers can reduce water circulation and organic matter buildup, thereby reducing environmental quality and interfering with animal

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health (Romeu & Mergulhão, 2023). In fisheries, biofouling causes fish quality to decrease, and even fish move away due to damage to cage nets (Vasileiou *et al*., 2024).

One particularly troubling type of biofouling is Barnacle (Rajitha *et al*., 2020). Barnacle *Balanus amphitrite* is one type of biofouling that adheres to surfaces using strong adhesives and forms hard, calcareous shells, making removing them difficult (Kim *et al*., 2019). Effective management and prevention strategies for biofouling are usually routine cleaning, anti-biofouling coatings, and chemical anti-biofouling agents (Müller *et al*., 2013). The anti-biofouling agents that exist today are highly toxic and can damage non-target organisms, including protected marine species and wider ecosystems. This toxicity can result in a decrease in biodiversity. Tributyltin (TBT) is one of the paints commonly used as an anti-biofouling. Still, it harms the environment because TBT contains toxic materials such as copper, lead, mercury, arsenic, and organotin (Qian *et al*., 2009; Sadan *et al*., 2022). Concerns about the bioaccumulation of toxic antifouling compounds, it is urgent to look for environmentally friendly anti-biofouling agents.

Marine organisms are reported to have non-toxic secondary metabolites, easily degraded in water, and harmless to the marine ecological environment (Qi & Ma, 2017). One marine organism with chemical compounds that can be used for various purposes is the sponge. The sponge can be an antiviral, antitumor, antioxidant, and antibacterial. Secondary metabolites such as steroids, phenolics, terpenoids, carotenoids, peptides, furanone, lactones, and alkaloids isolated from sponges have antifouling activity (Nalini *et al*., 2019).

The current study selected the sponge *C. pseudoreticulata* to investigate further its potential anti-biofouling based on the information that this species of sponge can produce secondary metabolite compounds with varieties of bioactivity and structural variation (Nurdin & Usman, 2017). This study aims to explore the potential anti-biofouling activity of *Callyspongia pseudoreticulata* extract components against the larvae of *Balanus amphitrite* barnacle and identify its chemical constituents. This research is important to find natural and sustainable antifouling agents that can inhibit the settlement or growth of *B. amphitrite*, which hinders the fishing industry.

MATERIALS AND METHODS

Experimental design

The material used is sponge *C. pseudoreticulata*, which was extracted using methanol 80%. Methanol is selected as a solvent for the extraction of bioactive compounds due to its high ability to dissolve a variety of substances, along with its relatively low cost and ease of accessibility. Furthermore, the extraction process using methanol can be performed with high time efficiency. *C. pseudoreticulata* was collected by diving at a depth of about 6 m in the waters around Barrang Lompo, South Sulawesi, Indonesia. Identify the sponge in the reference image (Figure 1) (Rosmiati *et al*., 2011). The sponge was collected and brought to the laboratory using plastic cooled with ice cubes. The sponge was stored in the freezer until extraction. Barnacle, *B. amphitrite* was obtained from around the mouth of the Maros River and collected with the substrate to which it was attached. Barnacles were transported using sacks that had been moistened with seawater. The Barnacle was then kept on seawater media with a salt content of 25 ppt, the same as its original environment. This study was designed using a Complete Randomized Design (CRD) with three treatments and three repetitions, namely: A) asbestos plate smeared with EMCp + varnish, B) asbestos plate smeared with varnish, and C) asbestos plate not smeared with EMCp and varnish (control). Asbestos can withstand high temperatures without degrading, making it ideal for environments where heat resistance is essential, and it is also resistant to many chemicals, including acids and alkalis, which ensures its durability in harsh chemical environments (Abú-Shams & Pascal, 2005; Cristina *et al*., 2024).

