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Leaves of *Rhizophora mucronata* hold quorum quenching moieties functional on the shrimp pathogen *Vibrio harveyi* as the prospective drug of choice in aquaculture

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ABSTRACT

The study is directed towards the effect of *Rhizophora mucronata* mangrove leaf extract on the quorum sensing capability of *Vibrio harveyi,* an aquaculture pathogen responsible for vibriosis in shrimp. A significant reduction of luminescence, biofilm formation and virulence have been detected with the 4th HPLC fraction of the extract, which resulted in the inhibition of the virulence of the pathogen. Anti-quorum sensing potential of *R. mucronata* fraction 4 in reducing the biofilm of *Vibrio harveyi* was evaluated through crystal violet assay and microscopy. A concentration of 0.5 to 1 µg/ µl of HPLC fraction 4 showed 49.6% to 59.5% biofilm inhibition, respectively. A visible reduction in the biofilm was evident using microscopy. The challenge trials with *Vibrio harveyi* showed a marked reduction in the mortalities of *Artemia* nauplii as the model organism, which summarized the anti-quorum sensing ability of *R. mucronata* extract fraction 4 in enhancing the survival rate of *Artemia* by decreasing the virulence of the pathogen. It has validated its quorum quenching abilities with *Chromobacterium violaceum* MTCC 2656, a model organism for QS research, thanks to its easily observable and quantifiable marker trait (violacein production). Moreover, on putting together all the information available, it has been evident that *R. mucronata* HPLC fraction 4 contains discrete compounds/molecules such as Shogaol, Naringin, Kaempferol-3-*O*-rutinoside, Rutin and Quercetin having quorum quenching property either independently or synergistically on *V. harveyi.* It has been concluded that this fraction can serve as the source of one or more quorum quenching compounds on *V. harveyi* as the drug of choice in aquaculture. This is the first report of quorum quenching property in the leaves of *R. mucronata* effective on the shrimp pathogen *V. harveyi*.

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1. Introduction

Mangroves are orchards of salt-lenient trees and shrubs along tropical and subtropical seashores. These patches of unique, productive ecosystems are storehouses of unexplored biological potential. Many species of flora and fauna endure with the substratum provided by the mangroves alone. They outspread the ecosystem possibilities above the water level as a canopy and below the water level as pools of nutrient-bedded accommodation, providing an abode for the aquatic organisms. The contributions of mangroves towards perpetuating offshore fish population by serving as nursery ground are substantial (Nagelkerken et al., 2008).

Tropical Indian mangroves are with staggering anti-inflammatory properties and can serve as sources of a wide variety of phytochemicals such as tannins, terpenoids, alkaloids and flavonoids that have been used to treat rheumatism, smallpox, ulcers, hepatitis, leprosy, asthma, snakebite, toothache, haematuria, diarrhoea, dysentery, and leprosy (Lalitha et al., 2019; Puspitasar et al., 2012). Among them, *Rhizophora* is the most abundant genus with a variety of species such as *Rhizophora apiculata*, *R. conjugata*, *R. mucronata*, *R. mangle*, and. *R. stylosa* having several medicinal properties (Balasubramanian et al., 2015), and *R. mucronata* found in Central Kerala (Ernakulam district) and Northern Kerala (Kasaragod, Kannur, and Malappuram districts (George et al., 2019) is the most abundant species. Alkaloid rich fraction of *R. mucronata* has inhibitory action against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Candida albicans, Aspergillus fumigatus* and *Aspergillus niger* (Kusuma et al., 2012; Nurdiani et al., 2012.

The ever-growing demand for food and nutritional security has helped aquaculture to gain momentum, transforming itself from an unorganized extensive to a well-organized semi-intensive and intensive mode adopting the best available management practices. Nevertheless, diseases caused by bacterial, fungal, and viral pathogens and metazoan and protozoan parasites still haunt the sector. Among the bacterial pathogens *V. harveyi, V. parahaemolyticus,* and *V. vulnificus* are the prominent ones responsible for the outbreak of vibriosis in aquaculture (Igbinosa and Okoh, 2008). Among them, the vibriosis caused by *V. harveyi* leads to massive losses in hatcheries as well as in grow outs. Over the years, farmers resorted to antibiotics as prophylactics and therapeutics since no alternatives were available. Their continuous and extensive use resulted in the development of multiple antibiotic resistance in bacteria in aquaculture systems (Preena et al., 2020). This has become a global concern as drug resistance is transferable and plasmid-mediated, and there are opportunities for this to happen in the case of human pathogens, too (Preena et al., 2020). In this context, management options other than antibiotics are in focus. Quorum sensing disruption (quorum quenching) has been one of the methods under consideration, as the pathways which regulate several virulent genes responsible for the pathogenicity of the bacterium are linked with it

(Bramhachari et al., 2019). The virulence of the pathogenic strains of *V. harveyi* is closely associated with the expression of luminescence (Karunasagar et al., 1994) and a typical transcriptional level control in their quorum-sensing system regulates the QS phenotype (Bassler, 1999). As anti-quorum sensing compounds can inhibit bacterial pathogenicity, it has been postulated that by inhibiting the quorum sensing system, the possibility of managing vibriosis in aquaculture can be very much utilized (Bramhachari et al., 2019).

Several works are centered on the anti-QS possibility and several such natural compounds are now known for their anti-QS potential. They include ethanolic extracts of red seaweed *Gracilaria fisheri* with immune-stimulatory and antimicrobial activity, protecting *P. monodon* from *V. harveyi* (Kanjana et al., 2011). A further study opened the natural compound behind the activity as N-benzyl cinnamamide, a potent AI-2 inhibitor (Karnjana et al., 2020). Another AI-2 channel inhibitor was isolated from a sponge *Leucetta chagosensis* (Mai et al., 2015). A non-psychoactive cannabinoid cannabigerol (CBG) present in the cannabis plant was identified for its anti-QS potential without affecting the planktonic bacterial growth (Aqawi et al., 2020). The antibacterial property in *R. apiculata*, *R. mucronata* and *R. mangle,* especially to *V. harveyi* has also been well documented (Manilal et al., 2015; Kannappan et al., 2018). Baskaran and Mohan (2012) reported the capability of *R. mucronata* to increase the survival rate of tiger shrimp, which helped protect them from diseases. Studies also have revealed the potency of phytochemicals in the plant as the safest possible quorum quenchers (Vattem et al., 2007). Even though the methanolic extract of *R. mucronata* is known for its anti-bacterial activity (Ramesh et al., 2014), its quorum quenching property has not yet been elucidated. This paved the way for conceptualizing the present work with *V. harveyi* as the target pathogen.

2. Materials and Methods

2.1. Collection and preservation of *R. mucronata*

Aerial foliage of *R. mucronata* was sourced from Fine Arts Avenue, near the boat jetty (9°57'51.1"N, 76°16'56.8"E) in front of the Lakeside Campus, CUSAT. The specimen was identified based on phenotypic characteristics (Yong, 2014) and was washed thoroughly in running tap water, shade dried and preserved at room temperature in an air tight zip lock cover.

