

ANTI-QUORUM SENSING POTENTIAL OF *LIMONIA ACIDISSIMA* (L.) AGAINST *VIBRIO HARVEYI* KUMB-VA4SRINIVASAN P^{1*}, RAJALAKSHMI M², DINESH KUMAR S¹

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ABSTRACT

Objective: This study aims to investigate the quorum-sensing inhibition (QSI) potential of *Limonia acidissima* L. against the biofilm forming *Vibrio harveyi* isolated from freshwater fish.

Methods: The present study evaluated the anti-QS activity of the *L. acidissima* methanol and ethyl acetate (LA-M and LA-EA) fruit extracts using *Chromobacterium violaceum* ATCC 12472 (wild) and *C. violaceum* CV026 (mutant) as biomonitor strains and biofilm formation using the crystal violet assay. *Vibrio* sp. were isolated from freshwater-cultured fishes and screened for biofilm formation property. Strong biofilm forming isolate were subjected to molecular characterization. *Limonia* fruit pulp was subjected to methanol and ethyl acetate extraction using cold percolation method and yield was calculated. In parallel to determining the QSI properties of the extract, minimum inhibitory concentration (MIC), biofilm inhibition concentration (BIC), antibiofilm properties, and metabolic activity of LA-M and LA-EA against the biofilm forming *V. harveyi* KUMB-VA4 was determined.

Results: The results of the present study demonstrated that the overall yield of methanol and ethyl acetate extract was 12.84% and 9.3% (w/w), respectively. Strong biofilm forming *Vibrio* isolate KUMB-VA4 was obtained from infected freshwater fishes and was subjected to molecular characterization. MIC of LA-M was 1510 µg/ml and LA-EA was observed to be 3000 µg/ml against the test pathogen, respectively. Biofilm inhibition assay revealed a BIC of LA-M at 250 µg/ml and LA-EA at 500 µg/ml. Both the plant extracts significantly reduced the biofilm formation of *V. harveyi* KUMB-VA4 and the metabolic activity in a dose-dependent manner. Light microscopy and scanning electron microscopy revealed that LA-M and LA-EA significantly altered 68.6% and 54.5% of the biofilm architecture at BIC. The QSI assay revealed that LA-M effectively reduced the violacein production of the biomonitor strains at sub-BIC (100–500 µg/ml) to 80% than LA-EA (43%) in a strong dose-dependent fashion.

Conclusions: The present study revealed the QSI property of *Limonia acidissima* against the biofilm forming *V. harveyi* isolated from infected fish.

Keywords: *Limonia acidissima*, *Vibrio harveyi*, *Chromobacterium violaceum*, Quorum-sensing inhibition.

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INTRODUCTION

The genus *Vibrio* and their closely related species are significant pathogens affecting the intensive rearing of finfish, molluscs, and shrimps [1]. Traditionally, antibiotics are used to control bacterial diseases in aquaculture sector. However, the frequent application, in many cases even as preventive measures, has resulted in the development and spread of resistance among the bacterial pathogens [2]. *Vibrios* are Gram-negative, rod-shaped bacteria, but they may also be curved or comma-shaped. They are non-sporulating, non-capsulated, facultative anaerobes, catalase-positive, and motile by means of a single polar flagellum [3]. In fish, the diseases include vasculitis, gastroenteritis, and eye lesions. With shrimp, the pathogen is associated with luminous vibriosis. Clinical signs of vibriosis are hemorrhage to intestines, body cavity, spleen and muscle, distended mucoid and necrotic intestine and petechiation, erosion and darkened coloration to the skin and fins. Changes to the eyes include distension and cloudiness and periorbital swelling. White/gray lesions can be found on the intestines and spleen and in fry, splenomegaly.

Decades of research has enabled the recognition of bacterial biofilms as the predominant bacterial survival tactics [4]. Biofilms play a vital role in the fouling process [5] and are known to have astounding rate of tenacious antibiotic resistance [6], one of the dominant obstacles in the

antimicrobial chemotherapy. The pathogenicity mechanisms of *Vibrio* are imprecisely understood, with likely mechanisms involving the ability to attach and form biofilms, quorum sensing (QS), various extracellular products including proteases and hemolysins, lipopolysaccharide, and interaction with bacteriophage and bacteriocin-like substances [7]. QS, or bacterial cell-to-cell communication, is a key process for bacterial colonization of substrata through biofilm formation, infections, and production of virulence factors. It involves the regulation of coordinated behaviors in bacteria as a function of population density which is achieved by the production, excretion, and detection of autoinducer (AI) molecules [8]. These AI molecules trigger the QS mechanism which aids the bacteria to optimize their energy metabolism and regulate its characteristic functions such as biofilm formation and expression of virulence factors. Biofilm formation by *Vibrio* sp. enhance the development of antibiotic resistance and sometimes the synthesis of bioluminescence pigment [9]. With the rapid developments in aquaculture, particularly in Asia, the organism has become a serious cause of disease in fish. Study on the QS inhibitors (QSIs) has gained prominence as the QSIs do not exert a selective pressure on the bacterial population and growth thereby successfully avoid the development of resistance toward antibiotic treatments [10]. Moreover, reports elucidate that QS signaling molecules interact directly with the biofilm forming bacterial colonies [11] and so QS disruption, directly or indirectly prevents the biofilm formation on underwater surfaces and

colonization of fish larvae [12,13]. Accordingly, the search for effective and QSIs has gained importance for research in herbal biomedicine, aquaculture and other fields where bacterial biofilms are causes of sanitary problems.

