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Influence of *Limonia acidissima* L. against the biofilm forming *Aeromonas hydrophila* isolated from fresh water fishes

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Abstract

Limonia acidissima is a medicinal plant commonly used for multitude of ailments. In this context, the validation of traditionally used medicinal plant that the fruit extracts of *L. acidissima* exhibit antibiofilm activity against the predominant fish pathogen *Aeromonas hydrophila* was assessed. Among ten isolates of *Aeromonas* spp, KUAH1 showed strong biofilm formation and was studied further. The methanol (LA-M) and ethyl acetate (LA-EA) fraction of Limonia fruit extract clearly demonstrated significant ($p \leq 0.005$) antibiofilm activity of 58 to 94% and 54 to 77% respectively. Furthermore, the potential of Limonia fruit extracts against some of the biofilm associated factors were tested by swimming and swarming motility assay, XTT and anti-haemolytic activity assay. Extracellular protein analysis revealed differential protein expression at molecular weight corresponding to 30-60 kDa. This is the first report on antibiofilm activity of *L. acidissima* fruit extracts, signifying the scope for development of complementary medicine to treat *Aeromonas* biofilm-associated infections.

Keywords: *Limonia acidissima*, *Aeromonas hydrophila*, Biofilm inhibitory concentration, Motility, Haemolytic activity.

Introduction

The genus *Aeromonas* are widespread in different parts of the world and consist of Gram-negative bacteria. *Aeromonas hydrophila* have been identified as causative agent for many infections with adverse effects in different parts of the world (Burke et al. 1983). Aeromonads are gram negative, rod to cocco bacilli, mesophilic, and motile with polar flagellum. *Aeromonas* infection is based on a multifactor model of pathogenesis which includes outer membrane proteins, lippopolysaccharides (LPS), the S-layer, polar flagella and pili. The clinical manifestation of *Aeromonas hydrophila* in fish disease includes skin ulceration, ophthalmic ulceration soft tissue infections, hemorrhagic septicaemia, and motile aero monad septicaemia (Merino et al. 1996; Rabaan et al. 2001; Kozinska and Guz 2004).

Most *Aeromonas* spp are opportunistic pathogens and cause infection to fish by entering through wound, while some Aeromonads are capable of infecting healthy fishes even at low bacterial counts (Reith et al. 2008). Outbreaks by *Aeromonas* often occur when fish immune system is compromised due to factors such as overcrowding, poor water quality, organic pollution, and hypoxia in conjunction with other diseases (Zhang et al. 2000).

Biofilm formation is one of the important virulence factors of *Aeromonas* spp. Biofilms are complex architecture of microbial cells embedded in a three dimensional matrix of extracellular polymeric substances (EPS) formed on biotic or abiotic surfaces (Flemming et al. 2007; Jahid and Ha 2012).The characteristics of biofilm is based on different factors such as the complexity of matrix, substrate availability, composition of EPS and the metabolic property of the micro organism (Gao et al. 2008, Simões et al. 2008). *Aeromonas* spp forms biofilm using peritrichous flagella and adhesin molecules which facilitates the attachment and colonization on substratum (Saidi et al. 2011; Pianetti et al. 2012). Biofilms formation are highly associated with resistance to environmental stresses such as starvation (Spector and Kenyon 2012), desiccation (Hingston et al. 2013), disinfectants (Moretro et al. 2012), and antimicrobial agents (Jahid and Ha 2012).

Studies have reported the emergence of multi drug resistant *Aeromonas* spp besides their classical resistance to β-Lactamic antibiotics. The bacteria are able to receive and transfer antibiotic resistance genes to other gram negative bacteria in the environment (Erika et al. 2012). Extensive use of antibiotics and chemotherapeutics to prevent the disease outbreak caused by *Aeromonas* in fishes has led to increased antibiotic resistance among the bacterial population (Rahman and Hossain 2010; Naviner et al. 2011; Prabhugouda et al. 2014; Sherajul et al. 2015). Recently, there has been increased

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interest on antimicrobial potential of plants due to their medicinal property and their use for treatment of infectious and non-infectious diseases. Herbal medicines have been reported to be safe and free of side effects with enhanced antimicrobial properties (Mohd Sajjad and Iqbal 2012). *Limonia acidissima* L. is a member of the Rutaceae family and widely distributed in India (Lim 2012). The fruits of *L. acidissima* are commonly known as wood apple and are of high nutritive value. The fruit is extensively reported for its wide medicinal property and is used in Ayurveda (Gupta et al. 2009). Wood apple has been widely reported to consist of carbohydrate, vitamins, amino acids, triterpenoids, phytosterols, saponins, coumarins (Kangralkar et al. 2010; Ilango and Chitra 2010; Teepica et al. 2013).

Recent studies show that the fruit has rich antioxidant, hepatoprotectant, anti-inflammatory, antipyretic and analgesic activities as well as for the antihelmintic activity, anti-bacterial and anti-fungal activity (Morton 1987; Adikaram et al. 2007; Ahamed et al. 2008; Teepica et al. 2013). The fruit pulp has been reported as potent growth enhancer for fresh water carps and enhances the survival rate (Teepica et al. 2014). Although the fruit has been studied extensively for its medicinal properties, little is known about its spectrum of action against the *Aeromonas* biofilm cells. Therefore, a systematic evaluation of the Limonia fruit against *Aeromonas* biofilm is needed to validate the scientific basis for determining their use against *Aeromonas* infections; especially biofilm associated mucosal and systemic infections in fishes.

We have studied the anti-bacterial activity of *Limonia acidissima* fruit extracts against different clinical pathogens (Srinivasan et al. 2015). Phytochemical analysis indicated the presence of compounds responsible for the anti-bacterial properties. Some other reports have also elucidated the anti-bacterial property of *Limonia acidissima* leaves and barks against different drug resistant pathogens (Neelamadhab Panda et al. 2013). As adherence and synthesis of complex matrix via signal transduction play a significant role in the initial phase of biofilm development we hypothesize that the wood apple extracts possessing anti bacterial properties might be active against *Aeromonas* biofilm development. Therefore, in this study, we evaluated two extracts of wood apple fruit for their ability to eliminate established biofilms and inhibition of biofilms at sub-MICs in *Aeromonas hydrophila* isolates.