Figure 1. *Callyspongia pseudoreticulata.*

Preparation of EMCp

The sponge was left at room temperature and washed with sterile seawater and fresh water to remove any adhering dirt. The sponge was prepared by cutting it into small sizes and drying it in the oven below 40°C. The sponge was then ground into powder form using a blender and extracted with 80% methanol using a forma orbital beater at 37°C until the residue became colorless. The methanol extract was filtered through Whatman No. 1 filter paper installed in Buchner's funnel and collected. The collected methanol extract was concentrated under low pressure using a rotary evaporator (Buchi type) to produce a dark gummy solid. Since sponges come from a saline environment, salts and waxes/lipids must be removed. Desalting was carried out using HP-20. Removing lipids/waxes was carried out by directly passing the salt-free methanol extracts through C-18 column chromatography activated with methanol before. The extract was then eluted with methanol to get interference material-free extract methanol. After concentrating the solvent, EMCp was obtained and continued for screening of anti-biofouling activity at the concentration of 10^3 ppm (Suryati *et al*., 2003).

Anti-biofouling activity Screening of EMCp

To ensure that EMCp had anti-biofouling activity, testing was carried out by mixing EMCp with varnish to determine the ability of bioactive compounds to protect structures from the attachment of biofouling organisms. Asbestos measuring 10 cm × 5 cm was used as a substrate for barnacle larvae settlement. Next, each piece of asbestos was smeared according to the treatment (A, B, and C) and dried for three days. Asbestos fragments were tied with ropes and installed 10 cm below the water's surface in an aquarium containing barnacle larvae (Figure 2) for eight weeks. The observation was done at the end of the study to calculate the percentage of surface cover of asbestos pieces by settled adult barnacles.

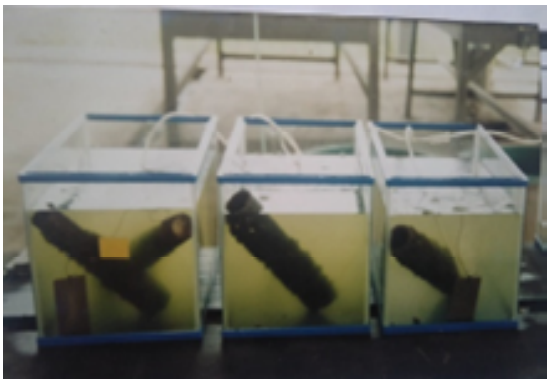

Figure 2. Screening of EMCp antibioufouling activity.

Barnacle culture

Barnacles were cultured using three aquariums containing 20 L seawater, each had been filled with seawater at a salinity of 25 ppt and equipped with aeration. Barnacles were fed plankton-type nannochloropsisevery day. Water changes were carried out as much as 50% every two days. Barnacle larvae were harvested every two days using plankton nets and observed under a microscope until sufficient density was obtained for toxicity and settlement assay.

Toxicity assay

Twenty barnacle larvae were collected by a Pasteur pipette and transferred to a clear bottle volume of 20 mL. The EMCp was diluted in filtered seawater until a stock solution was obtained with a 1,000 mg/L concentration. The EMCp was made using serial dilution from the stock to get final concentrations of 250, 200, 150, 100, 50, and 0 mg/L. These concentrations of EMCp were used to assess the toxicity against the barnacle larvae. In this study, the concentrations of EMCp were carefully chosen based on preliminary screening and established standards in antifouling research. Initial experiments were conducted to assess the baseline activity of the extract at various concentrations. This involved testing lower concentrations (e.g., 10, 50, 100 mg/L) to observe any significant bioactivity. The results indicated that while lower concentrations showed some activity, the higher concentration of 10^3 mg/L yielded the most consistent and significant inhibition of larval settlement. The bottles containing filtered seawater (without any extract) were used as a control. The mortality of larvae in each concentration was counted after 24 h. Each experiment was conducted in triplicate, and the mean percentage of mortality was calculated. The lethal EMCp concentration of 50% larvae was designated LC50 value.

Anti-Settlement activity assay

Evaluation of anti-settlement activity used barnacle larvae in a 24-well polystyrene plate. The polystyrene plate wells were filled with 5 mL EMCp with concentrations of 0, 50, 100, 150, 200, and 250 mg/L in filtered seawater. Next, ten larvae were transferred into each polystyrene plate well. The plate was transferred to darkness at 25 °C. The experiment was conducted in triplicates (for each concentration). The observation of anti-settlement activity was performed after 48 hours in the darkness. The number of barnacle larvae settled on the wells was counted under a dissecting microscope. The settlement larvae percentage was calculated by comparing the number of settled larvae with the number of transferred larvae × 100.