Natural products are vital sources of QSIs that can potentially inhibit QS mechanism. The presence of such compounds in medicinal plants is extremely interesting because, in most cases, medicinal plants are non-toxic to humans and readily available [14,15]. A wide variety of medicinal plants and their derived compounds have been reported in terms of structural diversity and biological activity as an effective inhibitors of not only the biofilm formation but also virulence factors in pathogens by interfering with QS mechanism in various pathogens [16,17]. Spices such as garlic, ginger, and turmeric have been reported for their QSI potential [18]. Similarly, the essential oils of cinnamon [19] and clove [20] are also known to possess QSI potentials. Moreover, the antibiofilm potential of Indian medicinal plants such as *Leucas aspera* (Lamiaceae), *Vitex negundo* (Verbenaceae), *Piper longum*, and *Piper nigrum* (Piperaceae) against biofilm-forming pathogens has been well established [21,22].

Limonia acidissima (Rutaceae), a traditional Indian medicinal plant used to treat blood impurities, leukorrhea and urinary problem. The plant belongs to a monotypic genus and is indigenous to Indian subcontinent. Recent study showed the presence of essential amino acids in the fruits of *L. acidissima* (common name-vilam palam) and its rich antioxidant potential Darsini et al. [23]. The fruit of *L. acidissima* has been evaluated for enhancing the immunity of freshwater fish fingerlings under *in vitro* conditions. The immunostimulatory potential of *L. acidissima* fruit as a feed supplement for freshwater fishes have elucidated increased growth, immunity, and survival of fishes against *Aeromonas hydrophila* infection [24]. In addition, research has substantiated the anti-biofilm potential of *L. acidissima* fruit against the biofilm forming *A. hydrophila* isolated from infected freshwater fishes [25]. The present study has been focused on the antibiofilm potential of *L. acidissima* on the biofilm-forming *Vibrio* sp.

METHODS

Sample collection

Samples were collected randomly from freshwater fishes cultured in and around Coimbatore, Tamil Nadu, India. The source of sample collection is represented in Table 1. Lesions from skin, gills, and scales of infected fishes were cut at 3 mm (diameter) using sterile scalpel. The cut tissues were resuspended in sterile 1× phosphate buffered saline (PBS, pH 8.2) and stored at 4°C until further use.

Bacterial strains

The tissue samples were taken and partially homogenized using microfuge tube tissue homogenizer (Tarsons, India). From this, samples were aseptically inoculated using spread plate technique on to thiosulfate citrate bile sucrose (TCBS) agar plates (Himedia, India). After inoculation, the plates were incubated at 37°C for 24 h. The isolates were then subcultured in nutrient broth (NB) (Himedia, India) for routine use. Glycerol stock was maintained at -20°C for long-term usage. Reporter strain *Chromobacterium violaceum* ATCC 12472 (wild type) and *C. violaceum* CV026 (mutant) were used to test the QSI potential of the plant extracts. Both the strains were cultured in Luria-Bertani (LB) broth at 35°C.

Characterization of bacterial isolates

The isolates were subjected to morphological characterization by media culture and microscopic morphology using Gram's stain. Motility was determined by microscopic examination of a hanging drop of NB culture incubated for 24 h at 37°C [26]. Biochemical tests included oxidase test, catalase test (H₂O₂ 5%), citrate utilization test, indole production test, hydrogen sulfide production test, MR-VP test, and carbohydrate fermentation test (1%w/v of glucose, xylose, sorbitol, sucrose, and arabinose) [27]. All the isolates were screened for *Vibrio* sp. and then subjected to further analysis.

Detection of biofilm formation

The *Vibrio* sp. Isolates were screened for their ability to form biofilm under static conditions in 24 well microtiter plates (MTPs) by following the method of Darsini et al. [23] with slight modifications. Briefly, 16 h cultures of isolates were prepared and gently resuspended in 5 ml NB (pH 7.3±0.2) (Himedia, India) medium and adjusted to an optical density of 1.0 at 620 nm. Then, the bacterial suspensions were aliquoted (1 ml) in each well of polystyrene 24 well MTPs (Tarsons, India) and incubated for up to 48 h at 37°C without shaking. After incubation, the isolates were analyzed for biofilm formation.

The planktonic cells were discarded and attached cells were gently washed twice with PBS, and fixed with glutaraldehyde for 15 min at room temperature and stained with 0.4% (w/v) crystal violet (Himedia, India) for 10 min at room temperature. Then, the crystal violet stained cells were solubilized with 1 ml of ethanol-acetone solution (8:2, v/v). The biofilm formation ability was scored as strong (+++), moderate (++), weak (+), and negative (-) by visually comparing the thickness of adherent layer and the results were tabulated. The isolates capable of strong biofilm formation were subjected for further studies.

Molecular characterization of biofilm-forming *Vibrios*

Isolation of genomic DNA

About 10 ml of bacterial biomass was collected by centrifugation at 10,000× g for 5 min. The cell pellets were resuspended in 400 µl of TE buffer (pH 8.0). Then, the mixture was lysed by adding 200 µl lysozyme (10 mg/ml) and incubated at 37°C for 1 h, followed by 40 µl of proteinase K (10 mg/ml) at room temperature for 10 min. The mixture was then treated with 1% SDS and incubated at room temperature until the solution turned clear. About, 80 µl of 0.5 M EDTA was added, mixed gently and the protein content was removed by sequential phenol: Chloroform: Isoamyl alcohol (24:23:1 v/v) extraction in which the aqueous layer containing the DNA was collected in a separate tube. Equal volume of isopropanol was added to the collected supernatant and centrifuged at 10,000× g for 10 min, and after centrifugation, 70% alcohol was used to wash the pellet. The tubes were blot dried and the pellet was dissolved in 500 µl TE buffer (pH 8.0).