2.2. Extraction of bioactive compounds from *R. mucronata*

The shade-dried leaves of *R. mucronata* were finely ground and subjected to defatting with 100% hexane (Merck, HPLC grade). The defatted leaves (100g) were extracted with 250 ml 100% methanol (Merck, HPLC grade) in Soxhlet apparatus at 60°-70°C for 4 hours. The concentrated methanol extract was further brought to n-butanol saponin based extraction by separating with water saturated n-butanol in a separating funnel following Silva and Lee (1998). It was then dried in a rotatory evaporator at 35°C, re-dissolved in fresh methanol and stored at 4°C.

2.3. Target bacterial isolates and growth conditions

The target pathogenic bacterium *V. harveyi* (MTCC 34380) was grown in ZoBell's Marine broth (pH 7.2± 0.2) at 27 °C. The experiment used 18-24-hour bacterial culture adjusted to Abs 0.1 at 600 nm (1.8×10^9 CFU/ml). The purity of the culture was checked by streaking on ZoBell's marine agar plate, Gram stained and observed under 100 X oil immersion objective (Olympus optical microscope).

2.4. Qualitative and quantitative quorum quenching analysis with crude methanolic extract

The shrimp bacteria *V. harveyi (*MTCC 3438) was used as the target pathogen for the anti-luminescence assay. The activity of crude methanolic extracts of *R. mucronata* was analyzed by agar disc diffusion. An aliquot of 1mg/ml extract-impregnated discs were placed over *V. harveyi* (Abs 0.1) swabbed plates, and after 12th hour of incubation observed for zones of luminescence inhibition and the diameter was measured. Simultaneously performed the quantitative bioluminescence inhibition assay, in which 2% (v/v) of *V. harveyi* broth culture (4µl; Abs 0.1 at 600nm) was inoculated to 200µl ZoBell's Marine broth in a 96 well plate and incubated at room temperature in the presence of *R. mucronata* crude extract which provided a final strength of 1µg/µl culture. Bioluminescence intensity was measured in terms of relative light units (RLU) in Varioskan LUX multimode microplate reader (Thermo fisher scientific) at 14th hour of incubation. The percentage inhibition was calculated based on the relative light Units (RLU) obtained.

2.5. HPLC-based separation of the active fraction

The crude extract of *R. mucronata* was subjected to fractionation in a semi preparatory HPLC column (Luna 5µ C18 (2) 100A 250×10.00mm 5-micron Phenomenex). An aliquot of 150µl of the sample was loaded and a linear gradient mobile phase containing water (A) and acetonitrile (B) was applied to have 0 to 25% B for first 5 minutes, held 90% B for the remaining 35 minutes, and in the next 2 minutes the gradient was brought back to 0. The flow rate was 2.5 ml/minute. The diode array detector wavelength was set at 254nm, 280nm, 210nm and 225nm, and the solvents were used after degassing in a sonicator. The peak pattern was analyzed using Chromeleon 6.8 software (Dionex, USA).

2.6. Identification of the active HPLC fraction

An aliquot of 4 µl (2 % (v/v) of *V. harveyi* cells at the aforesaid cell density (0.1Abs at 600nm) was inoculated into 200 µl appropriate growth medium (ZoBell's agar) and cultivated in the presence of each HPLC fractions having normalized concentration of 2µg/µl and incubated (27^0C) for 12 h. After incubation, the bioluminescence intensity was measured regarding relative light units (RLU) in Varioskan LUX multimode microplate reader (Thermo fisher scientific). The percentage of bioluminescence inhibition was calculated based on RLU reading.

2.7. Determination of minimum inhibitory concentration (MIC) of fraction 4

The MIC of fraction 4 of the extract was determined following the method of Thenmozhi et al. (2009).

Accordingly, 4 µl (2 % (v/v)) of the test pathogen (Abs 0.1 at 600 nm) was inoculated to ZoBell's Marine broth supplemented with diluted fraction 4 to attain final concentrations ranging from 1 to 0.00012 µg/µl in the wells of microtitre plate (MTP), and incubated for 24 h. The MIC was recorded in Varioskan LUX multimode microplate reader (Thermo fisher scientific) at 600nm, as the minimum concentration of the fraction with growth inhibition. The results were confirmed by diluting the samples up to 10^{-9} and the colonies were counted following the drop plate method in TCBS agar (Herigstad et al., 2001).

2.8. *Vibrio harveyi* bioluminescence inhibition assay

For bioluminescence inhibition assay, an aliquot of 4 µl (2 % (v/v) *V. harveyi* culture at the aforesaid cell density (0.1 OD) was inoculated into 200 µl appropriate growth medium (ZoBell's agar) and cultivated in the presence of fraction 4 (1- 0.00012 µg/µl) and incubated (27^0C) for 12 hours. After incubation, the intensity of bioluminescence was measured in terms of relative light units (RLU) in Varioskan LUX multimode microplate reader (Thermo fisher scientific). The percentage of bioluminescence was calculated based on the RLU reading (Pakiavathy et al., 2013).

2.9. Assessment of inhibition of biofilm formation *of V. harveyi*

Inhibition of biofilm development was evaluated through crystal violet (CV) assay (Musthafa et al., 2010). Concisely, 20 µl *V. harveyi* culture (Abs 0.1at 600nm) was added into 1ml ZoBell's growth medium in a six welled MTP and cultured in the presence of *R. mucronata* fraction 4 (1-0.00012 µg/µl) in static condition for 16 hours. The plates were washed three times with sterile deionized water to remove loosely attached cells following incubation, air-dried, stained with 200 µl of 0.4 % aqueous crystal violet for 15 minutes and washed three times with deionized water. The crystal violet in the stained cells was solubilized with 1 ml of 95 % ethanol and the absorbance was determined at 650 nm using a UV visible spectrophotometer (Hitachi U-2800, Japan). Percentage biofilm inhibition was calculated as the difference between the control and test Absorbance.

2.10. Confirmation of Quorum quenching property

2.10.1. Quorum quenching property in fraction 4 using *Chromobacterium violaceum* MTCC 2656.

The wild-type strain *Chromobacterium violaceum* MTCC 2656 with the two-component QS system was cultured in Luria Bertani (LB) broth and incubated for 24 h at 28 °C.

The activity of fraction 4 of *R. mucronata* were analyzed by quantitative violacein inhibition assay using 96 welled MTP, in which 2% (v/v) of bacterial broth culture (4µl; Abs 0.1 at 600nm) was inoculated to 200µl of Luria Bertani (LB) broth at room temperature in the presence of plant extracts which provided a final strength of 1 µg/µl. The absorbance was measured at 585nm in Varioscan LUX multimode microplate reader (Thermo fisher scientific) at the initial and final stages after 24 hours of incubation. The percentage of violacein inhibition was calculated based on the absorbance of untreated bacterial control. The percentage inhibition was calculated based on the absorbance of untreated bacterial control.

Results were further validated by qualitative agar disc diffusion assay for which aliquots of 1µg/µl extract-impregnated discs were placed over *C. violaceum* (O.D 0.1) swabbed plates. After that, plates were kept at 30°C for 24 h, followed by observation of clear zones, which indicated the inhibition of both growth and quorum sensing, and opaque zones, which indicated the inhibition of quorum sensing alone. All the zones were measured in millimeters (mm) against a violet background of bacterial growth. A negative control (1% DMSO) was included in every set of experiments.