Identification of chemical constituents

A chemical compound group of the active EMCp was identified using a thin-layer chromatography (TLC) plate. As a stationary phase, the TLC was performed on a pre-coated plate with Si.gel F254 (layer thickness 0.2 mm, Merck, Darmstadt, Germany). Liquid mobile phase was semipolar ($CHCl_3$: MeOH; 8:2 v/v). Visual detection was done in daylight and under UV light at 254 and 344 nm. The compounds were also detected by spraying with Dragendorff, anisaldehyde-sulfuric acid, Ferric chloride, and Ninhydrin reagent (Table 1).

Table 1. Detection of compounds by using chemical reagents

Reagents	Preparation	Detection
Dragendorff	Solution A: 0.85 g bismuth (III) nitrate was dissolved in 10 ml of glacial acetic acid and 40 ml of water. Solution B: 8 g potassium iodide was dissolved in 20 ml water. Stock solution: equal parts of A and B were mixed. The mixture can be stored in an amber bottle for longer.	Alkaloids and other nitrogen-containing compounds.
Anisaldehyde sulfuric acid -	Spray solution: before use, 1 ml of the stock solution was mixed with 2 ml of glacial acetic acid and 10 ml water. Spray solution: 0.5 ml anisaldehyde was mixed with 50 ml of glacial acetic acid, and 1 ml of 95 % sulfuric acid was added. Treatment: The plates were sprayed and then heated at 100 - 105 ºC until the maximal intensity of spots was observed. The background color may be brightened by water vapor. Lichen constituents, phenols, terpenes, sugars, and steroids turn violet, blue, red, grey, and green.	sugar, steroids, and other terpene compounds.
Ferric chloride	Spray solution: 1.0 g $FeCl_3$ was mixed with 50 ml of methanol, and 50 ml of H_2O was added.	Phenol
Ninhydrin	Spray solution: 1.0 g $FeCl_3$ was mixed with 50 ml of methanol, and 50 ml of H_2O was added.	Amino acids and Amines

Data analysis

The antifouling test data and the chemical constituent group identification were descriptively analyzed.

RESULTS AND DISCUSSION

Extraction of EMCp bioactive components

The sponge came from a saline environment, so it needed to remove salt and waxes/lipids. The percentage of its interference materials is shown in Table 2.

Table 2. Interference material percentage of *C. pseudoreticulata* methanol extract components (EMCp)

Marine sponge	Dry Weight (g)	Dry Weight of MeOH Extract (g)	Dry Weight after desalted and lipid removal (g)	Percentage of Interference materials (%)
C. pseudoreticulata methanol extract (EMCp)	100	9.92	6.94	30.04

Table 2 shows that interference materials (salt, lipid/waxes) obtained in the methanol extract of MECp were about 30%. Tian *et al*. (2024) supported this, who reported the presence of large quantities of inorganic salts in marine invertebrate extracts from sponges and tunicates.

Anti-biofouling testing of EMCp

C. pseudoreticulata methanol extract-mixed varnish coating (EMCp) can prevent the sticking of adult barnacles to pieces of asbestos. The percentage of closure of asbestos pieces by adult barnacles by each treatment eight weeks after smearing is shown in Table 3.