16S rRNA gene PCR

The 16S rRNA gene was amplified from the extracted genomic DNA using the 27 F and 1492 R universal eubacterial primers designed to target the conserved regions in the genomic DNA of the isolates and amplify approximately 1.3 kb length gene. The forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'-GGT TAC CTT GTT ACG ACT T-3' (Xcelris Lab Ltd, Ahmedabad, India) were used for amplification [28]. The PCR mix contained 5 µl of 10× PCR buffer, 4 µl of 25 mM MgCl₂, 5 µl of 10 pmol 27 F, 5 µl of 5 µM 1492 R, 5 µl of 1 mM dNTP's, 0.5 µl of *Taq* DNA polymerase (Thermo Scientific, India), and 2 µl (50 ng) of genomic DNA. The reaction volume was adjusted and made up to a final volume of 50 µl with sterile double-distilled water and amplified in an automated thermal cycler (Vapo protect Pro S, Eppendorf). The PCR conditions were an initial denaturation stage at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 60 s, extension at 72°C for 60 s, and a final extension step at 72°C for 10 min. Negative controls with no DNA template were included in all PCR experiments.

Collection of plant and solvent extraction

L. acidissima (LA) fruits were obtained from foothills of Vellingiri during December 2016. The fruits were washed and cleaned before processing. The outer shell was broken and the fruit pulp was scooped, shade dried, pulverized, and processed into fine powder. Then, 25 g of LA powder was soaked in 250 ml of solvents such as methanol (LA-M) and ethyl acetate (LA-EA) (1:4) for 48 h with constant mixing. Then, the concoction was subjected to cold percolation extraction method. The retentate was reextracted with respective solvents while the filtrate was subjected to rotary vacuum evaporation (Model: R150, Labomed,

India). The yield of dried extracts was calculated using the following equation,

$$\text{Yield (g/100 g of dry plant material)} = (W_1 \times 100) \div W_2$$

Where, W_1 was the weight of the extract after the evaporation of solvent, and W_2 was the weight of the dry plant material.

Effect of LA extracts on the biofilm forming test pathogen

Minimum inhibitory concentration (MIC) assay

The MIC of the LA-M and LA-EA extracts were determined against the test isolates by following the Clinical and Laboratory Standards Institute reference method of MTP assay [29] with slight modifications. Briefly, an overnight culture of *Vibrio* sp. isolate was subcultured in NB until a turbidity equivalent to 0.5 McFarland standard solutions (1×10^6 CFU/ml) was obtained.

This bacterial suspension was added to NB supplemented with LA extract serially diluted two-fold to give final concentration ranging from 31.25 $\mu\text{g/ml}$ to 8000 $\mu\text{g/ml}$ and incubated at 37°C for 24 h. The MIC was recorded as the lowest concentration, which showed complete inhibition of visible growth. The results were further confirmed spectrophotometrically at 620 nm.

Growth curve analysis

One percentage of overnight culture of test pathogens (1.0 OD at 600 nm) were inoculated in 250 ml conical flask containing 50 ml of LB broth supplemented with LA-M and LA-EA extracts (sub-MIC). The flasks were incubated at the optimum temperature of respective pathogens under 180 rpm in a rotatory shaker. The well density was measured in 96-well Multiplate reader (Biotek, ELx800, India) at every 1 hour interval up to 12 h [25].

Quantification of biofilm biomass

The effect of *L. acidissima* fruit extract treated *Vibrio* biofilm was performed according to [30], with slight modification. 20 μl of *L. acidissima* solvent extracts with concentrations ranging from 31.25 $\mu\text{g/ml}$ to 2000 $\mu\text{g/ml}$ (Sub-MIC) were incorporated in 1 ml of NB containing 20 μl of *Vibrio* sp. Isolates (1×10^6 CFU/ml) in 24 well MTP and incubated at 37°C for 48 h. After incubation, the biofilm was stained using 0.4% (w/v) crystal violet. The subinhibitory concentrations (0.5, 0.25, and 0.125 biofilm inhibition concentration [BIC]) of the solvent extracts were also tested against the biofilms by following the same protocol. Then, the absorbance for each well was measured at 570 nm in spectrophotometer (Model: ELx800, Biotek, India).

Metabolic activity assay

The metabolic activity of biofilm cells was determined by assaying the XTT reduction of the test pathogen in the presence and absence of the plant extracts at BIC. Briefly, overnight culture of *Vibrio* sp. was washed twice with PBS and then incubated with 0.5 mg/ml XTT (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide; Sigma-Aldrich) and 1 mM menadione (Sigma-Aldrich) in PBS at 37°C for 90 min under dark condition. Further, absorbance was measured spectrophotometrically at 450 nm for every 6 h intervals up to 24 h [25].

Light microscopic observation of biofilm

About 1% overnight culture of the test pathogens were added to 24-well microtiter plates containing 1 ml fresh TSB medium and cover glass of 1 cm^2 . The wells were then incorporated with different concentrations (31.25–1000 $\mu\text{g/ml}$) of LA-M and LA-EA extracts. Wells without plant extracts were treated as control. The plates were incubated for 48 h at 37°C and then the cover glasses were rinsed thrice with PBS to remove non-adhered planktonic cells. After rinsing, the cover glasses were stained with 0.4% (w/v) Crystal Violet solution for 1 min and washed with distilled water. Stained cover glasses with biofilms were air-dried

and visualized under light microscope at magnification of $\times 400$ (Nikon Eclipse E200, Tokyo, Japan) [31].

Scanning electron microscopy

The test pathogen was incubated on glass coverslips in LB medium at room temperature. The resulting biofilms were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h and then post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), dehydrated with ethanol (25–100%), critical point dried, and sputtered with gold-palladium alloy. Samples were examined with a scanning image observing device equipped with an electron microscope (Zeiss, Sigma V) [32].