2.10.2. Antimicrobial analysis of *Chromobacterium violaceum* MTCC 2656 through resazurin assay

To define the bacterial concentration in the sample, resazurin assay was performed. After overnight incubation of each extract treated bacterial cultures at 37°C, 5 µl resazurin (6.75 mg/ml) was added to all wells and incubated at 37°C for another 4 h. Changes of colour were observed and recorded. The fluorescence (RFU) of microbial-generated resorufin was recorded at λ_{ex} = 520 nm/λ_{em} = 590 nm after 4 h of resazurin dosing to all wells.

2.11. Microscopic analyses of biofilm

A comparative analysis of *V. harveyi* biofilm developed over the cover glass was done. Aliquots of *V. harveyi* culture adjusted to O.D 0.1 at 600 nm were incubated over a cover glass (1 cm^2) in the wells of MTP containing 1 ml ZoBell's broth at 27 °C for 24 hours in the presence of 1 - 0.5µg/µl fraction 4 of the extract. After incubation, the biofilm on the cover glass was stained in the pattern above and observed under the light microscope (Olympus).

2.12. Growth curve and bioluminescence kinetics

An aliquot of 100µl (2 % v/v) overnight culture of the test pathogen (0.1 OD; 600 nm) was inoculated into a test tube containing 5ml of ZoBell's broth supplemented with various concentrations (0.125-1µg/µl) of *R. mucronata* fraction 4. The test tubes were incubated at 27 °C for 24 hours. The cell density was measured using UV–visible spectrophotometer by checking Abs at 600nm from 12^{th} to 16^{th} hour along with luminescence which was measured in terms of relative light units (RLU) in Varioskan LUX multimode microplate reader (Thermo fisher scientific). The percentage of bioluminescence was calculated based on the RLU reading (Pakiavathy et al., 2013).

2.13. *In vivo* challenge experiment in *Artemia* nauplii

In vivo experiments were executed with freshly hatched nauplii of *Artemia franciscana*. A quantity of 5 milligram cysts was hydrated in 100 ml sterile seawater for 2 hours, decapsulated and well-aerated for 24 hours. The freshly hatched healthy *Artemia* nauplii were collected and used for the challenge test (Ravi et al., 2007; Brackman et al., 2008). Ten first instar *Artemia* nauplii were introduced to each well of the 6 well plates filled with 5 ml of filtered seawater having a salinity of 35ppm at room temperature. The three set wells were maintained with the test fraction 4 at a concentration of 0.25-1µg/µl for 24 hours in the rearing

medium to facilitate the intake of the anti-QS compound by *Artemia* nauplii. The treated *Artemia* nauplii were exposed to *V. harveyi* at the aforementioned cell density (1.8×10^9 CFU/ml) after 24 hours. Appropriate positive control with *Artemia* and *V. harveyi* and negative controls with *Artemia* alone were maintained. The experiments were performed in triplicate, and mortality in each set was recorded at an interval of 72 hours.

2.14. HPLC based separation and purification of 4th fraction

The 4th fraction of *R. mucronata* was separated with a semi preparatory HPLC column (Luna 5µ C18 (2) 100 A 250×10.00 mm 5-micron Phenomenex). An aliquot of 150 µl of the sample was loaded, and a linear gradient mobile phase containing water (A) and acetonitrile (B) was applied. In the gradient, 0 to 35% B was kept for the first 5 minutes, held 90% B for the remaining 42 minutes, and during the next 2 minutes the gradient was brought back to 0. The flow rate was 2.5 ml/minutes. The diode array detector wavelength was set at 254 nm, 280 nm, 210 nm and 225 nm and the solvents were used after degassing in a sonicator. The peak pattern was analyzed using Chromeleon 6.8 software (Dionex, USA).

2.15. Identification of the active HPLC sub-fraction using Quantitative assay

An aliquot of 4 µl (2 % (v/v)) of *V. harveyi* cells suspension at the aforesaid cell density (0.1 Abs at 600 nm) was inoculated into 200 µl of appropriate growth medium (ZoBell's agar) and cultivated in the presence of each HPLC fractions (4.1, 4.2.1, 4.2.2) having normalized concentration of 1µg/µl and incubated (27^0C) for 12 h. After incubation, the bioluminescence intensity was measured in terms of relative light units (RLU) using Varioskan LUX multimode microplate reader (Thermo fisher scientific). The percentage of bioluminescence inhibition was calculated based on RLU reading. Visible luminescence was also captured with the camera shutter speed adjusted at 1/30.

2.16. LC-MS analysis

Metabolite profiling of the plant crude extract by Ultra Performance Liquid Chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS). The system (Waters) consists of a TUV detector (J12TUV750A), a quaternary solvent manager (H12QSM632A), a column chamber (J12 CHA730G), and a sample manager FTN (K12 SDI069G). A reversed –phase BEH C18 column (of dimension 50 mm×2.1mm×1.7µm) with a flow rate of 0.3ml/min was used for chromatographic separation (Waters). The mobile phase was a mixture of water and acetonitrile, with 0.1% formic acid in a gradient mode. The UHPLC system was connected to the quadrupole time-of-flight mass spectrometer (Waters Xevo G2 Q-TOF) with an electrospray ionization (ESI) interface, which works in positive and negative ionization modes. The injection volume was 10µl. All samples were analyzed using ESI negative ionization mode, scanning the m/z range between 50 and 1000. The desolvation gas flow and the temperature were 900l/h and 350°C, respectively. The mass spectra were obtained using collision energy ranging from 5-30eV. The instrument control and data acquisition was done using Mass Lynx software (v4.1).

2.17. Statistical analysis

All experiments were performed independently in triplicates, and the data were analyzed statistically. The analyses were performed using SPSS software version 22, IBM Corp. Furthermore, the results were analyzed using one-way analysis of variance (ANOVA), followed by Turkey's method for pairwise comparison among the groups.

3. Results

3.1. Qualitative and quantitative quorum quenching analysis with crude methanolic extract

It is understood that the bioluminescence of bacteria is positively controlled by Quorum Sensing genes (Bassler, 1999). Hence on performing quantitative and qualitative anti QS potential assay of the crude methanolic extracts of the mangrove plant on *V. harveyi,* zones of luminescence inhibition ranging from 1-2cm without any growth inhibition were observed. Quantitative assay indicated luminescence inhibition by 59% to 94% at various concentrations from 0.4 to 2 µg/µl (Fig. 1).

3.2. HPLC based separation

Five fractions of the extract could be separated at wavelength 554 nm and collected manually at retention times 12.5 -14 min, 14 -14.8 min, 14.8 -16 min, and 16 -18 min respectively (Fig. 2). All the fractions were subjected to anti-QS assays through the above procedure after equalizing their concentration to 1 µg/µl.

3.3. Identification of the active HPLC fraction

In the experiment, fraction number 4 exhibited the best quorum quenching property with bioluminescence inhibition of 91.6% at the concentration of 2 µg/µl compared with all other fractions, this fraction was used for all further investigations at concentrations ranging from 0.001µg/µl to 1 µg/µl (Fig. 3).

