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Studies on Antifouling Activity of Methanolic Extract of the Mangrove *Rhizophora apiculata* against Micro and Macro Foulers

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Abstract: The antifouling activity and toxic properties of mangroves *Acrostichum aureum*, *Bruguiera gymnorhiza, Rhizophora apiculata,* and *Sesuvium potrulacastrum* are documented in this study. Among the extracts prepared using solvents, the methanolic form of *R. apiculata* had the highest antimicrofouling and antimacrofouling properties. The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of *R. apiculata* methanolic extract significantly reduced the development of all the investigated biofilm bacterial strains at 125 g ml^{-1} except for *Vibrio* sp1, *Vibrio* sp2, and *Pseudomonas* sp., where it registered MIC at 250 µg ml^{-1} to 350 g ml^{-1}. The extract's minimum bactericidal concentration (MBC) was determined to be between 250 and 750 µg ml^{-1}. Most of the fouling microalgal strains, such as *Nitzschia* sp., *Amphora* sp., *Tetraselmis* sp. and *Chaetocerous* sp. were found to have had their growth inhibited by the methanolic extract of *R. apiculata*. However, it registered noticeably higher MICs of 400 µg ml^{-1} for *Chaetocerous* sp. and *Dunaliella* sp. The methanolic extract of *R. apiculata* has shown promising molluscicidal activity by strongly reducing the adhesion of the limpet *Patella vulgate* with increasing doses (1-12 mg ml^{-1}). The methanolic extract of *R. apiculata* inhibited the growth of mussel (*Perna indica*) byssus thread and had an LC_{50} value of 424.47± 4.85 µg ml^{-1} after 72 h of exposure. The LC_{50} value for anticrustacean activity of *R. apiculate* methanolic extract was 39.4±1.45 µg ml^{-1} for larvae of *Artemia salina*. The potential antimicro and antimacrofouling activity of *R. apiculata* methanolic extract suggested that it is a good source of antifouling agent, and this knowledge was used to develop a potent eco-friendly antifouling molecule.

Keywords: Biofilm bacteria, *Rhizophora apiculata*, Antifouling, Mollusc foot adherence, Anticrustacean, Mussel bioassay

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Introduction

Aquaculture industries and other marine structures are affected by the complex structure of marine biofouling communities, which is global problem that has a significant ecological effect on marine field activities (Kumar *et al*., 2021; Silva *et al*., 2021). The formation of biofilm is created by bacteria and its key factors for marine fouling which serves as an attachment of sessile plans and animals (McDougald *et al*., 2012). On the contrary, the matured biofouling organisms are tremendously difficult to eradicate and creating wide-ranging complication in marine technology like roughness on the ships' hulls, reducing efficiency, speed and easy to mobility (Manilal *et al*., 2010). A common solution to prevent or avoid fouling is to make substrata unfit for settlement through usage of suitable antifouling paints containing environmentally friendly antifouling compounds. In the previous, mechanical cleaning techniques like brushing and scraping were used as the simplest method for treating fouling. However, this approach is more labor-intensive (Delauney *et al*., 2010). Inorganic and organometallic biocides, such as copper and tributyltin (TBT) compounds are combined with traditional methods to combat marine biofouling, that are primarily used by paints (Rosenhahn *et al*., 2008). On the other hand, numerous biocides have a variety of detrimental effects on the ecosystem and environmental problems. As a result TBT is no longer used in antifouling paints in the twenty first century; instead, copper-based paints are used, even though their toxicity is less than that of TBT. Mangrove plant extracts have historically been used extensively as antimicrobial agents (Shamsuddin *et al.,* 2013; Sivaperumal *et al*., 2022), and there is evidence that they have substantial antifouling activity (Chen *et al*., 2008; Abdel Salam *et al*., 2022). These plants have a various assemblage of bioactive compounds and it has a crucial factor in antifouling (AF) properties, and these components can be used as natural resources for antifoulers and are eco-friendly in nature (Sahoo *et al*., 2012; Mulyani *et al*., 2013; Nandhini and Revathi 2016; Syahputra and Almuqaramah 2019). Few mangrove species have been reported to possess biological properties such as antibacterial (Chandrakala *et al*., 2017), antiviral (Zandi *et al*., 2008), anti-diarrheal (Misra *et al*., 2011), antifeedant (Parthiban *et al*., 2022), insecticidal (Manilal *et al*., 2009) and cytotoxic impact (Han *et al*., 2007). In agreement, mangroves' secondary metabolites, including alkaloids, phenolics, steroids, and terpenoids have been identified. These chemicals have significance for toxicology, pharmacology and ecology (Bandaranayake *et al*., 2002). Very scant information is available on the isolation of antifouling activities from the mangrove and associated species. Therefore, the present study was undertaken to investigate the antifouling potential from three mangrove species (*Bruguiera gymnorhiza*, *Rhizophora apiculata* and *Acrostichum aureum*) and one mangrove associate species (*Sesuvium portulacastrum*) against the micro and macro biofouling organisms.

Materials and Methods

Collection and extraction of antifouling metabolites from mangrove plants:

For the present study, leaves of four different mangrove plants such as *Acrostichum aureum*, *Bruguiera gymnorrhiza*, *Rhizophora apiculata* and associated plant like *Sesuvium portulacastrum* were collected from Pichavaram mangrove (11° 29' N to 25° 55' N latitude and 79° 45' to 38.83' E longitude) forest from Kadaloor District and Manakudy patch mangrove (8.1034° N latitude and 77.4831° E longitude) from Kanyakumari District, Tamil Nadu, India. The collected leaves were thoroughly washed in running water to remove the soil and adherent dirt particles, and then completely dried in air. 100 g of each powdered plant materials were extracted individually with 500ml of three different organic solvents like methanol, ethyl acetate and acetone based on polarity. The extraction was performed in darkness at room temperature (32 ± 2°C). We combined the extracts and ran them through Whatman No. 1 filtration paper. Using a rotary

evaporator and decreased pressure, each filtrate was concentrated until dry.