Detection of QS inhibition by quantification of violacein production

The QSI properties of the LA solvent extracts were carried out preliminarily using *Chromobacterium violaceum* ATCC 12472. The solvent extract which exhibited the QSI property against the reported strain was further tested for the quantification of the violacein reduction using *C. violaceum* (CV026). The effect of LA solvent extracts on reduction of *C. violaceum* CV026 violacein production were quantified by spectrophotometric method according to Choo et al. [33]. Briefly, *C. violaceum* CV026 inoculated ($\text{OD}_{620\text{nm}} = 0.5$) in LB broth supplemented with N-hexanoyl-L-homoserine lactone (HHL, Sigma Aldrich) at a working concentration of 5 $\mu\text{mol/ml}$ was taken as control and LB broth incorporated with HHL and LA solvent extracts at different concentrations (100–500 $\mu\text{g/ml}$). The tubes were further incubated at 35°C for 24 h. Following incubation, 1 ml of the culture was centrifuged at 12,000 rpm for 10 min to precipitate the insoluble violacein. One ml of DMSO was added to the pellet and the solution was vortexed vigorously for 1 min to completely dissolve violacein. The solution was then centrifuged at 12,000 rpm for 10 min to precipitate the cells. The cell-free supernatant was collected and quantified spectrophotometrically (Hitachi U-2900, Japan) at 585 nm.

Statistical analysis

All experiments were performed in triplicates. The values were expressed as mean \pm standard error. Student's *t*-test was performed to determine the significant difference between the samples using SPSS software version 21.0 (USA). The values were considered statistically significant if $p < 0.05$.

RESULTS

Characterization of bacterial isolates

Among the colonies formed in the TCBS agar plates, translucent, raised pinpointed yellow colonies were predominant and were indicative of *Vibrio* sp. From this, a total of 6 isolates were selected for possible *Vibrio* colonies (Table 1), based on the morphological characteristics on the TCBS agar medium. The isolates were subjected to physiological and biochemical characterization. The results of the morphological and biochemical tests evidently showed that all 6 isolates were *Vibrio* sp. (Table 2).

Biofilm forming ability of *Vibrio* sp. Isolates

Out of 6 *Vibrio* sp. isolates, one isolate (VA4) (Fig. 1) formed moderate to strong biofilm which was evident from the thick slimy, dense cell architecture on the glass slides. Two isolates (VB3 and VA5) produced weak biofilm and the remaining isolates showed negative for biofilm formation (Table 3). The strong biofilm forming VA4 strain was subjected to further studies in the presence and absence the plant extracts.

Molecular characterization of the biofilm forming *Vibrio*

The results of the 16S rRNA gene PCR showed a prominent amplification for 1392 bp when subjected to 1% agarose gel electrophoresis (Fig. 2a). Phylogenetic analysis revealed that the test pathogen shared 99% gene

similarity with *Vibrio harveyi* with a statistical significance of $p < 0.1$ (Fig. 2b).

Solvent extraction and yield

The dried *L. acidissima* fruit powder was subjected to solvent extraction using methanol and ethyl acetate. The resulting filtrate was vacuum evaporated and the yield percentage was calculated. The methanol extract yielded 3.211 g (12.84%) per 25 g of the dry fruit powder, whereas ethyl acetate 2.316 g (9.3%) 25 g of the dry fruit powder.

Effect of *L. acidissima* fruit extracts on test pathogen

MIC assay

The MIC of methanol and ethyl acetate extracts of *L. acidissima* fruit were evaluated against VA4 at a concentration ranging from 31.25–8000 µg/ml using MTP assay. The results showed that the MIC of LA-M was 1510 µg/ml, whereas the MIC value of LA-EA was observed to be 3000 µg/ml against the test pathogen. The values were confirmed using the spectrophotometric method at 620 nm and the results were graphically represented (Fig. 3).

Growth curve analysis

Analysis on the growth of *V. harveyi* VA 4 planktonic cells was performed in the presence and absence of the LA-M and LA-EA at sub-MIC level to rule out the antibacterial effect of the extracts on the test pathogen. Results elucidated no significant difference between the absorbance values of the untreated and *L. acidissima* solvent extracts treated samples which was evident from the growth curve observed for 12 h time space. Furthermore, our results provided an additional information that the *L. acidissima* solvent extracts did not inhibit the growth of the test pathogen during the initial (log) phase of the growth (Fig. 4).

Table 1: Collection of samples from infected fishes

Host species	Tissue/organ	Place of collection
<i>Oreochromis mossambicus</i> (Tilapia)	Skin lesions	Fish reservoir (Ukkadam lake)
<i>Cyprinus carpio</i> (Common carp)	Gills	Fish farm (Bhavani Sagar)
<i>Cirrhinus mrigala</i> (Mrigal carp)	Skin lesion	Fish farm (Aliyar)

Table 2: Morphological and biochemical characterization of biofilm-forming test pathogen

Test	<i>Vibrio sp.</i>
Gram-stain	Negative
Colony morphology TCBS medium	Small, light green with dark green centers; yellow halo
Motility	Positive
Biochemical analysis	
Cytochrome oxidase	Positive
Catalase	Positive
Indole	Positive
MR	Negative
VP positive	Negative
Citrate utilization	Positive
H ₂ S production	Negative
Urease	Negative
5% NaCl	Positive
Acid and gas production	
Glucose	Acid and gas
Xylose	Negative
Sorbitol	Negative
Sucrose	Acid only
Arabinose	Acid only

TCBS: Thiosulfate citrate bile sucrose

Determination of biofilm inhibitory concentration

The effect of different concentrations LA-M and LA-EA on the biofilm forming ability of *Vibrio sp.* VA4 was determined. Interestingly, the LA-M extract showed a pronounced biofilm inhibitory effect, with greater than 50% inhibition against test pathogens at a concentration of 250 µg/ml (BIC), whereas LA-EA exhibited greater than 50% biofilm inhibition at a concentration of 500 µg/ml (BIC) (Fig. 5).