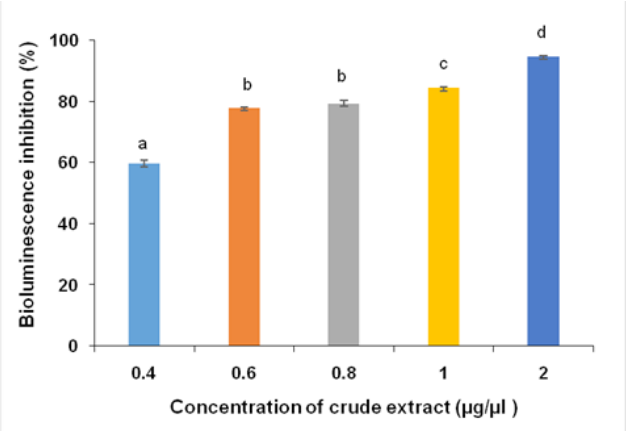

Fig. 1. Bioluminescence inhibition (Mean± SD) of *V. harveyi* bioluminescence in the presence of *R. mucronata* crude extract at various concentrations. One-way ANOVA analysis showed that there was significant difference ($p<0.05$) in the percentage of bioluminescence inhibition of the bacteria at different concentrations of crude extract of *R. mucronata*

3.4. Determination of minimum inhibitory concentration (MIC) of fraction 4

The absence of bactericidal activity was confirmed by counting the drop plated colonies of treated *V. harveyi.* A count of 1.6×10^9 CFU/ml was observed in the drop plate method, which was like the standardized colony count (1.8×10^9 CFU/ml) without the inhibitory compound. All the doses that were examined did not clearly hinder growth. Therefore, further experiments were conducted with the first three concentrations alone (Fig. 4).

3.5. Bioluminescence inhibition assay

The HPLC fraction 4 exhibited dose-dependent inhibition in bioluminescence after an incubation of 12 hours (Fig. 5). The concentrations from 0.125 µg/µl - 1 µg/µl of fraction 4 showed relevant inhibition throughout the luminescence period.

3.6. Assessment of biofilm — crystal violet staining assay

Fraction 4 of the extract exhibited concentration-dependent inhibition in bio-film formation (Fig. 6). The inhibition was maximum at 1 µg/µl (59.5%) and slightly less (49.6%) at 0.5 µg/µl of the fraction 4.

3.7. Quorum quenching property of *Rhizophora mucronata* in *Chromobacterium violaceum* MTCC 2656

The 4th fraction of *R. mucronata* plant extracts had 82.86% violacein inhibition activity and 30% anti-bacterial activity and disc diffusion assay showed opaque zones, which indicated the inhibition of quorum sensing alone (Fig. 7).

3.8. Microscopic analyses of biofilm developed on cover glasses

Evaluation of anti-biofilm effect of fraction 4 on *V. harveyi* showed a visible reduction in the thickness of the bio-film and the number of microbial colonies on coverslip when treated with 1- 0.5µg/µl of the extract (Fig. 8). Concentration dependent gradual decrease in the microbial colonies were observed and the cover slip treated with the extract at 1 µg/µl (Fig.8: c,f) showed a drastic reduction in the microbial colonies compared with the others.

3.9. Growth and bioluminescence kinetics assay

The bacterial growth kinetics assay was performed to confirm the non-antibacterial activity of specific. *R. mucronata* fraction 4 at the tested concentrations. Even after 16th hour of incubation, no significant differences were observed in the viable cell count between untreated control and treated cultures (Fig. 9a). But in the case of bioluminescence kinetics assay, a substantial decline in the bioluminescence of *V. harveyi* observed with increasing concentrations of *R. mucronata* fraction number 4 (Fig. 9b).

3.10. *In-vivo* challenge experiments with *Artemia* nauplii

The anti-infective potential of *R. mucronata* extract fraction 4 in protecting *Artemia* nauplii from *V. harveyi* infection was investigated. The pathogenic *V. harveyi* in the absence of the extract, caused total mortality of *Artemia* nauplii. The extract at 1 µg/µl demonstrated good survival rates, i.e., 93.3% of *Artemia nauplii,* while compared with 70% survival in *Artemia nauplii* treated with 0.5 µg/µl of plant extract (Fig.10) and the extract itself was found to be non-toxic to *Artemia.*

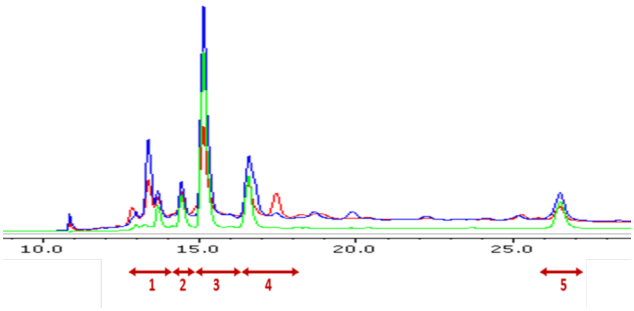

Fig. 2. HPLC chromatogram of the crude *R. mucronata* methanolic extract further separated into five fractions. Each peak was collected at its specific retention time

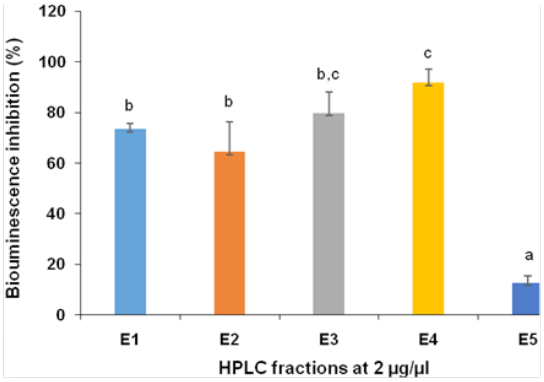

Fig. 3. Inhibition of V. harveyi bioluminescence (Mean± SD) in five different fractions at 2µg/µl of *R. mucronata* extract. One-way ANOVA analysis showed significant difference ($p<0.05$) in the percentage of bioluminescence inhibition of the bacteria at different HPLC fractions

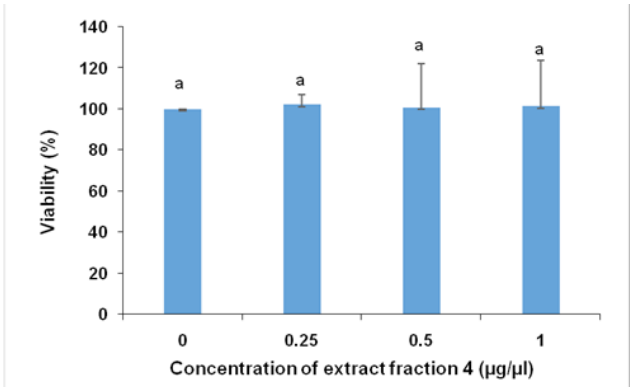

Fig. 4. Effect of *R. mucronata* fraction 4 (Mean± SD) on the growth of *V. harveyi*. One-way ANOVA analysis showed no significant difference in the bacteria's viability percentage at different concentrations of R. mucronata extract fraction 4.

3.11. Identification of the active HPLC fraction

In the experiment, as fraction number 4.2.2 exhibited the best quorum quenching property with bioluminescence inhibition of 91.6% at the concentration of 2 µg/µl compared with all other fractions, this fraction was used for all further investigations at concentrations ranging from 0.001 µg/µl to 1 µg/µl (Fig. 11).