Isolation of marine biofilm bacteria and identification:

By deploying various experimental panels submerged in water at the Chinnamuttom Fisheries Harbour in the Kanyakumari District of Tamil Nadu, samples of biofilm slime were gathered. Bergey's manual of determinative bacteriology was used to identify the separated biofilm bacterial colonies up to the genus level based on their morphological, physiological, and biochemical characteristics (Lau *et al*., 2002).

Antimicrofouling assay:

Agar is well diffusion technique to assess the antimicrofouling activity of selected mangrove plant crude extracts (Sahoo *et al.,* 2012). Following that, 100 ml of each individual extract (each having 300 mg of crude extract dissolved in DMSO) was added to the experimental wells, while 100 ml of DMSO alone was added to the control wells and both were then incubated for 24 h at 37°C. The test was performed three times, the methanolic extract of *R. apiculata* was found to have superior bioactivity, according to the results of the antimicrofouling activity. Therefore, further research was conducted by using *R. apiculata* methanolic preparation.

MIC of crude extract:

Using 96 well polystyrene plates, the MIC of the crude extract was ascertained by using the Marechal *et al*. (2004) technique. By dissolving in methanol, 100 g of various amounts of *R. apiculata* crude extract (10, 20, 40, 80, 160, and 320 µg ml^{-1}) were prepared. Wells devoid of extracts were used as the negative control and copper sulphate (2, 4, 6, 8, 10 and 12 mg ml^{-1}) as the positive control (culture alone). After adding 100 g of biofilm bacterial culture with a cell density of 2.10^8 cells ml^{-1} to the extract and copper sulphate coated plates, the bacteria were allowed to develop for 48 h at 30°C. The MIC was established as the lowest extract amounts at which no turbidity could be seen in 4 wells.

MBC of crude extract:

The method described by Hasson *et al*. (2011) was used to calculate the minimum bactericidal concentration (MBC) of methanolic extract of *R. apiculata* over biofilm bacterial strains. In a nutshell, a loopful of culture was plated onto newly prepared MHA plates and incubated for 24 h at 37°C for each MIC test dilution. MBC was defined as the maximum concentration in MHA plates where no bacterial strain exhibited signs of development.

Antimicroalgal assay:

According to the methodology described by Iyapparaj *et al.* (2013) *R. apiculata* was used to conduct antimicroalgal activity in 96-well plates. At various concentrations (50, 100, 200, 400 and 600 µg ml^{-1}) and used crude methanolic extract of *R. apiculata* in this test. Copper sulphate (2, 4, 6, 8 and 10 µg ml^{-1}) was used as a positive control and extract-free wells were used as a negative control (culture alone). The microalgal strains such as *Nitzschia* sp., *Amphora* sp., *Tetraselmis* sp., *Chaetoceros* sp. and *Dunaliella* sp. were used in this experiment. After 120 h, the MIC was determined (Thabard *et al.*, 2009).

Mussel bioassay:

The abyssal threads of every brown mussel like *Perna indica*, were completely scraped off prior to the experiment's commencement. According to Wilsanand *et al*. (1999) and Murugan and Ramasamy (2003), ten mussels were added to each beaker holding 100 ml of seawater with the appropriate extract concentrations (50–650 µg ml^{-1}), while seawater without extract served as the control. Three duplicates of the exercise were performed, and following a 24 h experiment. The MIC was measured in both the experimental and control groups as the mussels settled at different concentrations. In order to determine the EC_{50}, the experiment was repeated while considering the MIC discovered in the earlier experiment into account.

Mollusc foot adherence assay:

The assay was carried out in triplicate on a 100 mm petriplate with extract concentrations varying from 1 to 12 mg ml^{-1} and involved five limpets like *Patella vulgata*. One-third of the coated dishes received filtered seawater additions. According to Selvin and Lipton (2002), the immediate foot reflex and mobility were continuously noted higher than or equal to the foot until the foot was totally shrunk. Based on foot spreading or shrinking, the percentage of fouling were determined. The treated limpets were taken out after the exposure time and kept in a new petridish with fresh filtered seawater to observe the 21% recovery rate. The number of limpets that spread their foot relative to the total number of limpets introduced and recorded was used to determine the regaining capacity.

Anticrustacean assay:

The cytotoxicity assay was performed by passing ten *Artemia salina* nauplii through a thin capillary glass tube and placing them in a small test tube with 10 ml of diluted extract at different concentrations (5-320 µg ml^{-1}) that was kept at room temperature for 24 h while being illuminated (Meyer *et al*., 1984). The test was completed in three duplicates. Both the negative control (DMSO) and the positive control (copper sulphate) were used, with amounts ranging from 2.5 to 80 µg ml^{-1} for each. Following a 24 h exposure period, the number of larvae that persisted in each test concentration was enumerated and the LC_{50} values were calculated using a probit analysis.

Qualitative phytochemical analysis:

The qualitative analyses were performed to find out the presence of possible phytochemicals profile in the methanolic extract of *R. apiculata*. The test for Alkaloids, Glycosides, Saponins, Flavonoids, Terpenoids, Steroids, Tannins Carboxylic acids, Quinones, Coumarins, Resins and Phenolics were analysed by standard methods.

Statistical Analysis:

The data obtained in the present study were subjected to the following statistical analysis methods such as mean ± SD, two-way ANOVA, Regression Analysis and Probit analysis by using SPSS 16.0 (SPSS Inc., USA).