Metabolic activity assay

The effect of *L. acidissima* solvent extracts on the metabolic activity of *V. harveyi* VA4 was analyzed using the XTT metabolic assay. It was observed that during the initial growth phase (6 h) the solvent extracts did not hinder the metabolism of the test pathogen which could be elucidated from the absorbance readings of the LA-M and LA-EA treated pathogen as compared to the control (Fig. 6). However, prolonged exposure (12–96 h) to LA solvent extracts significantly ($p < 0.05$) reduced the XTT metabolism of *V. harveyi* VA4 which was evident from the reduced absorbance of the treated samples as compared to the respected control. Such reduction could be attributed to the fact that long-term exposure affected the metabolism of test pathogens which in turn prevented the organism from converting the XTT to colored end product as compared to their respective control.

Light microscopic observation of *Vibrio sp.* biofilms

Observation of biofilm by light microscopy revealed the biofilm disruption effect of *L. acidissima* extracts against the biofilms formed by *V. harveyi* VA4 over the matrix material. A thick layer of biofilm was formed over the control coverslips which was evident from the dense color of crystal violet on the biofilm (Fig. 7). However, the biofilms treated with LA-M and LA-EA showed concentration-dependent modification, which was noticeable from the reduction in biofilm architecture of *Vibrio* with increasing concentration. The light microscopic analysis elucidated that the maximum level of reduction in number of microcolonies was observed at the higher concentrations of LA-M and LA-EA against the test pathogens.

Scanning electron microscopy

The SEM analysis was performed to study the characteristics of *V. harveyi* VA4 biofilm obtained on the glass coverslips surface at optimal *in vitro* growth condition in the presence and absence of *L. acidissima* solvent extracts. Results elucidated smooth surface morphology in the untreated control samples, whereas the LA-M- and LA-EA-treated samples showed rough morphology with inundated surfaces and loosened cell architecture which might be due to the antibiofilm activity of *L. acidissima* solvent extracts. In both control and treated sample, large mucoid extracellular matrix was observed which could be attributed to the biofilm formation (Fig. 8).

QSI potential of *L. acidissima*

As both LA-M and LA-EA extracts showed significant antibiofilm property, they were further screened for anti-QS activity which was assessed from violacein production using *C. violaceum* CV026. The preliminary screening of LA-M and LA-E showed 82% and 68% significant reduction in violacein production as compared to *C. violaceum* ATCC 12472, respectively (Fig. 9a). Hence, both the

Table 3: Screening of *Vibrio sp.* isolates for their biofilm forming ability

Isolates	Source	Biofilm formation
VA1	Lesion on scales	-
VU2	Skin lesions	-
VB3	Lesion on scales	+
VA4	Skin lesions	++/+++
VA5	Lesion on scales	+
VU6	Skin lesions	-

-: No biofilm, +: Weak biofilm, ++: Moderate biofilm, +++: Strong biofilm

extracts were further tested against *C. violaceum* CV026. Results elucidated that LA-M and LA-EA exhibited concentration-dependent inhibitory activity, which was evident from reduction in the violacein production with increasing concentration (Fig. 9b). Simultaneously, the antibacterial activity of the LA-M was determined against *C. violaceum* CV26, by turbidimetric method at 600 nm after 24 h incubation at applied concentrations of 100–500 µg/ml. Results revealed that there was no significant reduction in the growth when compared with that

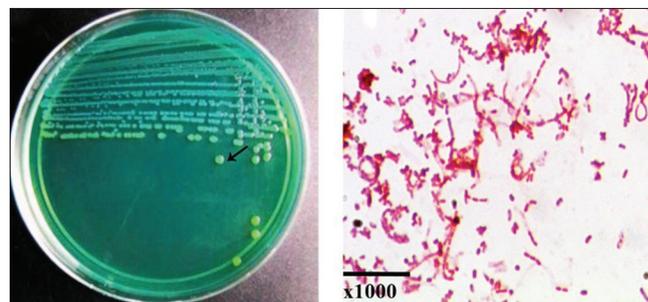


Fig. 1: Characterization of *Vibrio* sp. VA4 isolate (a) small, light green with yellow halo on thiosulfate citrate bile sucrose agar; (b) Gram-negative curved rods (scale bar=20 µm)

of control (Fig. 9c and d) which indicated that LA-M has no effect on the growth of *C. violaceum* CV026. Therefore, it is considered that LA-M exhibited considerable QSI activity.

DISCUSSION

In this study, the QSI and antibiofilm efficacy of *L. acidissima* fruit solvent extracts were evaluated against the biofilm forming *V. harveyi* VA4 at different concentrations (sub-MIC). Although previous study on the antibiofilm efficacy of *L. acidissima* fruit solvent extracts against the virulent and biofilm-forming fish pathogen *A. hydrophila* isolate AH1 demonstrated the significant antibiofilm efficacy of this wood apple fruit in disrupting the biofilm formation during the early phase [25], the biofilm-disrupting effect of the LA fruit extracts on the biofilm-forming *Vibrio* sp. has not been documented previously. *Vibrio* infections have gained significance in marine and cultured freshwater fish. *Vibrio* sp. is widespread in diverse habitats and causes infection to warm and cold-blooded animals [34]. Potentially, this pathogen can impact the fish yield and produce significant economic losses. Bacterial communities form biofilm by rapid proliferations which are associated with most of the infectious diseases [35]. *Vibrio* sp. has been widely studied for their biofilm formation and its associated virulence which enable them to proliferate, colonize the surfaces, infect fishes, and reduce fish yield by killing at early stages of their development [36]. Due to the emergence