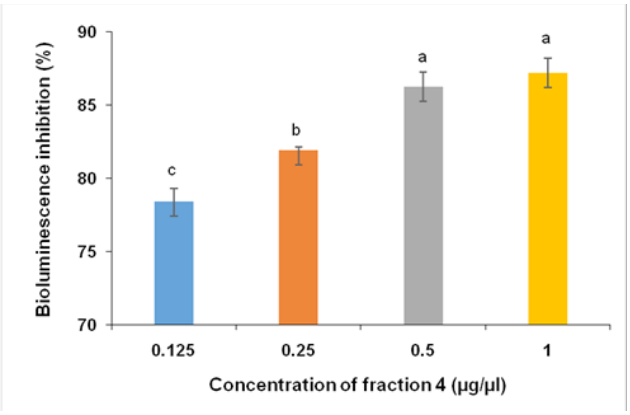

Fig. 5. Inhibition of *V. harveyi* bioluminescence (Mean± SD) in different concentrations of *R. mucronata* extract fraction 4. One-way ANOVA analysis showed that there was a significant difference ($p<0.05$) in the bioluminescence inhibition to varying concentrations of fraction 4

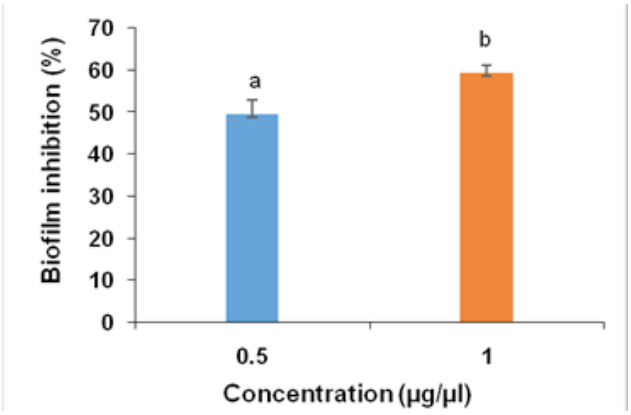

Fig. 6. Quantitative analysis of biofilm biomass inhibition by *R. mucronata* fraction 4 in *V. harveyi*. One-way ANOVA analysis showed a significant difference ($p<0.05$) in biofilm inhibition at different concentrations of *R. mucronata* extract fraction 4 at 95% confidence interval.

3.12. Determination of minimum inhibitory concentration (MIC) of fraction 4

There was no significant growth inhibition as all the tested concentrations showed 100% growth (Fig. 4). The absence of bactericidal activity was confirmed by counting the drop-plated colonies of treated *V. harveyi*. A count of 1.6×10^9 CFU/ml was observed, similar to the standardized colony count (1.8×10^9 CFU/ml). Therefore, further experiments were conducted with the first three concentrations alone (Fig. 12).

3.13. LC-MS analysis

A total of 28 compounds were initially identified using GC-MS analysis of the crude methanol extract. Additionally,

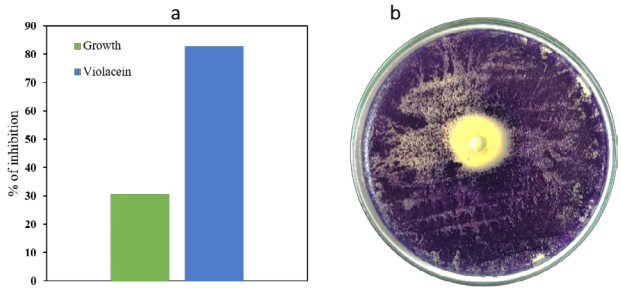

Fig. 7. Quorum quenching property of *R. mucronata* in C. violaceum MTCC 2656. a) Graph showing percentage of growth and violacein inhibition b) Disc diffusion assay showing Violacein inhibition activity of *Rhizophora mucranata* fraction 4 in C. violaceum MTCC 2656.

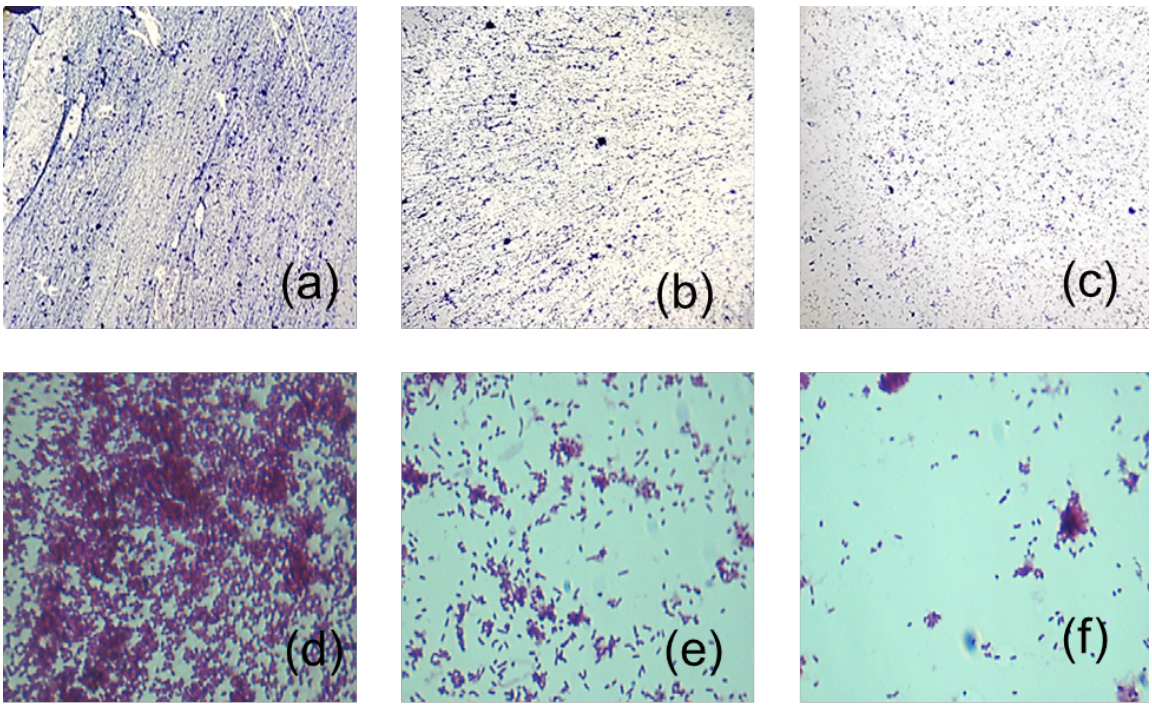

Fig. 8. Microscopic images showing biofilms of *V. harveyi* grown in the absence and presence of *R. mucronata* fraction 4. Light microscopic images in 10x and 40x magnification of untreated controls (a, d) and extract treated at 0.5µg/µl (b, e), 1µg/µl (c, f) biofilms of *Vibrio harveyi*. a, b and c were taken at 10 x magnification; d, e and f were taken at 40 x magnification