Materials and Methods

Sample Collection

Samples were collected from infected fishes cultured in different places 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 5 mm (diameter) using sterile scalpel. The cut tissues were resuspended in sterile phosphate buffered saline (PBS, pH 8.2) in wide mouth containers and transported to laboratory and stored at 4 °C.

Organisms and media

The tissue samples were taken and partially homogenized using microfuge tube tissue homogenizer. From this, samples were aseptically inoculated using spread plate technique on to Tryptone Soya Agar plates (TSA) (Himedia, India) supplemented with ampicillin sodium salt (0.5 mg/100 ml). After inoculation the plates were incubated at 37 °C for 24 h. The isolates were then sub cultured in Tryptone Soya broth (TSB) (Himedia, India) for routine use. *Aeromonas hydrophila* MTCC 1739 (IMTECH, Chandigarh, India) was used as reference culture. Glycerol stock was maintained at -20 °C for long term usage.

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 TSB culture incubated for 24 h at 37 °C (Jawetz et al. 2007). Biochemical tests included Oxidase test, Catalase test (H_2O_2 5%), Citrate utilization test, Indole production test, Hydrogen sulphide production test, MR-VP test, and carbohydrate fermentation test (1%w/v of glucose, fructose, sorbitol and galactose) (MaccFadin 2000). All the isolates were screened for *A. hydrophila* and then subjected to further analysis.

Detection of biofilm formation

The *Aeromonas hydrophila* isolates were screened for their ability to form biofilm under static conditions in 24 well micro titre plates by following the method of Gavin et al. (2003) with slight modifications. Briefly, 16 h cultures of isolates were prepared and gently resuspended in 5 ml TSB (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 micro titre plates (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 phosphate-buffered saline (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 solubilised 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.

Collection of plant and solvent extraction

Limonia acidissima fruits (LA) were obtained from foothills of Vellingiri during the month of September 2014. 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 100 ml of solvents such as Methanol and Ethyl acetate (1:4) for 48 h. Then the concoction was subjected to cold percolation extraction method. The retentate was re-extracted with respective solvents while the filtrate was subjected to rotary vacuum evaporation (Model: R150, Labomed, India). The yield of dried extracts were calculated using the following equation,

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

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.

Minimum Inhibitory Concentration of LA extracts against planktonic bacterial cells

The Minimum Inhibitory Concentration (MIC) of the LA methanol (M) and ethyl acetate (EA) extracts were determined against the test isolates by following the Clinical and Laboratory Standards Institute (CLSI) reference method of micro titre plate (MTP) assay (CLSI 2007) with slight modifications. Briefly, overnight cultures of *A. hydrophila* isolates were sub-cultured in TSB until a turbidity of 0.5 McFarland (1 $\times 10^6$ CFU/ml) was obtained. This bacterial suspension was added to TSB supplemented with LA extract serially diluted two fold to give final concentration ranging from 31.25 µg/ml to 8000 µg/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.

Agar well diffusion assay

The antibacterial activity of LA extract against *A. hydrophila* was performed by the well diffusion susceptibility test according to the CLSI guidelines (CLSI 2007). *A. hydrophila* (1×10^6 CFU/ml) cultures were uniformly spread over the surface of Muller Hinton Agar (MHA) (Himedia, India) using sterile cotton swabs. The plates were left undisturbed for 10 min for the absorption of excess moisture. Then, LA-M and LA-EA extracts were loaded on to the wells at MIC (2000 µg/ml and 4000 µg/ml respectively) and sub-MIC (500 µg/ml) concentrations. Ciprofloxacin (30 µg/ml) was used as antibiotic control and DMSO was used as negative control. Following addition, the MHA plates were incubated at 37 °C and the zone of inhibition was observed after 24 h.

Growth curve analysis

Overnight cultures of *A. hydrophila* (0.5 McFarland at 600 nm) were inoculated in 50 ml of TSB supplemented with sub-MIC concentrations (125 – 1000 µg/ml) of LA-M and LA-EA extracts. The cultures were incubated at 37 °C, under 156 rpm in a rotator shaker (Orbitek-LT, India). Absorbance was measured at 620 nm in UV-visible spectrophotometer (Shimadzu UV-3600 Plus, Japan) at 2 h interval up to 12 h.

Motility assays

The swimming and swarming motility activity of the LA extract treated *A. hydrophila* isolates were determined according to Christopher et al. (2013), with slight modification. Briefly, Luria-Bertani (LB) medium with 0.35% Bacteriological agar (Himedia, India) was used to prepare swim plates and determine swimming motility; whereas nutrient broth with 0.5% (w/v) of bacteriological agar (Himedia, India) and 0.5% filter sterilized D-glucose was used to prepare swarm plates. Both the swim and swarm plates were supplemented with different concentrations of LA-M and LA-EA extracts (250 µg/ml and 500 µg/ml each).

Swim plates were stab inoculated at the center of the LB agar medium and incubated at 32 °C for 16 to 18 h in upright position. Growth from the edge of the swimming zone within the agar of these plates was taken as inoculums for the swarming assay. Swarm plates were inoculated on the surface of the agar in the center and incubated at appropriate temperature in upright position. The swarming migration was observed by following swarm fronts of the bacterial cells.

Quantification of biofilm biomass

The effect of *L. acidissima* fruit extract treated Aeromonads biofilm was performed according to Gavin et al. (2003), with slight modification. *L. acidissima* solvent extracts with concentrations ranging from 31.25 µg/ml to 2000 µg/ml were incorporated in TSB containing *A. hydrophila* suspension of 1×10^6 CFU/ml in 24 well micro titre plate and incubated at 37 °C for 48 h. After incubation, the biofilm was stained using 0.4% (w/v) crystal violet. The sub inhibitory concentrations (0.5, 0.25 and 0.125 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).

Assessment of metabolic activity of biofilm cells

The metabolic activity of biofilm cells was determined by assaying the XTT reduction. Briefly, over night culture of *A. hydrophila* 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 for every 6 h intervals up to 24 h.

Light Microscopic Observation of A. hydrophila biofilms

About 1% overnight culture of the test pathogens were added to 24 well micro titer plates containing 1 ml fresh TSB medium and cover glass of 1 cm^2. The wells were then incorporated with different concentrations (61.5 µg/ml – 1000 µg/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 0.3% (w/v) Crystal Violet solution for 1 min and washed with distilled water. Stained cover glasses with biofilms were visualized under light microscope at magnification of ×400 (Nikon Eclipse E200, Tokyo, Japan) (Husain et al. 2013).