Table 3 shows that EMCp has anti-biofouling activity against barnacles larvae. It was indicated by the absence of barnacle larvae on plates treated with EMCp+Varnish after eight weeks of soaking. At the same time, two other controls (B and C) were obtained on the surface of full asbestos covered with adult barnacles. In the anti-settlement tests, the extract (EMCp) at a concentration of 10^3 mg/L effectively prevented the settlement of barnacle larvae on asbestos surfaces. In the treatment with EMCp + varnish, no barnacle larvae were able to attach after eight weeks, while the control exhibited 100% surface coverage by adult barnacles. There are two reasons why barnacles cannot settle. First, the smell of *C. pseudoreticulata* extract is very jolting, so barnacles do not like it, and second, the presence of *C. pseudoreticulata* bioactive compounds, which have anti-biofouling properties. It was reported that sponges produce a variety of natural chemical compounds that inhibit the settlement of the cyprid larvae of common fouling Barnacle, *A. amphitrite*, in laboratory assays (Haber *et al*., 2013; Hong *et al*., 2022). Some of these compounds can prevent the growth or replication of other organisms, such as bacteria, algae, or small animals (Karthikeyan *et al*., 2022; Martins *et al*., 2021). These compounds often have strong antibacterial, antifungal, or anticancer properties, which help prevent the attachment of organisms. The anti-biofouling activity of sponges has been widely reported (Aguila-Ramírez *et al*., 2014; Chen *et al*., 2023; Hosie *et al*., 2021; Ravi *et al*., 2023).

Table 3. Antibiofouling activity of *C. pseudoreticulata* methanol extract components (EMCp), varnish, and control against barnacle larvae

Code	Treatment	Average closure by attached barnacle larvae (%)
A	EMCp + varnish	0
B	varnish	100
C	Control (without EMCp and varnish)	100

Toxicity testing of EMCp

The mortality percentage of Barnacle larvae treated with EMCP at various concentrations is shown in Figure 3.

Figure 3 shows that barnacle larval mortality increased with increased EMCp concentrations. The LC50 value of the extract from (EMCp) against *B. amphitrite* larvae is 150 mg/L. This indicates that half of the larval population is expected to die at this concentration after 24 hours of exposure. The high LC50 shown by EMCp against the barnacle larvae indicated that the bioactive principles present in this extract might inhibit the larval barnacle settlement at a lower concentration. As obtained in the preliminary study, the concentration of 10^3 mg/L for EMCp showed no barnacle larvae settlement on the asbestos plate. It indicated that this concentration was toxic against barnacle larvae. The LC50 values found for the EMCp are much lower than the LC50 value for the crude methanol extracts of macroalgae (*Chaetomorpha linum* (199.32 ìg/mL), *Turbinaria ornate* (181.24 µg/mL), and *Sargassum polycystum* (174.737 µg/mL), respectively (Salama *et al*., 2018). On the contrary, the LC50 for EMCp was higher than the LC50 value for sponge *Clathria* sp., *Halichondria* sp., *Callyspongia* sp., and *Jaspis* sp, which were 27.99, 87.57, 112.62, and 117.63 mg/L, respectively (Suryati

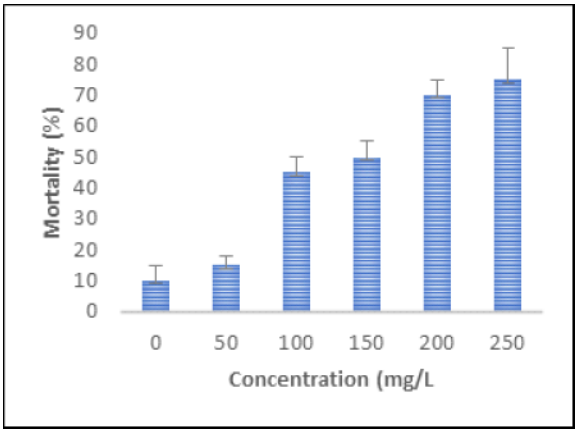

Figure 3. Percentage of Barnacle larvae mortality treated with different concentrations of EMCp.

et al., 2003). Two compounds isolated from *Callyspongia* sp. and identified as 6 NH_2-purine and 4-(1-phenylethyl) phenol were also reported to have a toxic effect on the cyprid larvae of the acorn barnacle *Balanus reticulatus,* which may play an important role in the antifouling activities of the sponge (Wenhao *et al*., 2012).

Barnacle larvae settlement assay

The settlement percentage of barnacle larvae treated with different concentrations at the laboratory condition is shown in Figure 4.