Results

In the present study, the results revealed antimicrofouling activity of methanol extract of chosen mangroves. Among the methanolic extracts tested, the *Rhizophora apiculata* exhibited maximum antimicrofouling activity than the other mangrove extracts tested by forming zone of inhibition. The forming zone inhibition ranged from 15 ± 0.82 to 25 ± 1.63 mm. It showed maximum growth inhibitory activity of 25 mm and 24 mm against *Escherchia* sp. and *Aeromonas* sp., respectively; whereas the minimum growth inhibitory activity of 15 mm noticed against *Pseudomonas* sp. (Fig. 1). The statistical two-way ANOVA test conducted for the data on antimicrofouling activity as a function of variation between different biofilm bacterial strains was statistically significant ($F = 8.5467$; $P < 0.001$), similarly variation between methanolic extracts of different mangroves was also statistically more significant ($F = 136.7734$; $P < 0.0001$).

The study was also extended to investigate the antimicrofouling activity with acetone extract, the extract was inferred comparatively lesser antagonistic activity than the methanolic extract. Among the selected mangrove species, the acetone extracts tested with *R. apiculata* have more antimicrofouling activity. The forming zone of inhibition ranged from 9 ± 0.82 to 22 ± 1.63 against the tested biofilm bacterial strains and it showed a maximum growth inhibitory activity of 22 mm against with *Escherchia* sp. However, it showed lesser level of zone of inhibition (9 to 10 mm) against the tested biofilm bacterial strains (Fig. 2). The statistical two-way ANOVA test conducted for the data on antimicrofouling activity as a function of variation between different biofilm bacterial strains as well as variation due to

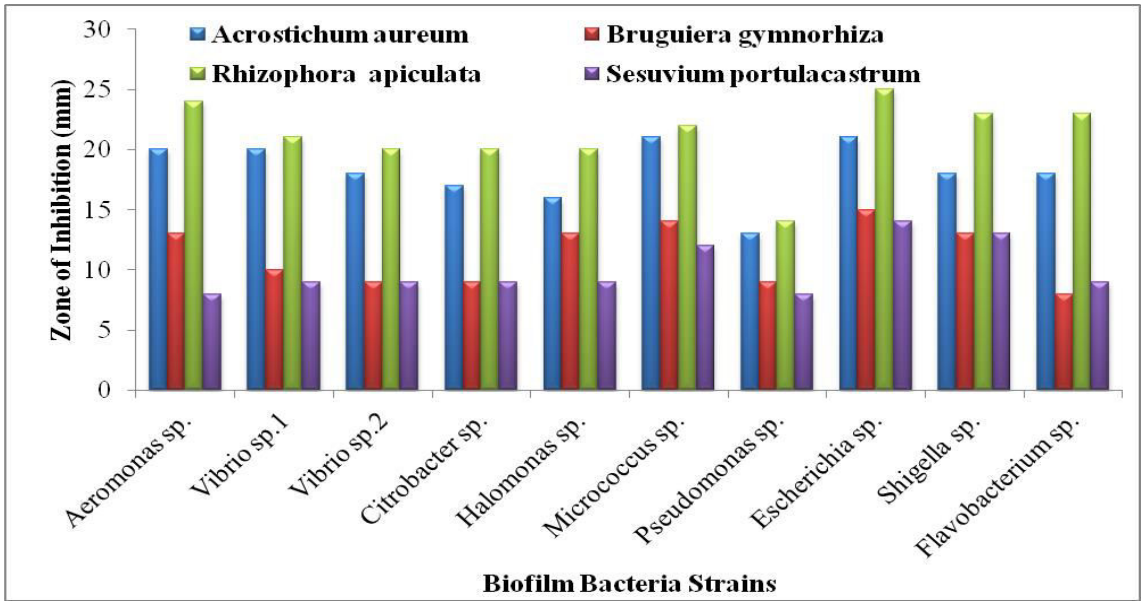

Fig. 1: Antimicrofouling activity of methanolic extract of chosen mangrove plants against biofilm bacterial strains (zone of inhibition in mm).

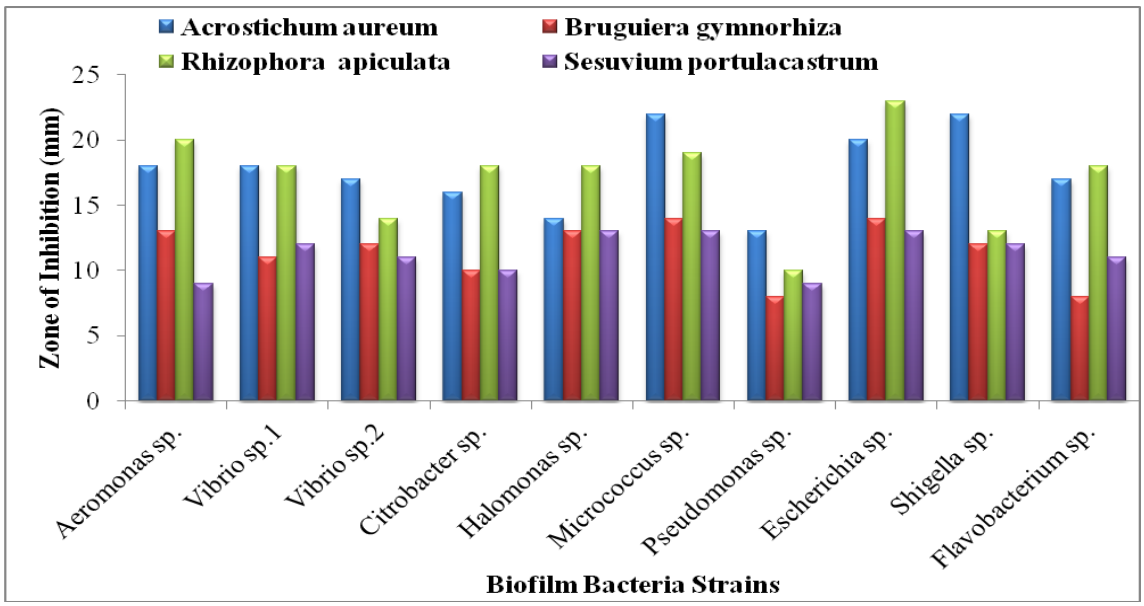

Fig. 2: Antimicrofouling activity of acetone extract of chosen mangrove plants against biofilm bacterial strains (zone of inhibition in mm).

acetone extracts of chosen mangroves were statistically more significant (F= 6.8570 and 56.7420; P < 0.001 to P<0.0001).