Haemolytic activity

The haemolytic activity of the biofilm forming *A. hydrophila* isolates and MTCC 1739 were detected by streaking the pathogens on TSA plates supplemented with 5% human blood (Lesmana et al. 2001).

Isolation of Extracellular proteins (ECPs) and SDS-PAGE analysis

Extracellular proteins of Aeromonads were isolated according to Anju et al. (2010). Briefly, the bacterial cultures were grown overnight in 5 ml LB broth containing LA-M and LA-EA extracts (500 µg/ml). Broth cultures without plant extracts served as control. Then, 100 µl of this culture suspension was added to 50 ml LB broth and incubated overnight at 37 °C at shaker speed of 150 rpm. Culture suspension was centrifuged at 8000 rpm at 4 °C for 15 min. Supernatant was precipitated by addition of trichloroacetic acid (TCA; 10% w/v) (Himedia, India) with overnight incubation at 4 °C. Further, centrifugation at 12000 rpm for 20 min resulted in pellet containing extracellular proteins which was resuspended in

50 µl of 1 mM Tris-HCl buffer (pH 8.0). The concentration of the protein was determined according to Lowry's method using Biophotometer plus (Eppendorf, India). Protein sample mixed with 1x sample buffer (10 µl) was analyzed by SDS-PAGE using 12 % poly acrylamide gel followed by silver staining. (Laemmli et al. 1970).

Statistical analysis

All experiments were conducted in triplicates. The statistical analysis was performed using SPSS (V 16, Chicago, USA). Values were expressed as mean ± S.E. Students' T test was used to compare parameters with control and the values were considered significant if $p \leq 0.05$.

Results

Characterization of bacterial isolates

A total of 10 isolates were selected for possible *Aeromonas* colonies from the tissue samples obtained from infected fishes (Table 1) based on the morphological characteristics on the TSA medium supplemented with Ampicillin. The isolates were subjected to physiological and biochemical characterization. The results of the morphological and biochemical tests evidently showed that all 10 isolates were *A. hydrophila* (Table 2).

Table 1: Collection of samples from infected fishes

Host species	Tissue/ organ	Place of collection
Catla catla (Catla)	Lesion on scales	Fish farm (Aliyar)
Oreochromis mossambicus (Tilapia)	Skin lesions	Fish reservoir (Ukkadam lake)
Cyprinus carpio (Carp)	Gills	Fish reservoir (Ukkadam lake)

Table 2: Morphological and Biochemical characterization of *Aeromonas sp* isolated from infected fishes.

Test	*Aeromonas sp*
Colony morphology	
Tryptone soy agar Medium	Pale white, translucent, raised, mucoid colonies
Gram-Stain	Negative
Motility	Positive
Biochemical Analysis	
Cytochrome oxidase	Positive
Catalase	Positive
Indole	Positive
MR	Positive
VP positive	Positive
H_2S test	Negative
Citrate	Negative
Urease	Positive
1% Nacl	Positive
Acid and Gas Production	Negative
Glucose	AG
Fructose	AG
Sorbitol	Negative
Galactose	A

AG — Acid and Gas, A—Acid

Biofilm forming ability of A. hydrophila isolates

Out of 10 isolates and one reference strain of *A. hydrophila*, 1 isolate (KUAH1) formed strong biofilm. Two isolates (KUAH3 and KUAH4) produced weak biofilm and the remaining isolates showed negative for biofilm formation (Table 3). The reference strain *A. hydrophila* MTCC 1739 formed moderate to strong biofilm. The strong biofilm forming KUAH1 and MTCC 1739 strains were subjected to further studies with the plant extracts.

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 2.018 g (8.1%) per 25 g of the dry fruit powder, whereas ethyl acetate 1.624 g (6.5%) 25 g of the dry fruit powder.

Effect of L. acidissima extracts on A. hydrophila

Minimal Inhibitory Concentration Assay

The MIC of methanol and ethyl acetate extracts of *L. acidissima* fruit were evaluated against KUAHI and *A. hydrophila* MTCC 1739 at a concentration ranging from 31.25 µg/ml – 8000 µg/ml using MTP assay.

The results showed that the MIC of LA-M was 2000 µg/ml, whereas the MIC value of LA-EA was observed to be 4000 µg/ml against the test pathogens.

The values were confirmed using the spectrophotometric method at 620 nm and the results were graphically represented (Figure 1a and Figure1b).

Antibacterial activity assay

The antibacterial activity of LA extracts against *A. hydrophila* isolates were determined using agar well diffusion assay. The results of the well diffusion assay showed no zone of inhibition at sub-MIC. However, at MIC (Figure 2a and 2b), both extracts showed inhibition zone, which confirmed that LA-M and LA-EA were non antibacterial at sub-MIC (Figure 2c and 2d).

Growth curve analysis

The non-antibacterial activity of *L. acidissima* at sub-MIC concentrations was elucidated by growth curve analysis. The observed results showed no changes in the cell densities between treated and untreated cultures (Figure 3a and Figure 3b), evidently showing that *L. acidissima* extracts does not exhibit antibacterial activity against the planktonic cells of Aeromonads at tested concentration (500 µg/ml).

Swimming and Swarming Motility Inhibition assay

The effect of *L. acidissima* extracts on the swimming and swarming motility of *A. hydrophila* were examined. It was observed that upon exposure to LA-M and LA-EA, there was a concentration dependent significant ($p \leq 0.05$) reduction in the swimming and swarming motilities of the tested bacterial cultures. However maximum migration was observed at 250 µg/ml test pathogens (Figure 4A and Figure 4B).

significantly ($p \leq 0.005$) decreased by 36-38% at 0.5 BIC, 18-21 % at 0.25 BIC, and 8-10% at 0.125 BIC. The LA-EA showed a significant ($p \leq 0.005$) decrease by 43-47% at 0.5 BIC, 27-29% at 0.25 BIC, and 5-7% at 0.125 BIC (Figure 5A and Figure 5B).