In the 24-well polystyrene plates, 83.3 % of barnacle larvae settled within 48 hours, as shown in the control (without EMCp) (Figure 4). Treatment of barnacle larvae with EMCp could inhibit the settlement on the polystyrene plate well. The percentage of barnacle larvae settled to the plate decreased as the concentration of EMCp given increased. The Barnacle larvae treated with EMCp at 250 mg/mL concentration exhibited the lowest settlement percentage (36.7%). The high mortality suspected of barnacle larvae is due to the toxic effect of EMCp at this concentration, as shown in the toxicity assay (Figure 2). From the data obtained, It could be suggested that the 100-150 mg/L concentration was the safe concentration for settlement assay against barnacle larvae in which LC50 of the EMCp was 150 mg/mL. The effectiveness of EMCp as an antifouling agent demonstrates significant promise when compared to other natural and synthetic antifouling solutions. This sponge-derived extract exhibited an LC50 value of 150 mg/L against *Balanus amphitrite* larvae and effectively prevented barnacle settlement at a concentration of 10^3 mg/L. These results highlight its potential as a viable alternative to conventional antifouling agents. Antifouling defense of this sponge may be due to the production of secondary metabolites on the unwanted epibiotic (Allchurch *et al*., 2022; Mol, 2016), as it is known that barnacles inhabit marine environments around the world. If their growth is not inhibited, it will be very easy to meet the body of ships or other potential substrates in the sea. The application of secondary metabolites of sponges allows cyprids not to find a suitable location to secrete protein cement to attach themselves to metamorphose into sessile barnacles (Rajitha *et al*., 2020).

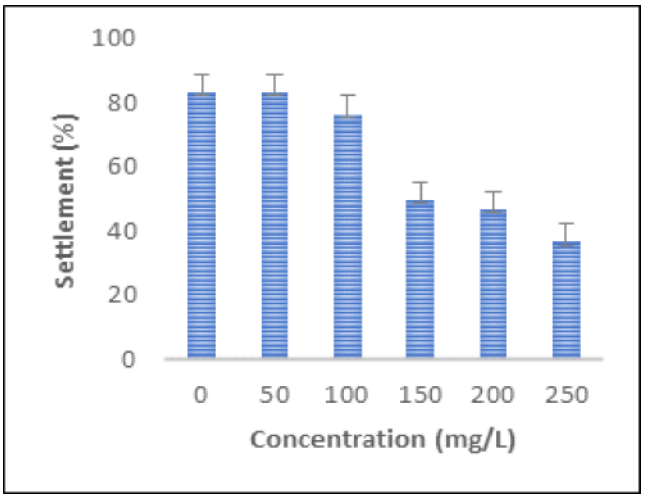

Figure 4. Percentage of barnacle larvae settlement (%) at the different concentrations of EMCp.

Identification of Chemical constituent

Detection of chemical compounds in the methanol extract of Callyspongia pseudoreticulata (EMCp with a UV lamp and spray reagent is shown in Figure 5.

In Figure 5a, the chromatogram under UV_{254nm} light shows spots indicating the presence of carbonyl, phenolic, or other groups with at least two conjugated double bonds. The Chromatogram under UV_{366nm} light showed greenish-yellow fluorescence patches (5b). As Agatonovic-Kustrin *et al*. (2022) reported, blue or green fluorescence spots under UV366 nm indicated a flavonoid group. The occurrence of spots showed the presence of a terpenoid group in the EMCp after being sprayed with anisaldehyde sulphuric acid (5g) (Irianti *et al*., 2011). The EMCp also contained alkaloid groups indicated by orange spots (Rosmiati *et al*., 2016). The anti-biofouling shown by EMCp was suspected due to a terpenoid compound group (Figure 5g) (Hirota *et al*., 1996). Terpenoids are a diverse group of compounds with antifouling activity against marine organisms. Terpenoids can prevent the attachment of marine organisms to submerged surfaces (Avila, 2020). How terpenoids prevent marine organisms from sticking underwater isn't entirely clear, but some research suggests that terpenoids may interfere with the way marine organisms stick and attach to objects underwater. Terpenoids preventing marine organisms from attaching to a substrate are thought to involve disrupting the organism's sensory and adhesive systems. For example, isocyanoterpenoids effectively prevent marine organisms from attaching by inhibiting barnacle larvae from attaching (Avila, 2020). Myrrh-derived terpenoids have also been shown to inhibit marine biofouling by preventing the attachment of marine organisms to surfaces (Pope *et al*., 2008). Additionally, terpenoids have been found to have antimicrobial activity (Guimarães *et al*., 2019) against both antibiotic-susceptible and antibiotic-resistant bacteria, which could also contribute to their antifouling activity (Núñez-Pons *et al*., 2020).

