BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, September 2018. Vol. **15**(3), p. 661-670

Influence of Linoleic Acid on Quorum Sensing in *Proteus mirabilis* and *Serratia marcescens*

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http://dx.doi.org/10.13005/bbra/2674

(Received: 04 July 2018; accepted: 08 August 2018)

Quorum sensing (QS) is a bacterial cell density dependent mode of communication involved in regulation of virulence in pathogens including biofilm formation. Accordingly, curbing QS might prove to be an anti-virulence approach of controlling nosocomial infections caused by multi drug resistant bacteria. The report presented here documents the QS inhibitory properties of linoleic acid against *Proteus mirabilis* and *Serratia marcescens* known to cause nosocomial infections. Urease assay, prodigiosin assay, protease assay, biofilm formation assay and growth curve analysis were performed to investigate the effectiveness of linoleic acid in controlling virulence of *P. mirabilis* and *S. marcescens*. 2.5mM linoleic acid reduced the urease activity and biofilm formation to 42.11% and 11.11% respectively in *P. mirabilis*; and prodigiosin synthesis, protease activity and biofilm formation to 0%, 65.91% and 33.33% correspondingly in *S. marcescens*. Therefore, analysis of QS inhibitory behaviour of linoleic acid substantiates its use as a plausible drug for anti-virulence therapy without subjecting the bacteria to discerning force of antibiotics.

Keywords: Quorum Sensing (QS), linoleic acid, virulence, biofilm, *P. mirabilis*, *S. marcescens.*

Communication among cells is a quintessential process synchronising assorted functions of higher organisms. Similar interaction also occurs amongst bacteria and is dependent on bacterial cell density. It is referred to as quorum sensing (QS). QS accounts for orchestrating bacterial functions and gene expression[1] and is mediated by small diffusible molecules, autoinducers. The nature of these autoinducers is oligopeptides in gram-positive bacteria and N-Acyl Homoserine Lactones (AHLs) in gram-negative bacteria[2]. Each bacterium produces autoinducers in small amounts. As the bacterial cell number increases so does the concentration of these autoinducers, thus reaching a threshold value. Subsequently, autoinducers bind to receptors on bacterial cell and trigger expression of certain genes while repressing other genes[3]. This behaviour helps in biofilm formation, horizontal gene transfer, protease and exoenzyme antibiotic synthesis.

Proteus mirabilis and *Serratia marcescens* are gram-negative opportunistic pathogens causing nosocomial infections. *P. mirabilis* is primarily found associated with urinary tract

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infections, characterised by swarm migration [4]. *P. mirabilis* forms biofilm in the host organism besides synthesising urease, hemolysin and other virulence factors[5]. *S. marcescens* frequently appears as nosocomial pathogen causing infections of wound, respiratory tract and urinary tract. This bacterium produces virulence factors like protease and prodigiosin and forms biofilm. Besides, this bacterium exhibits swimming and swarming motility[6]. Secretion of virulence factors and biofilm formation, under the control of QS facilitates bacteria to successfully establish infection in the host. Present technique of dealing with bacterial infection involves use of antibiotics that are lethal to bacteria, generating selective pressure that results in emergence of drug resistant strains[7]. Thus, impeding QS would be a favourable method of dealing with bacterial infections caused by multi-drug resistant (MDR) bacteria without causing selective pressure on them.

Fatty acids (saturated and unsaturated) and their derivatives have been reported to possess antimicrobial properties; specifically fatty acids containing two double bonds, like linoleic acid, is more potent bacteriostatic compound[8]. Moreover, conjugated linoleic acid has also been described to inhibit bacterial growth through lipid peroxidation in the membranes[9]. Recent studies made on the effect of conjugated linoleic acid on colon, has indicated its ability to offset the development of inflammatory lesions[10]. Linoleic acid and fatty acids like oleic acid, palmitic acid and stearic acid are capable of inhibiting autoinducer -2 (AI-2) activity consecutively affecting QS [11]. Considerable work has been done on antimicrobial nature of linoleic acid but very little work has been accomplished to scrutinize its QS inhibitory activity. Hence, analysis made in this report deals with QS inhibitory effect of linoleic acid on *S. marcescens* and *P. mirabilis*.