Assessment of metabolic activity of biofilm cells

The XTT reduction assay showed the metabolic activity of *A. hydrophila* control biofilm and *L. acidissima* extract treated biofilms at various time intervals. Higher XTT reduction was observed in control as compared to plant extract treated biofilms (Figure 6A and 6B). *L. acidissima* extracts (LA-M and LA-EA) evidently reduced the activity of Aeromonads biofilms and resulted in decreased biomass. The results showed that LA-M and LA-EA treated test pathogens showed decreased metabolic activity after 6th hour of treatment as compared to control which indicated that the plant extracts inhibited the biofilm formation at the initial stage from the 12th hour of the biofilm formation.

Table 3: Screening of *Aeromonas hydrophila* isolates for their biofilm forming ability.

Isolates	Source	Biofilm formation
KUAH1	Lesion on scales	+++
KUAH2	Skin lesions	-
KUAH3	Lesion on scales	+
KUAH4	Lesion on scales	-
KUAH5	Skin lesions	-
KUAH6	Skin lesions	-
KUAH7	Gills	+
KUAH8	Skin lesions	-
KUAH9	Gills	-
KUAH10	Gills	-
MTCC 1739 (Reference strain)	Water borne	+++

-: no biofilm, +: weak biofilm, ++: moderate biofilm, +++: strong biofilm.

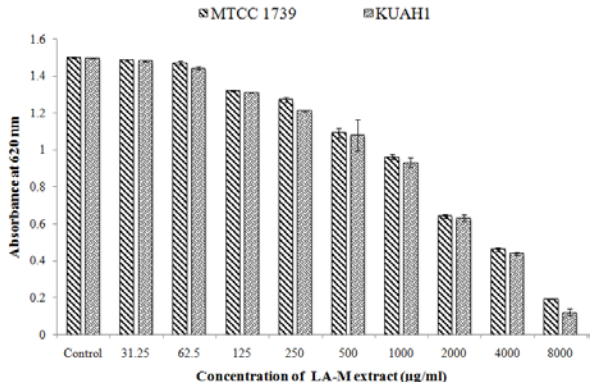

Figure 1a. Minimal inhibitory concentration of *L. acidissima* methanol extract (LA-M) against *A. hydrophila*.

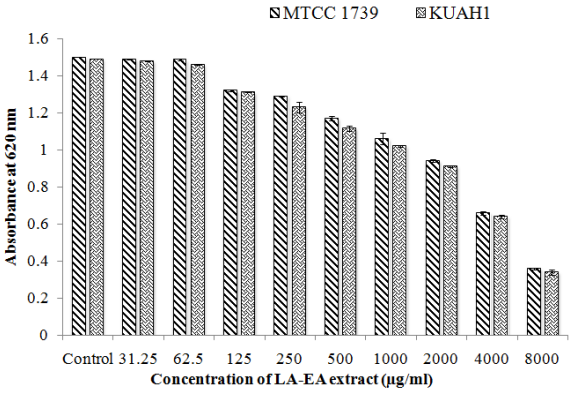

Figure 1b. Minimal inhibitory concentration of *L. acidissima* ethyl acetate extract (LA-EA) against *A. hydrophila*.

Biofilm biomass quantification assay

The effect of different concentrations LA-M and LA-EA on the biofilm forming ability of *A. hydrophila* KUAH1 and MTCC 1739 were 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). Further, to analyze the effect of *L. acidissima* extracts against biofilm formation, the biofilm inhibition assay was carried out at sub-inhibitory concentrations by diluting the extract via two fold dilution. It was observed that the biofilm inhibitory percentage of LA-M

Light Microscopic Observation of A. hydrophila biofilms

Observation of biofilm by light microscopy revealed the disruption effect of L. acidissima extracts against the biofilms formed by A. hydrophila KUAH1 and MTCC 1739 over the matrix material. A thick layer of biofilm was formed over the control coverslips which was evident from the dense colour of crystal violet on the biofilm (Figure 7). However the biofilms treated with LA-M and LA-EA showed concentration dependent inhibitory action, which was noticeable from the reduction in biofilm architecture of Aeromonads with increasing concentration. The light microscopic analysis elucidated that the maximum level of reduction in number of

micro colonies were observed at the concentration of 1000 µg/ml for LA-M and LA-EA against the test pathogens (Figure 8A-8D).

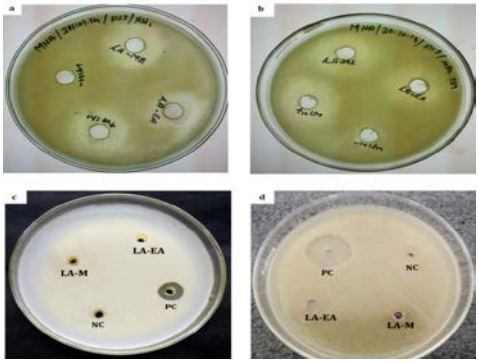

Figure 2: Antibacterial activity of *Limonia acidissima* solvent extracts a-b) LA-M (2000 µg/ml) and LA-EA (4000 µg/ml) MIC against KUAH1 and *A. hydrophila* MTCC 1739 respectively; c-d) LA-M and LA-EA (500 µg/ml) sub-MIC against KUAH1 and *A. hydrophila* MTCC 1739 respectively. PC-Positive control (Ciprofloxacin- 30 µg/ml), NC- Negative control (DMSO).