Furthermore, the investigation of Antimicrofouling activity in ethyl acetate extracts with chosen mangroves plants showed wide variation in biofilm bacterial growth inhibition. As like that of methanolic extract, acetone extract of *R. apiculata* exerted maximum growth inhibitory activity and attributed zone of inhibition ranged between 8 to 20mm.Moreover, the ethyl acetate extract of *B. gymnorrhiza* and *S. portulacastrum* evidenced moderate bioactivity with the zone of inhibition ranged from 9 to 14 and 9 to 13 mm (Fig. 3). The statistical two - way ANOVA test conducted for the data on antimicrofouling activity

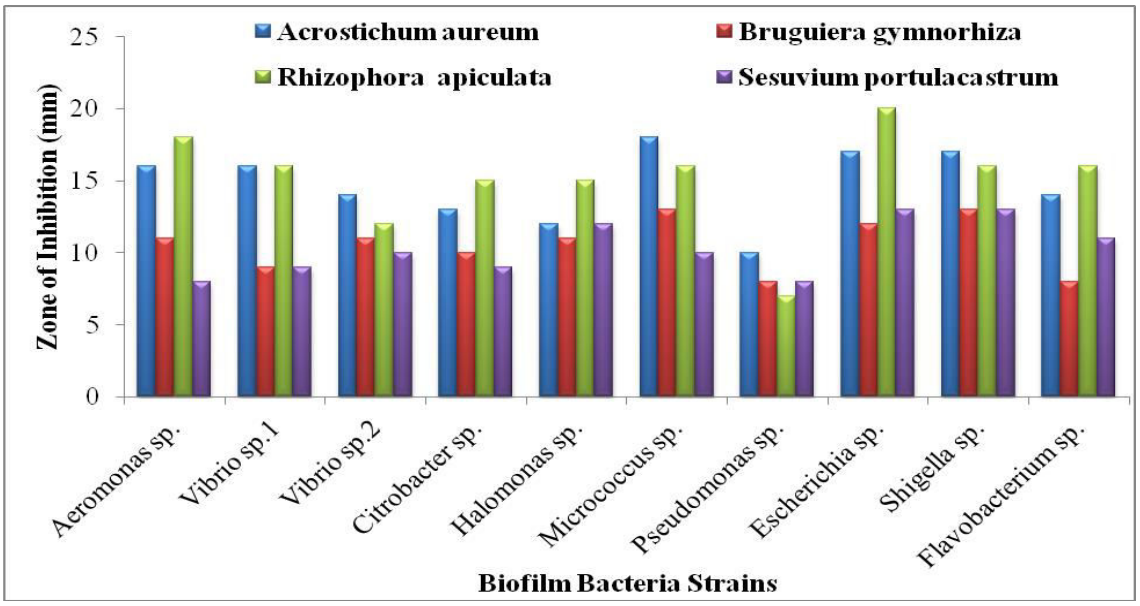

Fig. 3: Antimicrofouling activity of ethyl acetate extract of chosen mangrove plants against biofilm bacterial strains (zone of inhibition in mm).

as a function of variation between different biofilm bacterial strains was statistically significant ($F = 5.8201$; $P < 0.001$); whereas variation between ethyl acetate extract of different mangroves was statistically more significant ($F = 30.8314$; $P< 0.001$). Based on the results of antimicrofouling activity, it was found that methanolic extract of *R. apiculata* had a higher degree of bioactivity against biofilm bacterial strains than other tested extracts. Consequently, it was selected and subjected for further antifouling study.

The crude methanolic extract of *R. apiculata* was tested for its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against the tested biofilm bacterial strains and the results are shown in Figure 4. From the result, it was observed that the crude extract of *R. apiculata* was highly active against the biofilm bacterial strains such as *Aeromonas* sp., *Halomonas* sp., *Micrococcus* sp., *Escherchia* sp., *Shigella* sp. and *Flavo bacterium* sp. and recorded lowest MIC and MBC (125 and 250 µg ml^{-1}, respectively) and thus indicated that it was a significant antimicrobial potent extract. Besides, the same plant extract exhibited relatively increased MIC of 250 µg ml^{-1} and MBC of 500 µg ml^{-1} against other tested biofilm bacterial strains viz. *Vibrio* sp1and *Vibrio* sp2. Whereas, the biofilm bacterial strain *Pseudomonas* sp. was found to have recorded highest MIC and MBC (375 and 750 µg ml^{-1}, respectively).

The antimicroalgal activity (MIC) of crude methanolic extract of *R. apiculata* was tested against microalgal strains. From the result, it was noticed that, the concentration of crude extract required for complete inhibition of microalgal strains ranged between 50 to 400 µg ml^{-1}. Among the tested micro algal strains, *Nitzschia* sp., *Amphora* sp. and *Tetraselmis* sp. were found to be the most sensitive strains (MIC of 200 µg ml^{-1}) than the others (MIC of 400 µg ml^{-1}) strains. Copper sulphate (positive control) exhibited 100% micro algal growth inhibition at the least concentration of 10 µg ml^{-1} (Table 1).