MATERIALS AND METHODS

Linoleic acid, free acid ($C_{18}H_{32}O_2$, CAS no. 60-33-3) was purchased from Himedia Laboratories, India. 357mM Stock solution was prepared by dissolving linoleic acid in DMSO and stored at 4p C. Different concentrations of linoleic acid were tested against *P. mirabilis* and *S. marcescens* based on earlier made reports[11, 12]. Concentrations of 1.0mM to 2.5mM of linoleic acid were found to be non – inhibitory and hence were used for further assays.

Bacterial strains and growth conditions

S. marcescens was obtained from Vishakha Labs Pvt. Ltd.,Nagpur and *P. mirabilis* was obtained from Institute of Microbial Technology (IMTech), Chandigarh. The bacteria were grown and maintained on nutrient agar (peptone 5g/l, sodium chloride 5g/l, beef extract 1.5g/l, yeast extract 1.5g/l, agar 15g/l and pH 7.4±0.2) slants and stored at 4p C till further use.

Urease Assay (for *P. mirabilis*)

Effect of linoleic acid on urease activity of *P. mirabilis* was ascertained by quantifying the amount of hydrolysed urea in urea - Luria Bertani (LB) broth [13]. Sterile LB broth (casein enzyme hydrolysate 10g/l, yeast extract 5g/l, sodium chloride 10g/l and pH 7.5±0.2) tubes containing urea were prepared. Different concentrations of linoleic acid (1mM, 1.5mM, 2mM and 2.5mM) were added to these tubes. 1% overnight broth culture was inoculated in these tubes and incubated for 48 hours at 37p C. After incubation, amount of hydrolysed urea was determined by comparing with a suitable control.

Prodigiosin assay (for *S. marcescens*)

Overnight culture of *S. marcescens* was used for development of assay. 1% of broth culture was inoculated in sterile nutrient broth tubes containing different concentrations of linoleic acid (1.0mM – 2.5mM) and incubated overnight. After incubation the cells were pelleted by centrifugation at 10,000 rpm for 10 minutes and resuspended in acidified ethanol solution (96ml of ethanol containing 4% of 1M HCl) for extraction of prodigiosin. The prodigiosin extracted was determined by reading absorbance at 534nm[14,15]. The relative prodigiosin concentration was determined using the following equation:

$$Relative\ Prodigiosin\ concentration\ per\ cell = \frac{A_{534} ml^{-1}}{OD_{600} unit}$$

Protease assay (for *S. marcescens*)

Effect of linoleic acid on protease synthesis was analysed using skimmed milk agar assay [16]. *S. marcescens* was inoculated in sterile broth tubes containing different concentrations of linoleic acid (1.0mM – 2.5mM) and incubated overnight. After incubation, cells were pelleted by centrifugation at 10,000rpm for 15 minutes. The supernatant obtained was loaded into wells of

skimmed milk agar (skimmed milk powder 28g/l, casein enzyme hydrolysate 5g/l, yeast extract 2.5g/l, dextrose 1g/l, agar 15g/l and pH 7.0±0.2) plates. Plates were incubated overnight at 37p C and observed for zone of clearance using suitable control.

Biofilm formation assay

Influence of linoleic acid on biofilm formation ability of *P. mirabilis* and *S. marcescens* was investigated using crystal violet assay [15]. 1% of overnight broth culture (OD_{600} = 0.4) was inoculated in LB broth and 1 ml of freshly inoculated broth was then transferred into wells of microtiter plate. Different concentrations of linoleic acid (1.0mM – 2.5mM) were then added to these wells and incubated overnight. After incubation, planktonic cells were removed from the wells by washing twice with sterile water and biofilm was stained with 0.4% crystal violet. Excess of stain was removed by washing with sterile water. Crystal violet bound cells were solubilised using 1ml of ethanol and absorbance was read at 570nm.

Microscopic analysis

Microscopic analysis of biofilm formation by *P. mirabilis* and *S. marcescens* in presence of linoleic acid was studied using air-liquid interface assay as described by Merritt *et. al.* [17] . 0.1% inoculum was added to sterile LB broth and 250¼l of this freshly inoculated broth was transferred to wells of microtiter plate placed at an angle of 30 - 50p . 50¼l of different concentrations of linoleic acid (1.0mM – 2.5mM) were then added to these wells and incubated overnight in the angled position. Then the spent medium was removed carefully followed by washing twice with sterile medium. 200¼l of sterile medium was then added to each of the wells and plate was gradually lowered on the flat surface of inverted microscope and cells of biofilm were visualised.