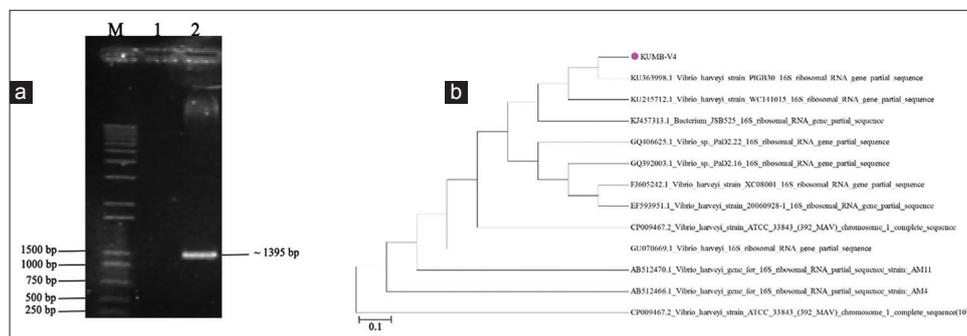


Fig. 2: Molecular characterization of biofilm-forming *Vibrio* isolate KUMB-VA4 (a) 1% agarose gel representing 16S rRNA gene amplification (M- 1Kb ladder; Lane 1 - Negative control; Lane 2 - 16S rRNA amplicon of VA4) and (b) Phylogenetic tree of 16S rRNA gene representing the similarity between the test pathogen and standard *V. harveyi* strains

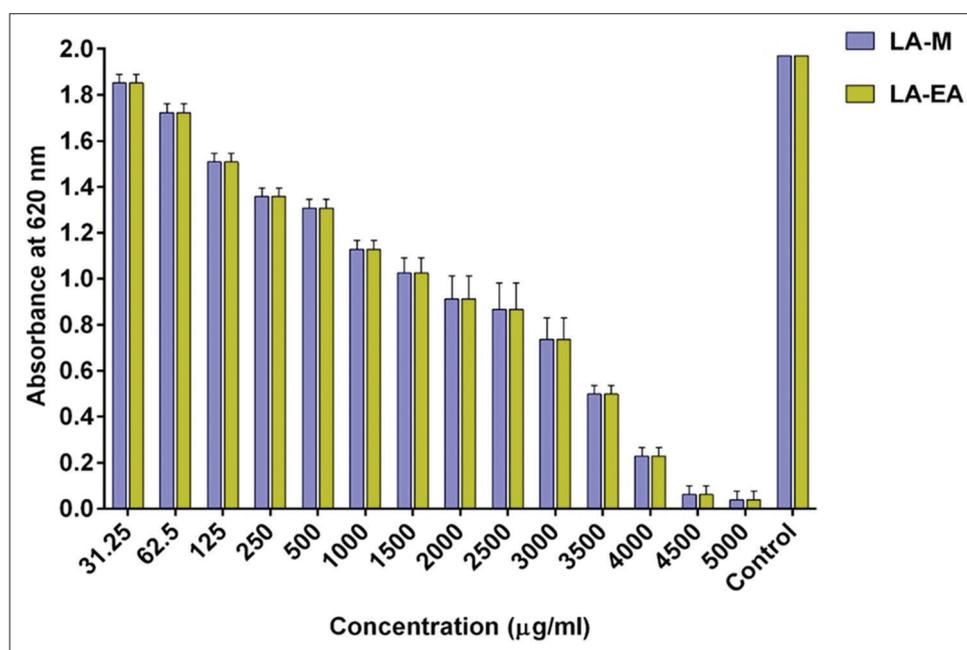


Figure 3: Minimum inhibitory concentration assay of LA-M and LA-EA against biofilm forming *Vibrio harveyi* VA4. Values are represented as mean±standard error of independent biological triplicate experiments

of drug resistance in *Vibrio* biofilms, the need for an alternative medicine with low toxicity and side effects has become essential. Medicinal plants constitute as an alternative source for antibiotics with low side effects. It has also been found that bacteria living in the biofilm mode of growth are often up to 1000 times more resistant to antibiotic than their planktonic counterparts [37]. Several studies have elucidated the role of medicinal plants and derived compounds against *Vibrio* sp. planktonic cells [30]. However, relatively few studies have been conducted on the effect of herbal plants against *Vibrio* biofilms. *L. acidissima* plant is known for its significant medicinal properties such as antioxidant [23], antibacterial [38], hepatoprotective [39], and anti-inflammatory [40] activity.

Among 6 isolates of *Vibrio* sp. VB3, VA4, and VA5 showed biofilm-forming potential (Table 3). Interestingly, the isolate VA4 exhibited moderate to strong biofilm forming characteristics. Strong biofilm forming potential plays an important role in the establishment of infection, enhanced pathogenesis, and drug resistance [30]. Molecular characterization of the strong biofilm forming isolate VA4 using 16S rRNA gene amplification revealed the pathogen as *V. harveyi*. Our results substantiated the prevalence of *V. harveyi* among the cultured freshwater fishes. Mishra et al. [41] also reported the prevalence of *V. harveyi* in the cultured freshwater fishes and prawns in India.

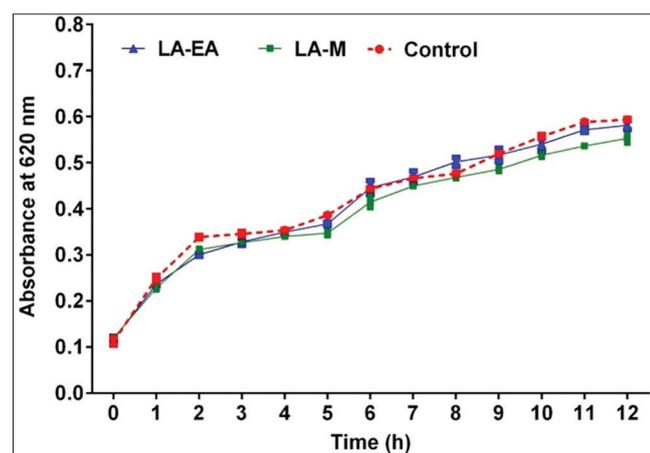


Fig. 4: Growth curve analysis of *Vibrio harveyi* VA4 planktonic cells treated using random sub-minimum inhibitory concentration of LA-M and LA-EA extracts compared to the untreated control. Values are represented as mean±standard error of independent biological triplicate experiments