The result of antimacrofouling activity revealed that the crude methanolic extract of *R. apiculata* had significantly inhibited the byssal thread production and attachment of mussel *P. indica*. The minimum inhibitory concentration (MIC) of the crude extract of *R. apiculata* was recorded as 286 ± 4.27 µg ml^{-1}. However, in the case of control group normal byssal thread production and attachment was noticed. On the

Table 1: Antimicroalgal activity (MIC) of crude methanolic extract of *R. apiculata* and copper sulphate

Microalgal strains	Minimum Inhibitory Concentration (µg ml^{-1})	
	Methanolic extract	Copper sulphate
Nitzschia sp.	200	10
Amphora sp.	200	10
Tetraselmis sp.	200	10
Chaetocerous sp.	400	10
Dunaliella sp.	400	10

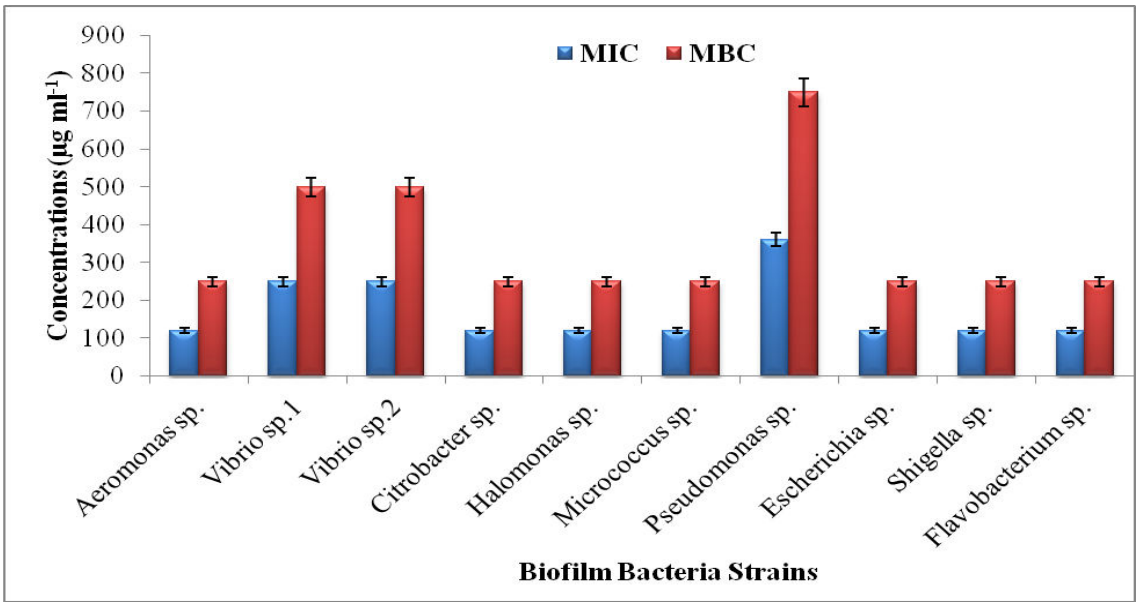

Fig. 4: MIC and MBC of crude methanolic extract of *R. apiculata* against biofilm bacterial strains.

Table 2: Mussel byssal thread attachment and toxic profile of methanolic extract of *R apiculata*

Conc. of the extract (µg/ml)	No. of Mussels exposed	Mussels Unattached (%)	Mussels Attached (%)	Mortality after 24h (%)	Mortality after 48h (%)	Mortality after 72h (%)	LC_{50} (After 72 h)
Control	10	0	100	0	0	0	
50	10	0	100	0	0	0	
100	10	90	0	0	0	0	
150	10	30	70	0	0	10	
250	10	50	50	0	10	20	LC_{50} = 424.47± 4.85
350	10	60	40	0	10	40	
450	10	90	10	10	20	60	
550	10	100	0	20	30	80	
650	10	100	0	30	40	100	

other hand, the effective concentration (EC_{50}) of *R. apiculata* for 50% inhibition of byssal thread production and attachment of mussel was estimated as 263 ± 6.51 µg ml^{-1} after 24 h of exposure. The same mussels subjected to toxic study inferred that after 72 h of exposure, 50% lethal concentration (LC_{50}) value was 424.47 ± 4.85 µg ml^{-1} (Table 2).

The fouling (%) and regaining (%) ability of foot of common limpet *Patella vulgata* against crude methanolic extract of *R. apiculata* and the result obtained is shown in Table 3. The results inferred that *P. vulgata* exposed to 1 and 12 mg ml^{-1} concentration of the extract coated plate

Table 3: Molluscicidal activity of methanolic extract of *R. apiculata* against Limpet *P. vulgata*

Conc. of the crude extract (mg/ml)	Fouling (%)	Regaining (%)	Behavioural changes of Limpets observed during exposure and after in fresh sea water
1	100 ± 0.00	100 ± 0.00	No shrinkage and mobility were noticed. Regained spreading of foot and attached firmly to the substrata.
2	100 ± 0.00	100 ± 0.00	No shrinkage and mobility were noticed. Regained spreading of foot and attached firmly to the substrata
4	90.0 ± 3.62	100 ± 0.00	Shrunken foot was observed in very few limpets on the extract coated plate. After transfer to fresh seawater majority of the limpets showed normal foot spreading and settlement
6	80.36±3.15	100±0.00	Shrunken foot and lack of firm adherence was noticed in few limpets. However, after transfer to fresh seawater adherence of limpets were observed.
8	62.86 ± 2.44	92.62 ± 4.38	In coated plates mobility was noticed at the beginning, but after transfer to fresh seawater >50% regained
10	44.75 ± 2.12	80.24 ± 3.18	In the extract coated plate, lack of foot spreading and adherence of limpet was observed. Foot of few limpets became shrunken and lost their sense of spreading. After transfer to fresh seawater poor regaining of foot was noticed.
12	6.43 ± 1.16	68.30 ± 2.64	Lack of foot spreading and adherence of limpets was observed in the extract coated plates. Foot of considerable numbers of limpets became shrunken and lost their sense of spreading after transfer to fresh seawater.
Copper sulphate (Control-1mg/ml)	26.00 ± 2.00	100±0.00	Shrunken foot and loss of settlement over copper sulphate coated plate was noted. After transfer to fresh sea water regaining and spreading of foot was noted