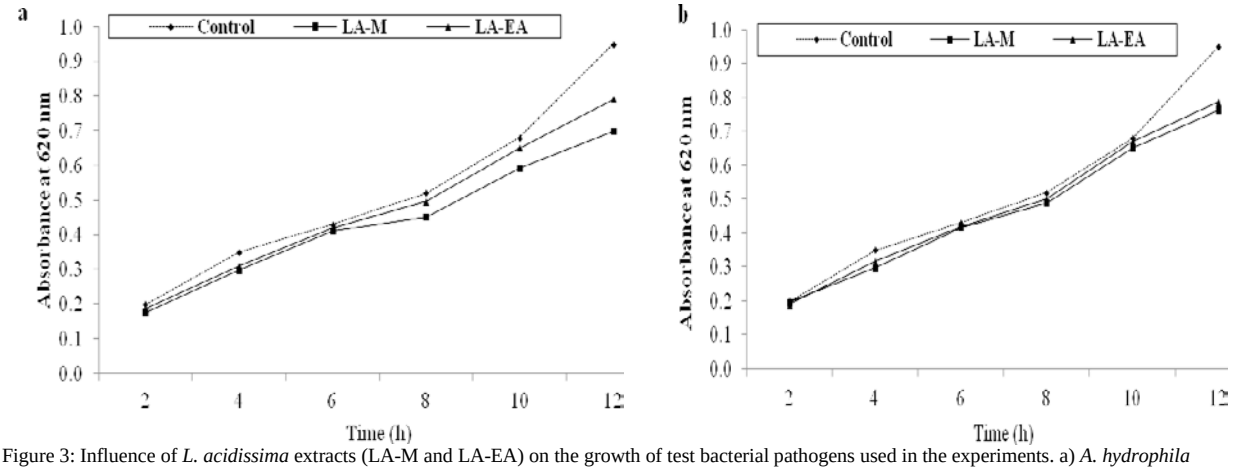

Figure 3: Influence of *L. acidissima* extracts (LA-M and LA-EA) on the growth of test bacterial pathogens used in the experiments. a) *A. hydrophila* KUAH1, b) *A. hydrophila* MTCC 1739.

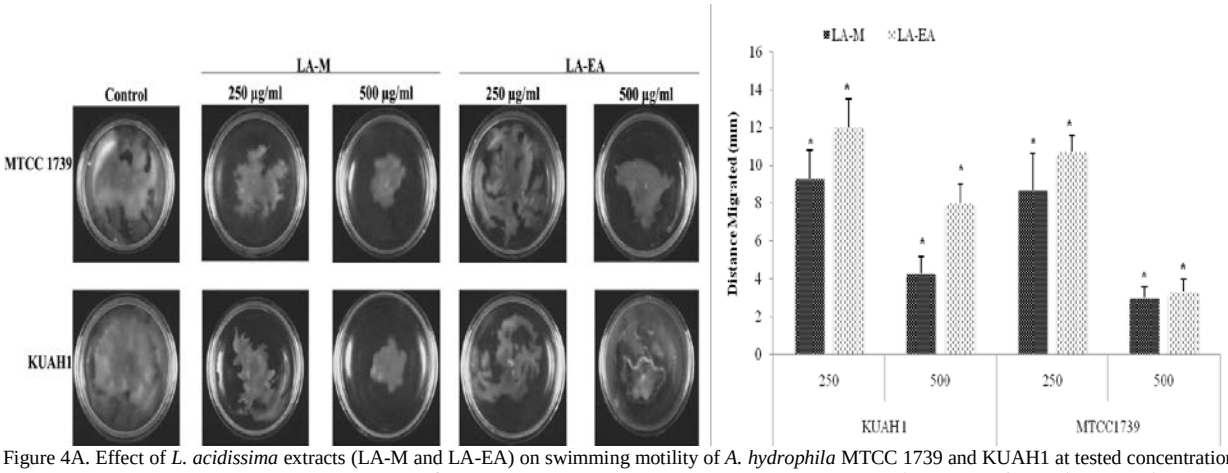

Figure 4A. Effect of *L. acidissima* extracts (LA-M and LA-EA) on swimming motility of *A. hydrophila* MTCC 1739 and KUAH1 at tested concentrations as compared to their respective control. Mean values of triplicate independent experiments and S.E are shown. *p ≤ 0.05, of treated compared to control as determined by T-test.

Haemolytic activity

A. hydrophila KUAH1 and 1739 demonstrated β haemolytic activity which was observed in the form of a zone along the streak on blood gar plate within 24 h of incubation at 37 °C (Figure 9). Isolate KUAH7 did not show haemolytic activity which was evident from absence of haemolysis zone on blood agar plate.

SDS-PAGE analysis of extracellular proteins

The protein profile of the extracellular fraction of *L. acidissima* extract treated Aeromonads showed the presence of protein bands ranging between 25 to 70 kDa with band patterns variation between 30 to 50 kDa (Figure 10).

The protein bands of Limonia extract treated samples were less prominent as compared to control which was evident from prominent band patterns of control corresponding to 40 kDa region of protein marker.

Discussion

Aeromonas infections have gained significance in marine and cultured freshwater fish. *Aeromonas hydrophila* are wide spread in diverse habitats, and causes infection to warm and cold-blooded animals (Palu et al. 2006). Presence of this bacterium is more profound in water bodies, which enables the pathogen to cause disease outbreaks in aquaculture and reduce the productivity (Yousr et al. 2007). Bacterial communities

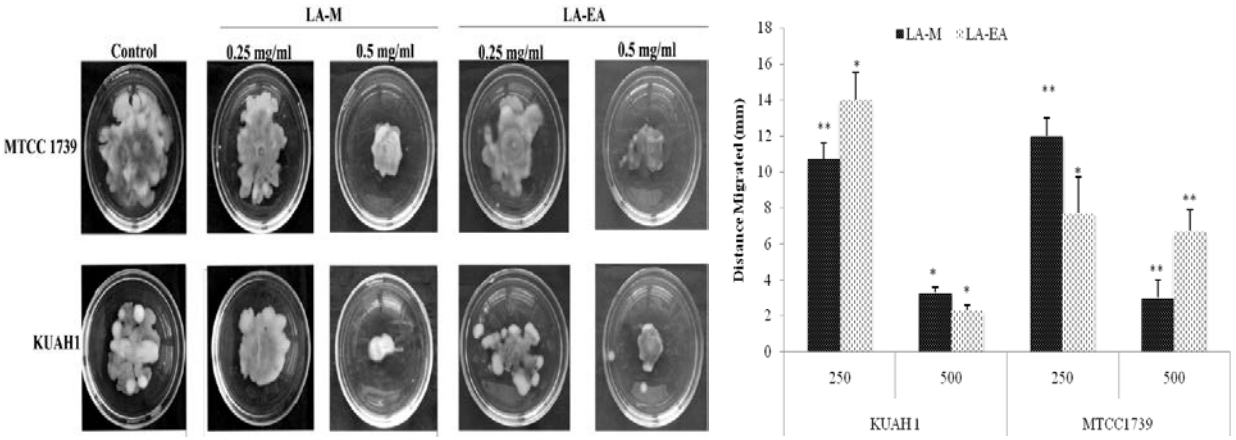

Figure 4B: Effect of *L. acidissima* extracts (LA-M and LA-EA) on swarming motility of *A. hydrophila* MTCC 1739 and KUAH1 at tested concentrations (250 µg/ml and 500 µg/ml) as compared to their respective control. Mean values of triplicate independent experiments and S.E are shown. *p ≤ 0.05, **p ≤ 0.005 of treated compared to control as determined by T-test.