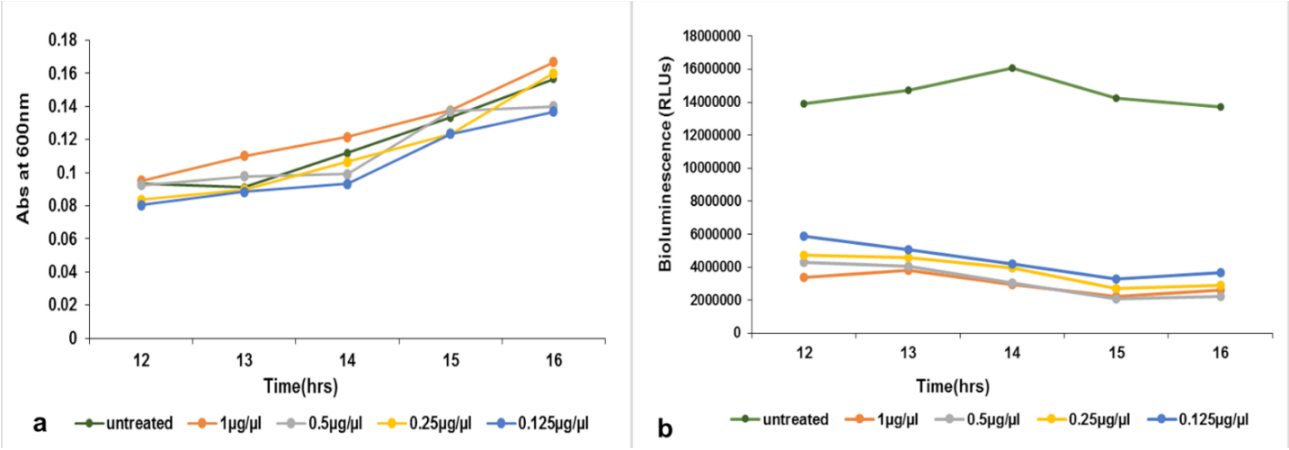

Fig. 9. Effect of *R. mucronata* fraction 4 at various concentrations (0.125, 0.25, 0.5 and 1µg/µl) on growth and bioluminescence of *Vibrio harveyi*. a) Growth analysis of *Vibrio harveyi* in the presence and absence of fraction 4. Cell density was quantified by measuring the absorbance at 600nm. b) Bioluminescence kinetics of *V. harveyi.*

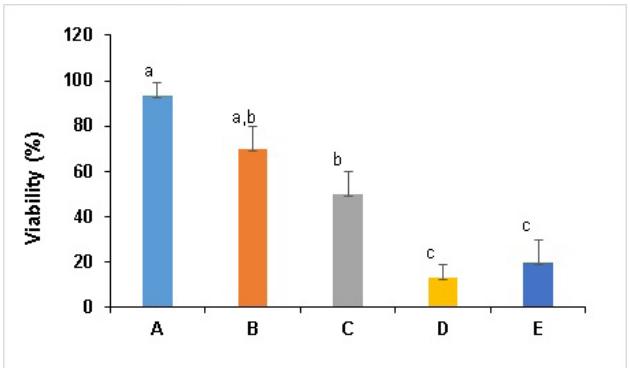

Fig. 10. Anti-infective potential of fraction 4 of mangrove R. mucronata in enhancing the survival of Artemia nauplii from *V. harveyi* infection. (A) *V. harveyi* + Artemia + extract (1µg/µl); (B) *V. harveyi* + Artemia + extract (0.5µg/µl); (C) *V. harveyi* + Artemia + extract (0.25µg/µl); (D) *V. harveyi* + Artemia; (E) Artemia alone. One-way ANOVA analysis showed that there was a significant difference ($p<0.05$) in the viability of Artemia nauplii at different concentrations of *R. mucronata* extract fraction 4 at 95% confidence interval.

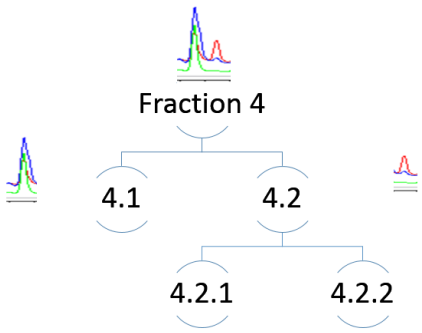

Fig. 11. Peak separation of fraction 4 and further purification based on specific peak collection

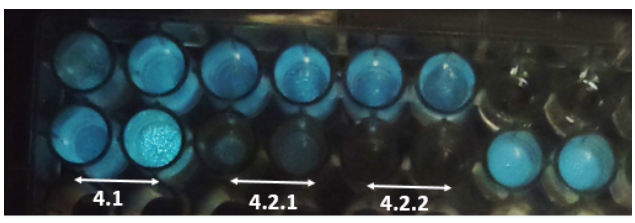

Fig. 12. Absence of bioluminescence in wells treated with 1 µg/µl of fraction 4.2.2

a few phytochemicals were identified using UPLC–Q–TOF– MS/MS from the active fraction 4.2 (4.2.1 and 4.2.2) also. Representative UPLC–QTOF–MS/MS total ion chromatograms (TIC) in the negative ion mode are shown in Fig. 13-17. The compounds were tentatively identified by matching retention times (RT), m/z values, MS/MS fragments with compounds from the reported data in the literature and database resources. Accordingly, it detected the presence of compounds such as Shogaol, Naringin, Kaempferol-3-*O*-rutinoside, Rutin and Quercetin.

4. Discussion

Marine plants as the sources of several natural products and biomaterials occupy a unique position pertaining to their potential to offer remedial measures to health-related issues in aquaculture other than that of humans and domesticated animals. In the present context, phytochemicals are unique in their capabilities against multi drug resistant pathogens to be applied instead of antibiotics. Among them, mangroves, occupy a pivotal position however, they have not explored adequately. This prompted us to undertake the present investigation to elucidate anti-vibrio properties of *R. mucronata*. Such an approach shall pave for finding a solution to the plasmid-mediated drug resistance to human pathogens, a threat delimiting the scope of antibiotic treatment in aquaculture systems (Preena et al., 2020). In this context, the disruption of microbe's quorum sensing mechanism, a widely discussed concept, is considered as a novel and promising tool to control such pathogens (Rutherford and Bassler, 2012). Quorum sensing system of *Vibrio harveyi* regulates its biofilm formation (Kadirvel et al., 2014), flagellar motility (Yang and Defoirdt, 2015) and expression of its virulence factors such as type III secretion, extracellular toxin, metalloprotease and siderophore (Defoirdt et al., 2008; Manefield et al., 2000; Henke and Bassler, 2004). In addition, QS also manages the formation of bioluminescence, a major cellular function, which plays an obtrusive role in *V. harveyi* symbiosis with another organism (Guerrero-Ferreira and Nishiguchi, 2007; Guerrero-Ferreira et al., 2013). Therefore, the disruption of QS system can be effectively practiced for the inactivation of *V. harveyi.* Several phytochemicals as well as plant