The use of sponge extracts, such as those derived from *Callyspongia pseudoreticulata*, offers several ecological benefits compared to traditional synthetic antifouling agents. These benefits not only enhance marine health but also align with the growing emphasis on sustainability in marine industries such as biodegradability and reduced toxicity to non-target organisms. Harvesting sponge extracts can be conducted sustainably, especially when managed properly. This ensures that the natural populations of sponges are not depleted and can continue to provide their valuable bioactive compounds. In contrast, the production of synthetic antifouling agents often involves resource-intensive processes that can deplete natural resources and contribute to environmental degradation.

While this study demonstrates the promising antifouling activity of *Callyspongia pseudoreticulata* extract, there are several limitations of this study namely; the chemical composition of sponge extracts can vary significantly based on factors such as the sponge's growth conditions, geographical location, and the extraction method used. This variability may impact the consistency of antifouling efficacy across different batches of extract and the study primarily focused on the antifouling activity against *Balanus amphitrite* larvae. While this species is a relevant model organism, the effectiveness of the extract against other fouling organisms remains untested.

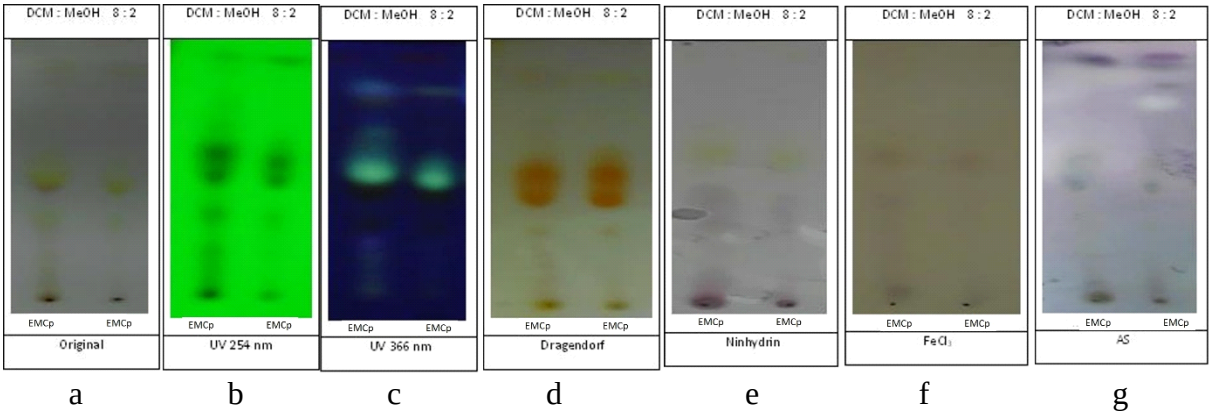

Figure 5. TLC profiling of EMCp at original (a), under UV_{254} nm (b), and UV_{366} nm (c) and after being sprayed with Dragendorf (d), ninhydrin (e), ferric chloride (f), and anisaldehyde sulphuric acid (g).

CONCLUSION

In conclusion, the extract from *Callyspongia pseudoreticulata* demonstrates significant effectiveness as a natural antifouling agent, exhibiting a notable capacity to inhibit the settlement of *Balanus amphitrite* larvae. With an LC50 value of 150 mg/L and successful prevention of barnacle attachment at a concentration of 10^3 mg/L, this sponge-derived extract presents a promising alternative to synthetic antifouling agents. Its environmentally friendly profile, characterized by biodegradability and reduced toxicity to non-target organisms, positions *C. pseudoreticulata* as a viable solution in the quest for sustainable antifouling strategies. Further exploration of its active compounds could enhance its application in marine coatings and aquaculture, paving the way for more eco-conscious practices in managing biofouling.

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