form biofilm by rapid proliferations which are associated with most of the infectious diseases (Lewis 2007). *A. hydrophila* has been widely studied for their biofilm formation and its associated virulence (Williams 2007).

studies have elucidated the role of medicinal plants and derived compounds against *Aeromonas hydrophila* planktonic cells with special reference to antibacterial activity (Direkbusarakom 1998; Harikrishnan 2003; Bhuvaneshwari

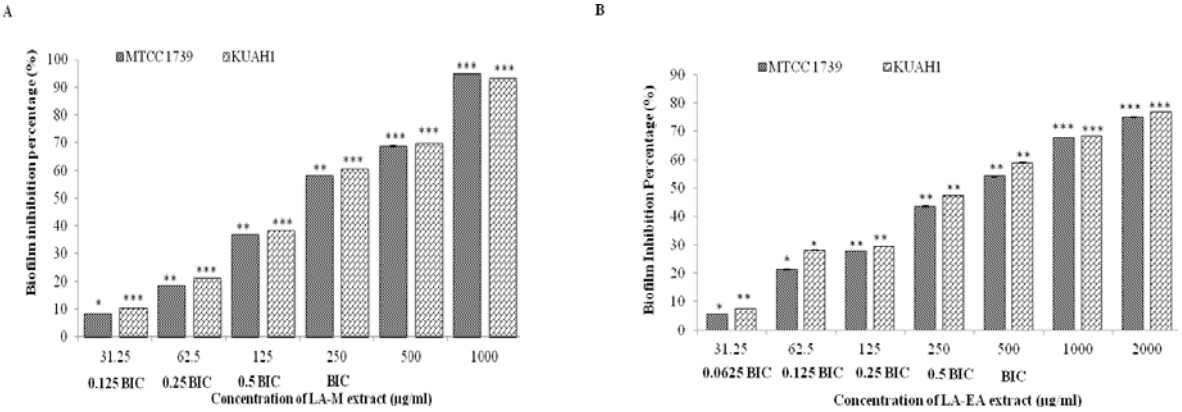

Figure 5: Percentage inhibition of *A. hydrophila* (KUAH1 and MTCC 1739) biofilm formation by varying concentrations (BIC, 0.5 BIC, 0.25 BIC, 0.125 BIC, 0.0625 BIC) of *L. acidissima*, A) Methanol extract (LA-M); B) Ethyl acetate extract (LA-EA). Mean values of triplicate independent experiments and S.E are shown. *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0001 of treated compared to control.

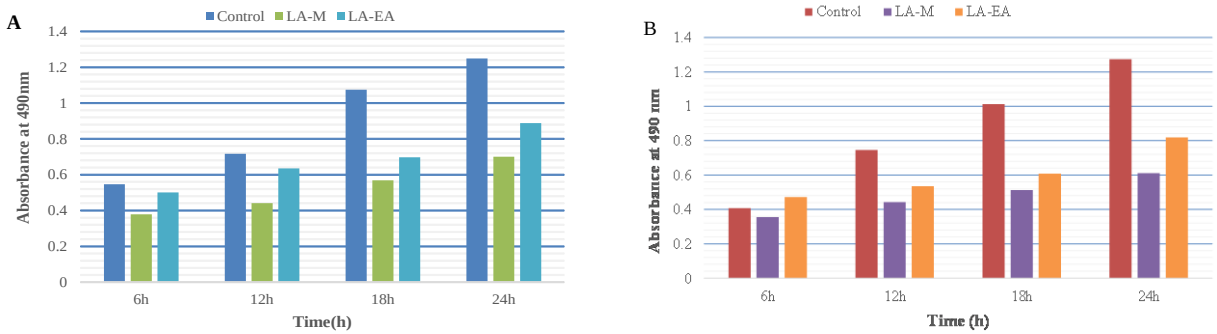

Figure 6: Effect of *L. acidissima* extracts on A) *A. hydrophila* KUAH1 and B) *A. hydrophila* MTCC 1739 bacterial growth using XTT reduction assay at different time points (6 h, 8 h, 12 h, and 24 h).

Recent reports have revealed that *Aeromonas hydrophila* biofilms cause higher infection rate in fishes and develop resistance against broad spectrum antibiotics (Olaimat and Holley 2012; Jahid et al. 2013). Due to the emergence of drug resistance in *Aeromonas* 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. Several

and Balasundaram 2006; Gilles et al. 2010; Oliveira et al. 2010; Husain et al. 2013).

However, relatively few studies have been conducted on the effect of herbal plants against *Aeromonas* biofilms. Although several medicinal properties such as antioxidant (Teepica et al. 2013), anti-bacterial (Shipra et al. 2014; Srinivasan et al.

2015), hepato protective (Ilango and Chitra 2010), anti-inflammatory (Ahamed et al. 2008) effects of *L. acidissima* have been investigated so far, its ability to inhibit biofilm formation has not yet been reported. Our present investigation, has attempted to evaluate the efficacy of *L. acidissima* fruit extract as an alternative medicine, against the biofilm forming *Aeromonas*.

Among ten isolates of *A. hydrophila*, KUAH1, KUAH3 and KUAH7 showed biofilm forming potential (Table 3). Interestingly the isolate KUAH1 exhibited strong biofilm forming characteristics.

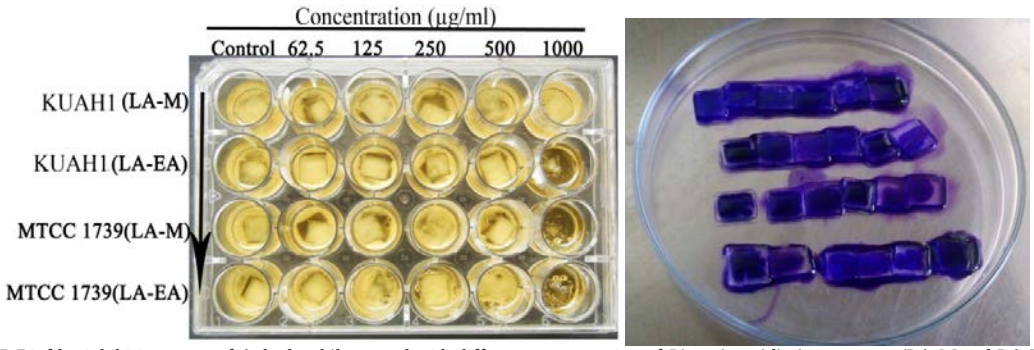

Figure 7: MTP Biofilm inhibition assay of *A. hydrophila* treated with different concentration of *Limonia acidissima* extracts (LA-M and LA-EA) followed by 0.3% crystal violet staining for microscopic visualization.