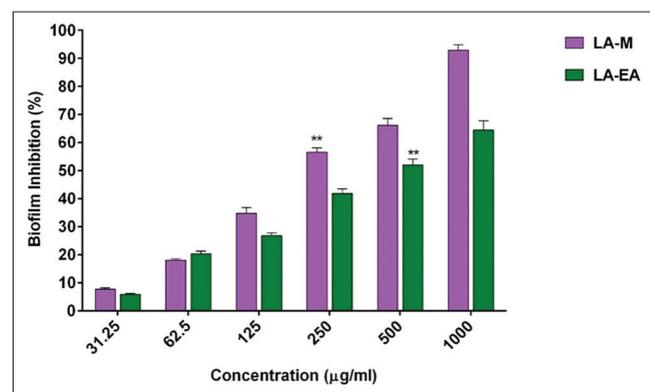


Fig. 5: Biofilm inhibitory concentration percentage of LA-M and LA-EA against biofilm forming *Vibrio harveyi* VA4. Values are represented as mean±standard error of independent biological triplicate experiments. **p<0.01 significantly different as compared to the control with ≥50% biofilm inhibition

The minimal inhibitory concentrations for the *L. acidissima* solvent extracts showed prominent inhibitory activity against VA4 at lower concentrations. Further, the antibacterial property of the *L. acidissima* solvent extracts were ruled out by studying the growth curve of the planktonic cells in the presence and absence of the extracts which showed that the extracts were not bactericidal at the tested sub-MIC level. In continuation of the results obtained from the MIC assays, *L. acidissima* extracts were further studied, for its ability to alter *Vibrio* biofilm formation. Interestingly, the biofilm formation was reduced in the presence of LA extracts in a dose-dependent manner. Moreover, the solvent extracts significantly modified the metabolic rate of the test pathogen which evidently showed that at BIC the solvent extracts of *L. acidissima* were not only disrupting the biofilm formation but also modifying the metabolism of the cells. Decrease in the XTT reduction could be correlated to the decreased metabolic rate of the organism and alteration in the enzyme process of the microorganism [42].

It was also observed that the plant extract treated samples showed significant difference in the biomass inhibition as compared to their respective untreated control. In addition, this study elucidated that LA-M showed significant BIC as compared to LA-EA extract which may be attributed the presence of active biological compounds in methanol extract. Enhanced biofilm reduction of LA-M as compared to LA-EA extract was evident from the altered biomass morphology when observed under the light microscopy (Fig. 7). Moreover, SEM analysis showed typical difference in the phenotype of the test pathogen in the

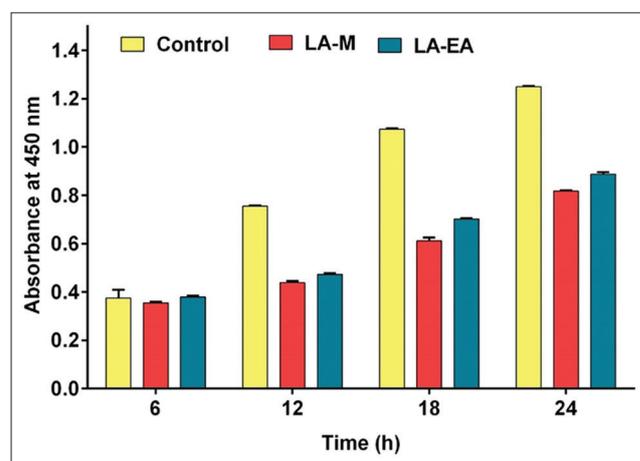


Fig. 6: Metabolic activity assay of LA-M and LA-EA against biofilm forming *Vibrio harveyi* VA4 at different time points. Values are represented as mean±standard error of independent biological triplicate experiments

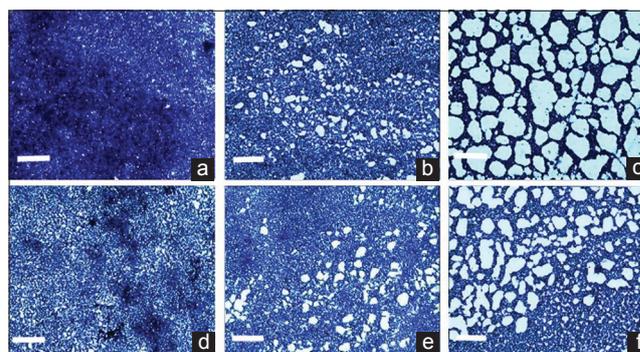


Fig. 7: Biofilm biomass inhibition of *Vibrio harveyi* VA4 (a and d) untreated control; (b and e) 31.25 µg/ml LA-M and LA-EA extract treated biofilm, respectively; (c and f) 500 µg/ml LA-M and LA-EA treated, respectively. Images were taken at ×400 magnification using Nikon Eclipse E200 series microscope. Scale bar = 20 µm.

presence and absence of *L. acidissima* extracts. Similar results were observed by Alhede *et al.* [43] who studied the biofilm formation of Gram-negative bacteria on the different substratum and reported that the bioactive components of plant extract result in the hydrophobicity of the biofilm interaction leading to alteration in the structural morphology of the biofilms. Mature biofilms are notably difficult to control and represent a source of infection that is recalcitrant to antibiotics. Planktonic population of the pathogen can be susceptible to a wide range of antibiotics, but sessile cells exhibit phenotypic changes which render antibiotics ineffective against biofilms. Biofilm formation in *Vibrio* is regulated by cell to cell signaling systems and virulence factors which play a significant role to cause disease in most fishes [44].

In addition, biofilms facilitate chronic wound infections by creating barriers against the immune system of host and antibiotics [45].