Growth Curve Assay

Effect of linoleic acid on quorum sensing was studied using growth curve assay described by Hall *et. al.* with few modifications [18]. Sterile nutrient broth tubes containing different concentrations of linoleic acid were inoculated with 0.1% overnight culture of *P. mirabilis* and *S. marcescens*. 100ml of this freshly inoculated broth of each tube was transferred to different wells of microtiter plate. The plates were covered and immediately the absorbance of the plate was read at 600nm using plate reader. The plates were

Table 1. Effect of Linoleic acid on Urease Activity of *Proteus Mirabilis*

Concentration of Linoleic acid (mM)	Urea remaining in reaction mixture after incubation (10^{-3} ìmoles) Mean ± SD	Percentage of Urease Activity
Control	7.6±2.82	100.00
1.0	8.4±0.14	90.48
1.5	9.7±1.05	78.35
2.0	11.2±0.14	67.86
2.5	18.9±0.28*	40.21

Experiments were performed in triplicates and *p<0.02.

Table 2. Effect of Linoleic acid on Prodigiosin Synthesis and Protease Synthesis of *S. Marcescens*

Concentration of Linoleic acid (mM)	Prodigiosin Synthesis		Protease Synthesis	
	Relative Prodigiosin Synthesis Mean ± SD	Percentage of Prodigiosin Synthesis	Zone of Clearance (cm) Mean ± SD	Percentage of Protease Activity
Control	0.09±0.03	100.00	2.20±0.10	100.00
1.0	0.06±0.02	67.98	1.70±0.07#	77.27
1.5	0.02±0.02*	21.91	1.58±0.04	71.82
2.0	0.00±0.00^	0.00	1.50±0.10$	68.18
2.5	0.00±0.00^	0.00	1.45±0.05&	65.91

Experiments were performed in triplicate and *p<0.04, ^p<0.01, #p<0.005, $p<0.003 and &p<0.001

then transferred to shaker incubator and OD_{600} was read at 2, 4, 6, 8 and 24 hours and compared with control.

Statistical Analysis

All the experiments were performed in triplicates and the results are expressed in the form of mean ± standard deviation (SD). Statistical

Table 3. Effect of Linoleic acid on Biofilm Formation in *P. Mirabilis* and *S. Marcescens*

Concentration of Linoleic acid (mM)	*P. mirabilis*		*S. marcescens*	
	O. D. $_{570}$ Mean ± S. D.	Percentage of Biofilm Formation	O. D. $_{570}$ Mean ± SD	Percentage of Biofilm Formation
Control	0.009±0.0039	100.00	0.012±0.0043	100.00
1.0	0.008±0.0000	88.89	0.010±0.0023$^{\&}$	83.33
1.5	0.008±0.0012$^{\#}$	88.89	0.008±0.0041	66.67
2.0	0.005±0.0026$^{\$}$	55.56	0.006±0.0013*	50.00
2.5	0.001±0.0056$^{\wedge}$	11.11	0.004±0.0017*	33.33

Experiments were performed in triplicate and $^{\#}p<0.02$, $^{\$}p<0.03$, $^{\wedge}p<0.01$, $^{\&}p<0.006$ and $^{*}p<0.001$ and O.D. $_{570}$ – Optical Density at 570nm

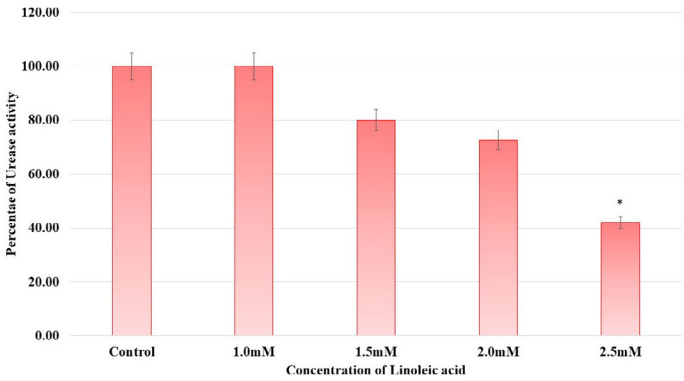

Fig. 1. Effect of Linoleic acid on Urease activity of *P. mirabilis.* Experiments were performed in triplicates and *p<0.02 with respect to control