Strong biofilm forming potential plays an important role in the establishment of *A. hydrophila* infection, enhanced pathogenesis and drug resistance (Husain et al. 2013). The minimal inhibitory concentrations for the *L. acidissima* solvent extracts showed prominent inhibitory activity against KUAHI and MTCC 1739 at lower concentrations. In continuation of the results obtained in the MIC assays, we tested *L. acidissima* extracts further, for its ability to inhibit biofilm formation.

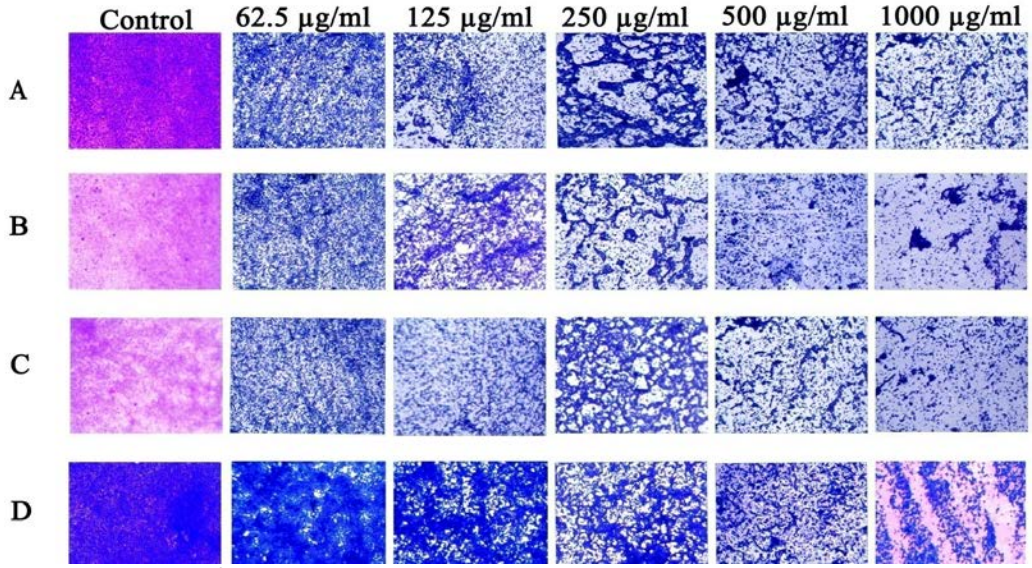

Figure 8: Light microscopic images of A) *A. hydrophila* MTCC 1739 treated with LA-M; B) *A. hydrophila* MTCC 1739 treated with LA-EA; C) *A. hydrophila* KUAH1 treated with LA-M; D) *A. hydrophila* KUAH1 treated with LA-EA, at different concentrations (62.5 µg/ml - 1000 µg/ml) respectively. Untreated test pathogens served as control biofilm. All images were taken at ×400 magnification using Nikon Eclipse Microscope (Model: E200, Tokyo, Japan).

However, in order to rule out the antibacterial property of the *L. acidissima* extracts over biofilm inhibition, we performed growth curve analysis and agar well diffusion assay against the test pathogens at sub-MIC. Evidently, LA-M and LA-EA did not inhibit the growth of test pathogens at sub-MIC concentrations (Figure 2 and Figure 3).These findings corroborated with the earlier reports on medicinal plants (Adonizio et al. 2008) and essential oil (Husain et al. 2013) against the biofilm forming *P. aeruginosa* and *A. hydrophila*.

Further, treatment of *A. hydrophila* KUAH1, MTCC 1739 with LA-M and LA-EA (250-500 µg/ml) evidently revealed concentration dependent reduction in the swimming and swarming motility of the test pathogens (Figure 4A and Figure 4B). Bacterial motility, facilitated by polar flagella, may enhance the invasion of host cell barriers by *Aeromonas hydrophila* and establish infection (Merino et al. 1997). In addition, motility is an important trait in establishment and development of biofilm biomass on host surface (Tomich et al. 2002). Significant ($p \leq 0.05$) decrease in the swimming and swarming motility of test pathogens as compared to control, indicated that LA-M and LA-EA extracts indirectly affected a significant trait on the biofilm formation, in part by interfering with its ability to reach the substratum and initiate cell-to-surface attachment at sub-MIC. Moreover, swarming or pili dependent motility plays a vital role in enhancing virulence as it is involved in biofilm formation and mass translocation of cells (Daniels et al. 2004; Kearn 2010). Reduction in the swarming motility in the present study evidently showed that

Limonia extracts have the potential to reduce the virulence of *A. hydrophila* KUAH1 and MTCC 1739 at sub-MIC without affecting the growth of the bacteria. Similar reports have indicated the efficacy of different plant extracts and biologically active phyto-compounds inhibiting one or more characteristics of biofilm forming gram negative pathogens (Taganna et al. 2011; Husain and Ahmad 2013).

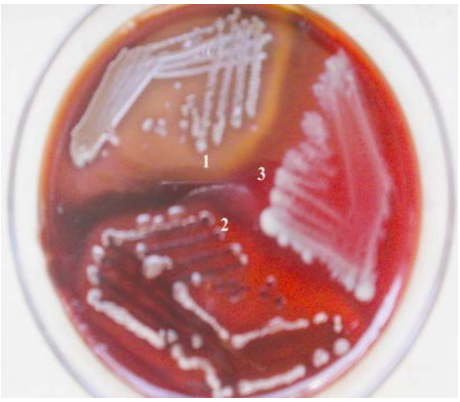

Figure 9: β Haemolytic activity of *A. hydrophila* isolates on blood agar 1) KUAH1, 2) MTCC 1739 and 3) KUAH7 (non haemolytic).