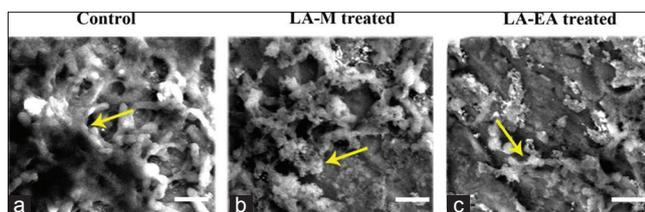


Fig. 8: Scanning Electron Micrographs representing the biofilm biomass reduction of (a) untreated Control; (b) LA-M; and (c) LA-EA extract treated *Vibrio harveyi* VA4 biofilms, respectively. Images were taken at $\times 20,000$ magnification (using Zeiss, Sigma V Scanning Electron Microscope). Scale bar = 100 μm

C. violaceum is reported to produce violacein which is regulated by QS process. Inhibition of QS-regulated pigment production is by is often used for the QSI screening studies [46]. As the acyl homoserine lactone (AHL) molecules synthesized by *C. violaceum* differ in their chain length, a wild type (CV ATCC 12724) and a mutant (C026) has been used to assess the QSI activity of LA extracts in the present study. Interestingly, the present study revealed that both the plants extracts were able to inhibit the violacein synthesis irrespective of the *C. violaceum* strains at BIC without affecting the growth of the cells. Other reports have substantiated the anti-QS activity in plant extracts of *Myristica cinnamomea* [47], *Panax notoginseng* [48], *Ocimum sanctum*, *Ananas comosus*, *Musa paradisiaca*, and *Manilkara zapota* [49] against *C. violaceum*. Our results are in affirmation with previous studies on bacterial biofilm inhibition by plant extracts [50-52]. Reports have evidently shown that *Vibrio* forms biofilms in the larval tanks by forming biomass on to the substratum, water lines, and also on recirculation system leading to higher recurrence of infection and fish yield loss [53]. However, complex processes related to genetic and ecological parameters are involved in the biofilm development and establishment mechanisms [54].

CONCLUSION

The present study is an effort to determine the QSI property of *L. acidissima* against the biofilm forming *V. harveyi* isolated from infected fish. The findings of the present study are in overall consistence with previous studies on the effect of medicinal plants against biofilm-forming fish pathogens. Apparently, the present study shows that *L. acidissima* may possibly be used to control the biofilms of *vibrios*. However, further studies are needed to explore the effect of *Limonia*

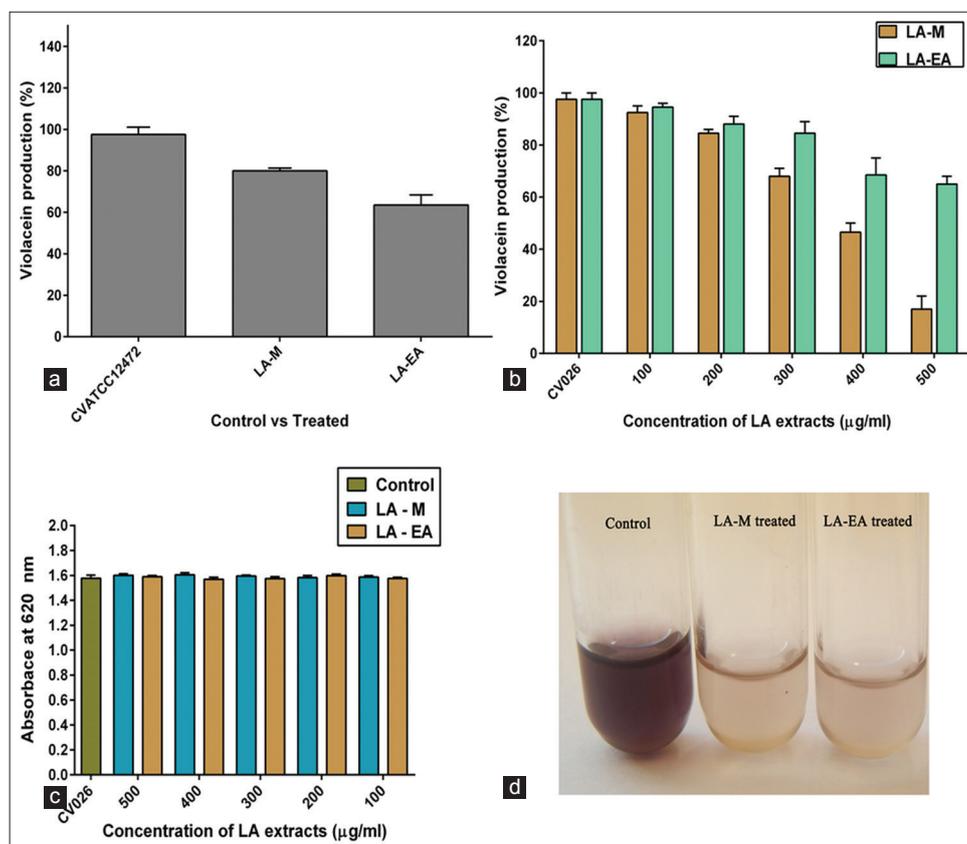


Fig. 9: (a) Inhibition of violacein production by LA solvent extracts at their BIC as compared to the reported strain CV ATCC 12472. Data are represented as the percentage of violacein production. (b) Inhibition of violacein production by LA-M and LA-EA extracts at different concentrations. Data are represented as the percentage of violacein production. (c) Influence of LA-M and LA-EA on the growth of *Chromobacterium violaceum* CV026 used as a control in the experiment. Cell density was quantified by measuring the optical density at 620 nm after 24 h. Mean values of the triplicate independent experiments and standard error are shown. (d) Inhibition of violacein production in CV ATCC 12472 in the presence and absence of LA extracts

fruit extracts on the expression of virulence genes in the biofilm forming *Vibrio*.

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AUTHOR CONTRIBUTIONS

SP, RM designed and executed the work, DKS proof read the data.

CONFLICTS OF INTEREST

None declared.

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