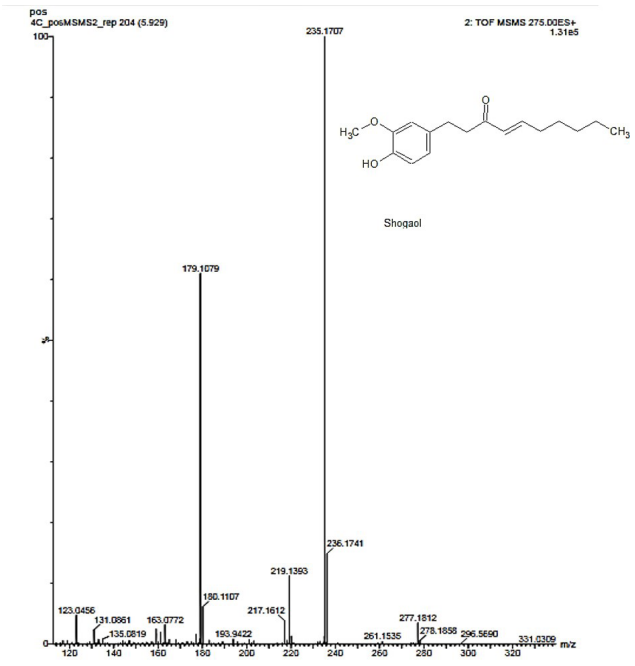

Fig. 13. LC-MS chromatogram showing fragments of shogoal

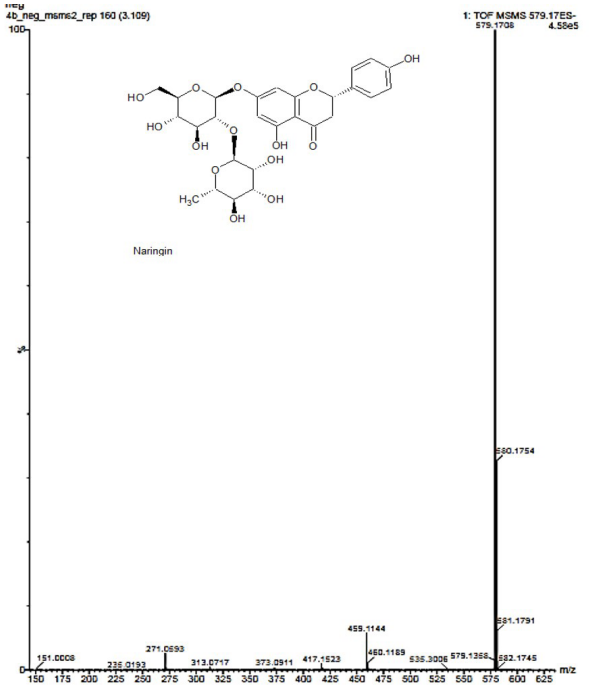

Fig. 14. LC-MS chromatogram showing fragments of naringin

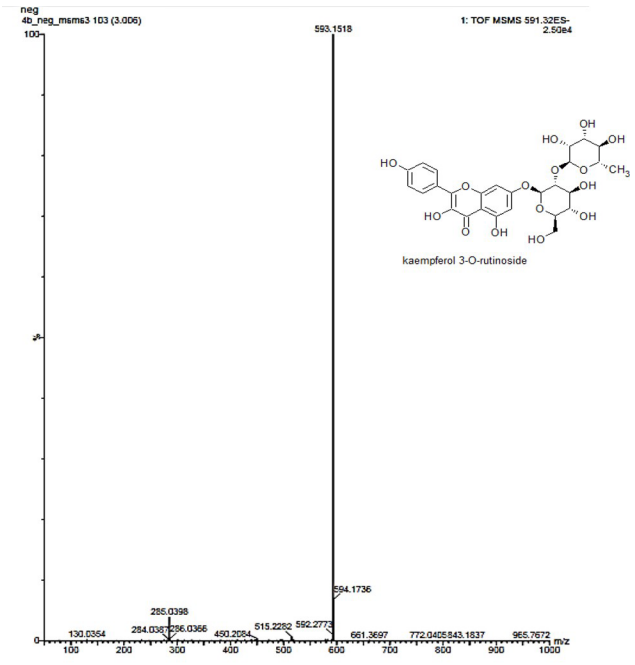

Fig. 15. LC-MS chromatogram showing fragments of kaempferol-3-rutinoside

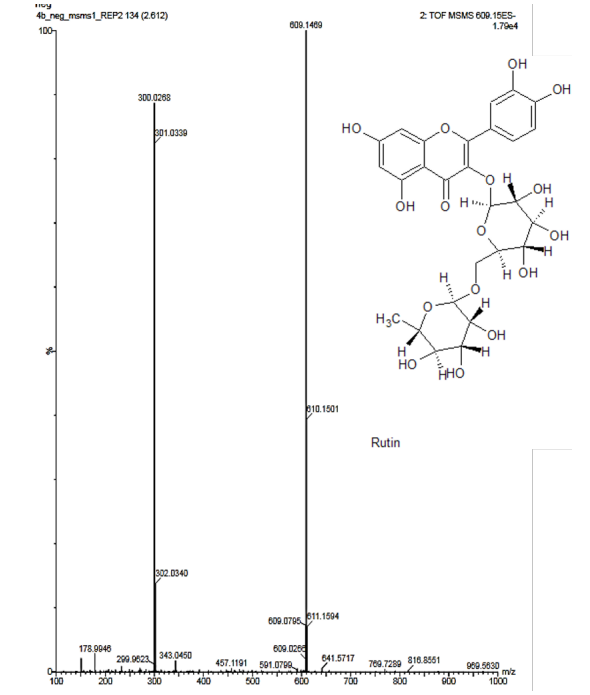

Fig. 16. LC-MS chromatogram showing fragments of Rutin

byproducts have been reported as anti- QS compounds against *V. harveyi*. Curcumin, green algal sulphated polysaccharides, cannabigerol, N-benzyl Cinnamamide from *Gracilaria fisheri* are some of such anti QS compounds (Packiavathy et al. 2013; Aqawi et al. 2020; Karnjana et al., 2020). In addition, their QS inhibition mechanism has also been reviewed recently (Chen et al., 2020). In the present work the potential of *R. mucronata* leaf extract is analyzed for its QS regulated bioluminescence quenching property and its efficiency against biofilm formation. The QS status of bacteria can be assessed by its bioluminescence (Ng and Bassler, 2009; Soni et al.,2015) and hence bioluminescence assays offer to be a good method to study the effect of crude extracts on QS.

The results obtained from bioluminescence inhibition assays showed that even crude leaf extract of *R. mucronata* at 0.6µg/µl exhibited more than 70% inhibition of bioluminescence, having a positive correlation between the concentration of the extract and bioluminescence inhibition. The inhibition can be due to hindrance in any of the QS pathways supported by the findings of Karnjana et al. (2020) in which N-benzyl Cinnamamide from

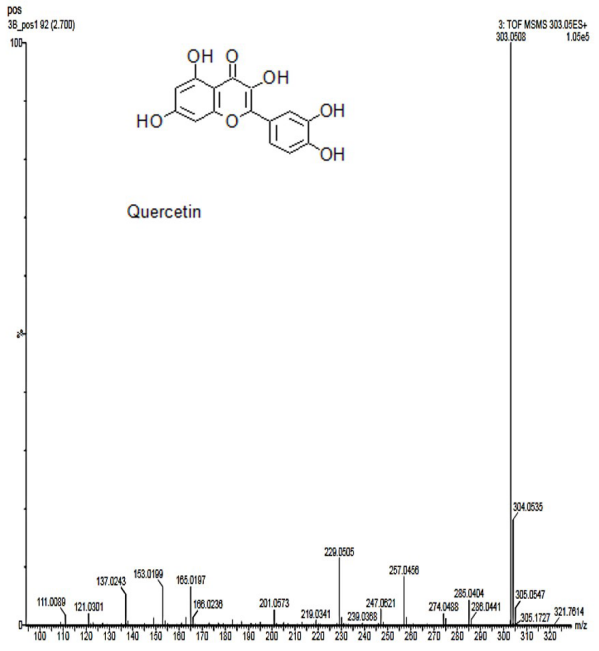

Fig. 17. LC-MS chromatogram showing fragments of quercetin

Red seaweed *Gracilaria fishery* exhibited a significant inhibitory effect on the bioluminescence of *V. harveyi* strains 1114 and BAA 1116 by hindering at Autoinducer 2 (AI-2) QS pathway. Similarly, isolated alkaloids from the sponge *Luecetta chagosensis* were identified as inhibitors of QS pathways of *V. harveyi* (Mai et al., 2015).