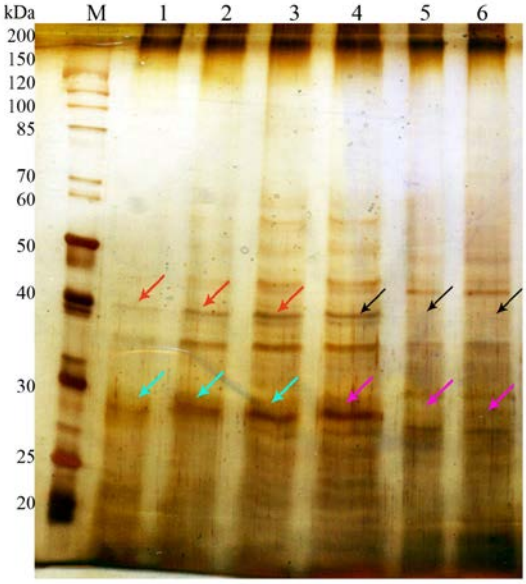

Figure 10: SDS-PAGE analysis of extracellular protein fraction (~25 µg). Lane M- 200 kDa Protein molecular weight marker (~20 µg), Lane 1) *A. hydrophila* MTCC-1739 treated with LA-M , Lane 2) *A. hydrophila* MTCC-1739 treated with LA-EA, Lane 3) *A. hydrophila* MTCC-1739 Control, 4) *A. hydrophila* KUAH1 Control, 5) *A. hydrophila* KUAH1 treated with LA-M, Lane 6) *A. hydrophila* KUAH1 treated with LA-EA. Arrow heads indicate the differential protein bands in plant extract treated samples as compared to control.

Biofilm formation in *A. hydrophila* is regulated by cell to cell signalling systems and virulence factors which play a significant role to cause disease in most fishes (Lynch et al. 2002; Khjanchi et al. 2010). In addition, biofilms facilitate chronic wound infections by creating barriers against the immune system of host and antibiotics (Thurlow et al. 2011). Since *L. acidissima* extracts exhibited effective inhibitory activity against some of the virulence characteristics such as motility of KUAH1 and MTCC1739, we further hypothesized that LA-M and LA-EA may also influence the biofilm formation in test pathogens. Our results support the hypothesis as the extracts inhibited the biofilm biomass significantly ($p \leq 0.005$) in a dose dependent manner (Figure 5 and Figure 6) without influencing the growth of the bacteria thereby preventing the development of resistance.

Further, our study elucidated that LA-M showed good biofilm inhibition (BIC) as compared to LA-EA extract which may be attributed the presence of active biological compounds in methanol extract. The biofilm inhibitory activity of *L. acidissima* extracts were significant at sub-BIC (0.5 BIC, 0.25 BIC and 0.125 BIC) elucidating the antibiofilm potential of the plant extracts at minimal concentrations. Our results were in affirmation with previous studies on bacterial biofilm inhibition by plant extracts (Choo et al. 2006; Harijai et al. 2010; Kavanaugh and Ribbeck 2012; Dineshbabu et al. 2015).

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 (Swift et al. 1999). In order to confirm the concentration dependent inhibition of biofilm formation visual analysis we performed light microscopy of *A. hydrophila* KUAH1 and MTCC 1739. Our study revealed intact biofilm formation by untreated (control) cells in 48 h whereas treated cells exhibited disorganization of biofilm stages with increasing concentration of LA-M and LA-EA extracts. Therefore, it is expected that treatment of KUAH1 and MTCC 1739 with sub-MICs of LA-M and LA-EA resulted in the formation of weak biofilms possibly by reducing the surface adhesion and micro colony formation (Figure 7 and Figure 8). However, complex processes related to genetic and ecological parameters are involved in the biofilm development and establishment mechanisms (Kjelleberg and Molin 2002).

Therefore, the exact mechanism of action of Limonia extracts on specific stages of biofilm formation is to be worked out. Results of the present study are in agreement with Khajanchi et al. (2010) and Husain et al. (2013) who studied the effect of plant products on molecular mechanism in biofilm formation.
A. hydrophila have been reported to synthesize two haemolytic toxins, haemolysin and aerolysin. Among the haemolytic protein isolated from pathogenic bacteria, β haemolysins are one of the important bacterial virulence factors. Haemolysins contain cystathionine β synthase enzyme that function by forming membrane- spanning pore on the host cell surface (Bolch and Monteil 1989). The present study showed that *A. hydrophila* KUAH1 and MTCC1739 exhibit β haemolytic activity (Figure 9).

The molecular mass of the bacterial haemolysins and associated sub units ranges from 30-60kDa (Buckley 1992; Aoki and Hirono 2006). In the present study, we have demonstrated presence of prominent protein band of approximately 30-45 kDa in the extracellular protein fraction of KUAH1 and MTCC1739 untreated controls which indicate the presence of the bacterial toxin hemolysin (Figure 10). Interestingly, LA-M and LA-EA treated test pathogens showed less prominent band patterns at 30-45 kDa which suggest that the LA extracts might have reduced the intensity of virulence by suppressing the toxin synthesis. It is well documented that the polyphenols and flavonoids present in plants have antihaemolytic properties (Peralta et al. 2013). *L. acidissima* fruits have been extensively reported to be rich in phenols,

flavonoids, amino acids and medicinally useful bioactive compounds (Teepica et al. 2013; Shipra et al. 2014; Srinivasan et al. 2015). It can be suggested that polyphenols and flavonoids contents in the Limonia extracts might play a vital role in reducing the haemolytic activity of the *A. hydrophila* which is evident from the differential protein band patterns of test pathogens.

Conclusion

In conclusion, the study reveals the anti-biofilm activity of *L. acidissima* extracts against *A. hydrophila* isolated from infected fresh water fishes. The findings of the present study are in overall consistence with previous studies on the effect of medicinal plants against biofilm forming pathogens. Apparently our study shows that *L. acidissima* may possibly be used to control the biofilms of Aeromonads. Considering the antibacterial activity at minimal concentrations and absence of toxicity, it is envisaged that *L. acidissima* in combination with conventional antibiotics could be used in the treatment of drug resistant *A. hydrophila* in fish cultivation systems. However, it remains for further studies to explore the effect of Limonia fruit extracts on the molecular mechanism involved in Aeromonads biofilm formation.

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