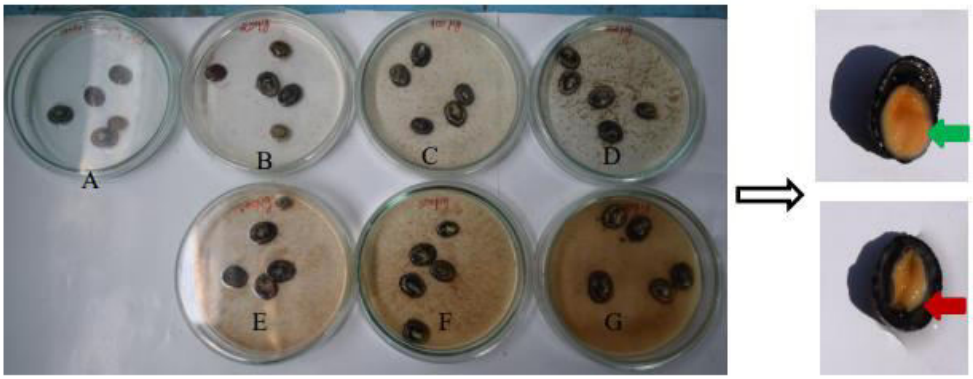

Fig. 5: Mollusc foot adherence assay of methanolic extract of *R. apiculata* against common limpet *P. vulgate*
A – Control (Mollusc - limpet *P. vulgata*) in sterile sea water (without extract); B - 200 µg ml^{-1}; C - 400 µg ml^{-1}; D - 600 µg ml^{-1}; E - 800 µg ml^{-1}; F - 1000 µg ml^{-1} and G - 1200 µg ml^{-1}.
Limpet showing normal spreading of foot and adherence over substrata
Dead limpet showing foot shrinking and lack of adherence over substrata

Table 4: Anticrustacean (*Artemia nauplii*) activity of various concentrations of methanolic extract of *R. apiculata*

Concentration of the extract (µg ml^{-1})	No. of nauplii exposed	No.of responding	LC_{50} (µg ml^{-1})
Control	10	0	39.4±1.45
5	10	0	
10	10	2	
20	10	3	
40	10	5	
80	10	7	
160	10	9	
320	10	10	

Table 5: Anticrustacean activity of copper sulphate over brine shrimp *A. salina* nauplii

Concentration of copper sulphate (µg ml^{-1})	No. of nauplii Exposed	No. of responding	LC_{50} (µg ml-1)
Control	10	0	9.35±0.75
2.5	10	2	
5.0	10	4	
10	10	5	
20	10	6	
40	10	8	
60	10	10	
80	10	10	

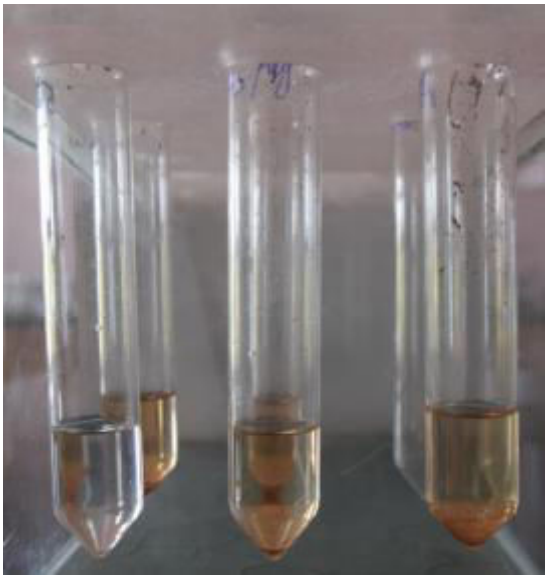

Fig. 6: Anticrustacean assay – Experimental setup.

Table 6: Phytochemical analysis of methanolic extract of *R. apiculata*

S. No.	Phytochemical tests	Results
1	Alkaloids	+
2	Glycosides	-
3	Saponins	-
4	Phenolics	+
5	Flavonoids	+
6	Terpenoids	+
7	Steroids	-
8	Tannis	-
9	Carboxylic acid	+
10	Quinones	+
11	Resins	-
12	Coumarins	+

+ Present; - Absent

showed 100% fouling. The same limpets exposed to fresh seawater after the specified time interval (10 min) showed 100% regaining of foot spreading and adherence. Besides, *P. vulgata* exposed to 4, 6 and 8 mg ml^{-1} of extract coated plate showed 90.0±3.62%, 80.36±3.15% and 62.86±2.44% fouling, respectively. However, after transfer to fresh seawater, the limpets showed 100% regaining of foot spreading and adherence except 8 mg ml^{-1} concentration it showed 92.62 ± 4.38% of regaining of foot spreading and adherence in the substrata *P. vulgata*. Further increasing concentration of the extract up to 10 to 12 mg ml^{-1}, the reduction of fouling rate was 44.75±2.12% to 6.43±1.16% over substrata, but considering regaining was showed 80.24±3.18% to 68.30±2.64% of foot spreading and adherence in the substrata. Copper sulphate was used as positive control, and 1% copper sulphate coated plates showed a 26±2.00% fouling of *P. vulgata,* whereas only 10% fouling was recorded when exposed to a progressive increase in concentration of 2 mg ml^{-1} of copper sulphate, but the foot of limpets shrunken well and did not show any positive regaining (Fig. 5).