The HPLC-based separation and collection of 5 fractions in different retention time brought out a convincing effect with fraction 4 while comparing with the others with 91.6% bioluminescence inhibition. Studies with fraction 4 at various concentrations revealed concentration-dependent bioluminescence inhibition on *V. harveyi* without hindering growth. Both the bioluminescence inhibition and concentration of fraction 4 were found positively correlated. The high value of bioluminescence inhibition showed by fraction 4 at 1µg/µl (91.6%) falls in line with the finding of Pakiavathy et al., (2013) where curcumin exhibited dose dependent bioluminescent inhibition without affecting the growth of the pathogen.

Noticeably, *R. mucronata* fraction 4 showed a rare QS activity without bacterial death in all the tested concentrations where bacterial growth was 100% (Fig 4). More over colony count of the extract treated *V. harveyi* (1.6×10^9 CFU/ml) was also similar to the normal standardized bacterial count (1.8×10^9 CFU/ml). This finding reveals the possibility of an effective QS inhibitor presents in fraction 4 of *R. mucronata* extract, as the QS inhibitors target mainly the bacterial QS system and attenuate virulence alone without directly affecting the viability of bacteria, which will make the bacteria more susceptible to removal by the host immune system (Chen et al., 2019).

The key factor which aids in the expression of virulence of a bioluminescent bacterium is its biofilm property. They act as an external digestive system, recycling centre and shelter against antibiotics (Defoirdt et al., 2008). Biofilm can be correlated with its pathogenicity and it even allows *V. harveyi* to foster resistance against antibiotics (Manefield et al., 1999; Yildiz and Visick, 2009). Hence understanding biofilm formation and its inhibition is inevitable in developing an appropriate drug for *V. harveyi*. In the present study, a 0.5 - 1 µg/ µl concentration of the extract showed 49% to 59% biofilm inhibition, respectively. A very clear and visible reduction in the number of microbial colonies, as well as biofilm, was more evident by using both light and compound microscopes. The findings are consistent with the previous reports, wherein grape fruit limnoids suppressed biofilm formation in *V. harveyi* which is also in a non-growth inhibitory fashion (Vikram et al., 2010).

The kinetics focusing on optical density and bioluminescence demonstrated the effect of *R. mucronata* fraction 4 on bioluminescence and not on the bacterial viability measured in terms of absorbance at 600nm. Hence it is realized that the biofilm reduction and constant reduction in bioluminescence are possibilities due to the QS hindering activity of *R. mucronata* fraction 4.

An effort was also made to evaluate the efficiency of *R. mucronata* fraction 4 in protecting *Artemia* nauplii, as a model organism, from the pathogenic *Vibrio harveyi*. In the presence of *R. mucronata* fraction 4, at the concentration of 1 µg/µl, a marked reduction in mortality of *Artemia* nauplii was observed on challenging with *V. harveyi.* This suggested that the quorum quenching ability of fraction 4 enhanced the survival of *Artemia* nauplii by decreasing the virulence of *V. harveyi* in vivo. The anti-infective potential of the fraction 4 of *R. mucronata* demonstrated through the survival of *Artemia* nauplii in the presence of *V. harveyi* suggested that *R. mucronata* fraction 4 did not have any lethal effect on *Artemia* nauplii indicating that the extract was safe enough for aquatic application. Earlier studies have shown that, among the three bioluminescent regulatory pathways, both AI-2 and CAI-1 mediated pathways were necessary for sufficient production of LuxRvh transcriptional regulator to allow expression of virulence factors that are essential to kill *Artemia nauplii*. But HAI-1 mediated pathway will not affect the organism and may not trigger mortality which could be either due to environmental conditions or host-derived enzymes that could destabilize the specific AHL (Defoirdt et al., 2008). Accordingly, it is hypothesized that, *Artemia* nauplii have been protected from *V. harveyi* due to the suppression of any of the AI-2 or CAI-1 mediated QS pathways of the pathogen by one or more of the compounds present in the HPLC fraction 4 of *R. mucronata.*

Globally, mangroves are one of the most vigorous and vulnerable ecosystems positioned within the tropics and subtropics intertidal zones. The mangrove ecosystem of India is the only one with the highest record of biodiversity, flourishing with the mangrove genetic bliss at Bhitarkanika and unprotected wildlife species in the Sundarbans. They act as guardians from storm surges and tsunamis, which can be an important source of livelihood for coastal communities and protect juvenile fish stock. One of the most endangered ecosystems in the world is the

mangroves which are vulnerable to various anthropogenic activities such as industrialization, agriculture, solid waste dumping, construction of aquaculture ponds, construction of buildings and other infrastructures (Gurjar et al., 2019). Mangrove structure development in Kerala was inadequate, as evidenced by the low complexity Index (Ic) value (10) found in seven out of ten coastal districts (George et al., 2019). Cochin mangrove habitats were highly influenced by the toxic concentration of heavy metals (Ag, Cd, Cr, Cu, Hg and Ni) that were above NOAA ERL standards, notably Ag and Ni above ERM. There is a higher probability that these metals (Ag, Ni) impart toxic effects on mangrove vegetation and dependent benthic macrofauna, meiofauna and microfauna and alter community structure (Joseph et al., 2019). The present study provides a strong indication of quorum quenching moieties in mangrove plants, which can be developed as a drug of choice to control vibriosis in shrimp. Likewise, there is variety of valuable bio-actives present in mangroves as a whole and *R. mucronata* in particular. Hence, the inclusiveness of the mangrove ecosystem must be the developmental agenda with community participation.

Quorum sensing is the key mechanism that modulates the virulence and phenotypic properties of *V. harveyi*. Hence targeting QS is the best way to tackle the problem of its extreme pathogenicity. As the mode of function of the quorum quenching molecules is altering signaling molecules rather than killing or inhibiting bacteria, the possibility of the development of resistance, just one that happens with traditional antibiotics, may not take place. Hence, the therapeutic application of such compounds has a longer mean life than the other antimicrobial products (Rutherford and Bassler, 2012). Moreover, on putting together all the information available, it has been evident that *R. mucronata* HPLC fraction 4 contains discrete compounds/molecules such as Shogaol, Naringin, Kaempferol-3-*O*-rutinoside, Rutin and Quercetin having quorum quenching property either independently or synergistically on *V. harveyi,*. It has been concluded that this fraction can serve as the source of one or more quorum quenching compounds on *V. harveyi* as the drug of choice in aquaculture. This is the first report of quorum quenching property in the leaves of *R. mucronata* effective on the shrimp pathogen *V. harveyi*.

Acknowledgement

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