The anticrustacean assay was used against *Artemia salina* (nauplii) to test the toxicity of methanolic extract of *R. apiculata.* The LC_{50} value for crude extract was recorded as 39.4 ± 1.45 µg ml^{-1} (Table 4). On the other hand, anticrustacean assay carried out with copper sulphate (positive control) recorded toxicity (LC_{50}) at the least concentration of 9.35 ± 0.75 µg ml^{-1} (Table 5, Fig. 6). However, negative control (DMSO) did not show cytotoxic activity (LC_{50}) > 1000 µg ml^{-1} against *A. salina*. The phytochemical analysis of methanolic extract of *R. apiculata* inferred the presence of phytochemicals such as alkaloids, phenolics, flavanoids, terpenoids, carboxylic acids, quinones and coumarins (Table 6).

Discussion

The microfouling organisms play a critical role in the settlement of macro foulers such as macro algae, barnacle, mollusks, bryozoans, polychaetes, tunicates, coelenterates etc. The formation and attachment of micro and macro fouling communities are influenced by environmental factors and surface nature of immersed substrata (Immanuel *et al.*, 2005). In the present investigation, results of antimicrofouling activity of methanolic extract of chosen mangroves inferred that *R. apiculata* had 100% bioactivity with the zone of inhibition ranged between 15 and 25 mm. Besides, methanolic extract of *A. aureum* exhibited better antagonistic activity with the zone of inhibition ranged from 14 to 21 mm.

However, methanolic extract of other mangroves viz. *B. gymnorhiza* and *S. portulacastrum* tested for bioactivity recorded only 9-16 mm to 9-14 mm growth inhibitory activity against the biofilm bacterial strains. In agreement with the results of the present study, Vadlapudi *et al.* (2009) examined methanol, chloroform and hexane extract of *E. agallocha* against bacterial and fungal strains and inferred that methanolic extract had greater degree of antibacterial activity than chloroform and hexane extracts. Nandhini and Revathi (2016) investigated the antifouling potentials of methanolic extracts of mangroves *R. apiculata* and *B. cylindrica* against *Vibrio parahemolyticus* and *Exiguobacterium profundum* with the zone of inhibition as 24 mm, and the methanolic extract of *R. apiculata* had a higher antagonistic activity in those species. Consequently, Ramasubburayan *et al.* (2015a) and Ramasubburayan *et al.,* (2017b) have also reported that, methanolic extract of leaf of *E. agallocha* exerted good antibacterial spectra against a panel of fouling and pathogenic bacterial strains.

In the present investigation, MIC and MBC recorded for crude methanolic extract of *R. apiculata* against biofilm bacterial strains showed significant variation. Among the ten biofilm bacterial strains, *Escherchia* sp., *Micrococcus* sp., *Aeromonas* sp., *Shigella* sp., *Flavobacterium* sp. and *Halomonas* sp. were the most sensitive to crude extract of *R. apiculata*, which recorded lowest bacteriostatic (MIC) and bactericidal (MBC) concentrations of 125 and 250 µg ml^{-1} respectively. Besides, the same plant extract exhibited relatively increased MIC of 250 µg ml^{-1} and MBC of 500 µg ml^{-1} against other tested biofilm bacterial strains viz. *Vibrio* sp1 and *Vibrio* sp2, whereas the biofilm bacterial strain of *Pseudomonas* sp. have to be found in highest MIC and MBC (375 and 750 µg ml^{-1}). Scherrer and Gerhardt (1971) reported that gram-positive bacteria are more sensitive than gram-negative bacteria towards different antimicrobial compounds, because of the difference in the structure of their cell walls. But in the present study, crude extracts of *R. apiculata* was found to be effective against both gram positive and gram-negative bacteria. In the present study copper sulphate was used as positive control. Indeed, it is one of the most commonly used biocides in the antifouling paints. The result implied that, the biocide copper sulphate had recorded MIC and MBC at very low concentration of 6 and 10 µg ml^{-1}, respectively against majority of the test biofilm bacterial strains. The observed MIC and MBC values were too low when compared to the crude extract of *R. apiculata*, which indicated the toxic effect of copper sulphate.

Thus, in the present study, the copper sulphate concentrations affecting the test biofilm bacterial strains were in the ranges reported in the literature. Marine algae play an important role in fouling with the wide range of immersed artificial substrata, particularly in shallow water and it was sufficient light to permit the active growth of algae. Although there are many diverse phyla of marine algae, only a limited number are considered to be important as fouling organisms. According to Fletcher (1995) who stated that, the *Chlorophyta*, *Rhodophyta* and *Cyanophyta* are three major phyla which were described as primary colonizers succeeding biofilm bacterial strains during the biofouling process. Both toxic and environmentally friendly antifouling paints tend to be effective against most of the fouling organisms, yet fail badly to inhibit diatom slimes (Molino and Wetherbee, 2008). Concerning the antimicroalgal activity, present study clearly proved that the crude methanolic extract of *R. apiculata* had effectively inhibited the growth of test micro algal strains with the MIC's ranged from 50 to 400µg ml^{-1}. Reports related to antimicroalgal activity from marine plants have demonstrated that seaweed extracts potent source towards growth inhibition of fouling microalgal strains. Therefore, this might be the first study which evidently substantiated that crude methanolic extract of *R. apiculata* may be a potent source of antimicroalgal activity. Thus, the promising

antimicroalgal activity exhibited by methanolic extract of *R. apiculata* ascertained that, the polar compounds localized in the methanolic extract has actively participated in growth inhibition of test microalgal strains. On the other hand, effect of varying concentration of biocide copper sulphate against microalgal strains apparently demonstrated remarkable reduction of microalgal growth and recorded MIC at 10 µg ml^{-1}. Toxicity is expressed as LC_{50} value, which is the concentration of a compound that kills 50% of the target organisms when administered as a single exposure. Toxicity bioassays are compulsory to get toxicological information of antifouling compounds. For antifouling formulations, toxicity tests are usually performed towards a wide range of organisms including microalgae, *Artemia* sp., oysters, barnacles, mussels, sea urchins, ascidians, fish larvae and cells of mammals. To be selected as a new promising antifouling compound, the new products need to have an effective concentration $EC_{50}<LC_{50}$ (Dahms and Hellio, 2009; Marechal and Hellio, 2009).

Microbial films have generally been examined as a stimulus for the settlement of macrofouling organisms (Crisp, 1974). Among macrofoulers, mussels are one of the rapidly attaching secondary colonizers, which cause serious problems by settling on man-made surfaces (Waite and Tanzer, 1980). Once firmly attached mussels strongly resist detachment by wave action and currents (Cope *et al*., 1997). Laboratory assays using mussels as model organisms in fouling studies have been reported during the last two decades (Da Gama *et al.*, 2003). Mussels are used as one of the bio-indicators to study the antifouling potency against the macro organisms. Therefore, assessment of toxicity of marine bioactive compounds is most vital for induction of compounds in antifouling paints (Murugan and Ramasamy, 2003). The mussel bioassay carried out in the present study is most rapid and reliable assay method in which inhibition of byssal thread production and attachment was alone taken into account. Also, this method has the advantage to evaluate several concentrations of the extract at the same time.

In the present investigation, the crude methanolic extract of *R. apiculata* has completely inhibited byssal thread production and attachment of brown mussel *P. indica* and recorded MIC at 424.47 ± 4.85 µg ml^{-1}. The result of the present study was corroborated by the findings of Ramasamy and Murugan (2007) and Rittschoff *et al.* (1992) who have reported that comparison between LC_{50} and EC_{50} values provided a relationship between toxicity and settlement inhibition. Toxic biocides such as copper and tributyltin were found to have similar LC_{50} and EC_{50} values (Wilsanand *et al.*, 1999). Furthermore, the result of recovery assay evidenced 100% recovery of experimental mussels that were exposed to test concentration for EC_{50} assay within 24 h transfer to extract free fresh seawater. This result supports the previous findings of Wilsanand *et al.* (1999), as they examined antifouling efficacy of extracts of several sedentary invertebrates over green mussel *P. virdis* and observed 100% recovery of test mussels that had been exposed to extracts of gorgonian coral *Gorgonella sanguinolenta* and *Anthipatharian* sp. (Unidentified species No. 2) and substantiated non - toxic nature of the extract.

In the present study, marine crustacean *Artemia salina* nauplius was used as model organism to study the cytotoxic property of the methanolic extract of *R. apiculata*. Many earlier studies that have been carried out using *Artemia nauplii* indicated that it could be used as reference organism to evaluate the toxicity of plant extracts (Meyer *et al.,* 1982), antifouling paints and toxic biocides (Barahona and Sanchez-Fortun, 1999). Toxicity against *Artemia* may be an indication of potency against marine fouling organisms, more specifically; crustacean foulers (Persoone and Castritsi-Catharios, 1989). From the experimental result, it was found that methanolic extract of *R. apiculata* tested for toxicity over *A. salina* nauplii had LC_{50} value of 39.4 ± 1.45 µg ml^{-1} after 24 h of exposure. Many of the secondary metabolites

biosynthesized by the marine plants are well known for their cytotoxic property (Manilal *et al.,* 2009). In general, production of secondary metabolites (phytochemicals) in marine plants was reported to be changing with respect to ecological condition and nutrients availability. Therefore, this may be the reason for variation in lethality (LC_{50}). Anticrustacean study carried out with copper sulphate (positive control) exhibited toxic effect at least concentration of 9.35 ± 0.75 µg ml^{-1}. This was corroborated by the findings of Ortega-Morales *et al.* (2008), who recorded the LC_{50}value of copper sulphate (l%w/v) at very low concentration of 3.2 µg ml^{-1}. Mollusc foot adherence assay is a reliable assay methodology determining the antifouling activity of marine natural products. At different concentrations (2 to 12 mg ml^{-1}) of crude extract coated plate exposed to limpet *P. vulgata*; it was observed that with increase in progressive concentration, there was a gradual increase in inhibition of fouling from 0 to 30%. Similarly, considering the regaining percentage with increase in concentration (1 to 12 mg ml^{-1}) of extract, there was a consistent decrease in regaining from 100 to 26.43%. From the result it was noticed that *P. Vulgate* exposed to 12 mg ml^{-1} concentration coated plate had lost its mobility, undergone shrinking of foot and hence it could not adhere to the substrata firmly. The significant fouling deterrence (44.75-80%) exhibited by the methanolic extract of *R. apiculata* against *P. vulgata* at 10 – 12 mg ml^{-1} concentration could thus prove that it has molluscicidal activity. The result obtained in the present study was in consonance with the result of Manilal *et al.* (2009), who pointed out that, methanolic extract of mangrove *A. marina* has shown 100% molluscicidal activity at 6 mg ml^{-1} concentration, at the same concentration it has also shown 80% regaining of foot spreading and adherence. Extracts from mangrove plants and associates have been used worldwide for medicinal purposes, and having been recorded around 349 metabolites turned out to be a rich source of steroids, triterpenes, saponins, flavanoids, alkaloids and tarmins (Bandaranayake *et al*., 2002; Pakhathirathien *et al*., 2005; He *et al.*, 2007). In the present study, phytochemical analysis of crude methanolic extract of *R. apiculata* revealed the presence of various biologically active substances such as alkaloids, phenolics, flavanoids, terpenoids, carboxylic acids quinones and coumarins. In accordance to the result of the present study, Behbahani *et al.* (2017) repoted the presence of phytochemicals such as tannins, saponins and alkaloids in the extract of mangrove leaf of *A. marina.*

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