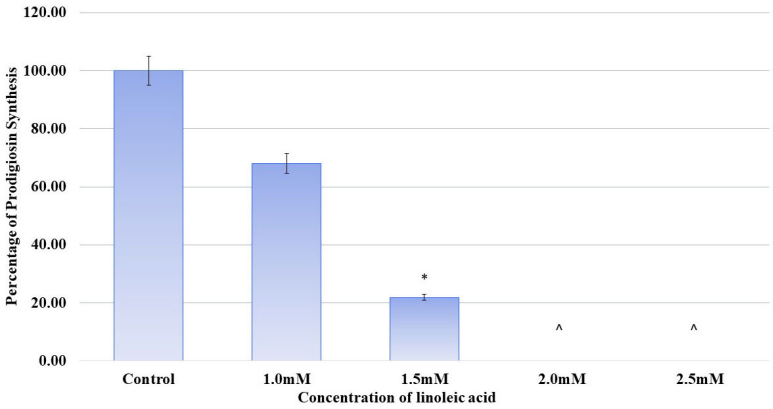

Fig. 2. Effect of Linoleic acid on Prodigiosin synthesis of *S. marcescens*. Error bars indicate that experiments were performed in triplicates; *p<0.04 and $^{\wedge}p<0.01$

significance was determined by Student's t-test using Microsoft Excel (Version 16.4.1) and p value < 0.05 was considered to be significant.

RESULTS AND DISCUSSION

QS is a bacterial communication synchronizing biofilm formation and virulence factor synthesis [19]. Work presented here documents the influence of linoleic acid on formation of biofilm and production of virulence factors in *P. mirabilis* and *S. marcescens*.

Urease activity of *P. mirabilis* decreased with increasing concentration of linoleic acid. While, urease activity was marginally affected at 1.0mM of linoleic acid, highest inhibition was seen at 2.5mM linoleic acid, where the activity of urease was reduced to 40.21% with respect to control. Urease activity and its percentage inhibition in presence of linoleic acid are presented in Table 1. The effect of linoleic acid on urease activity is graphically represented in Fig 1. Previous studies of napthoquinones from plant *Diospyrus lotus*[20], allicin from garlic[21], curcumin[22] and

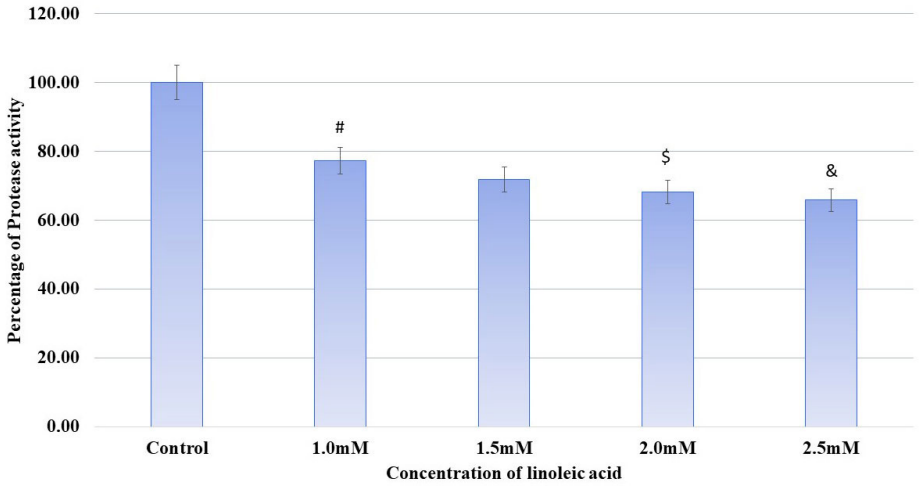

Fig. 3. Effect of Linoleic acid on Protease activity of *S. marcescens*. Error bars indicate that experiments were performed in triplicates; #p<0.005, $p<0.003 and &p<0.0001

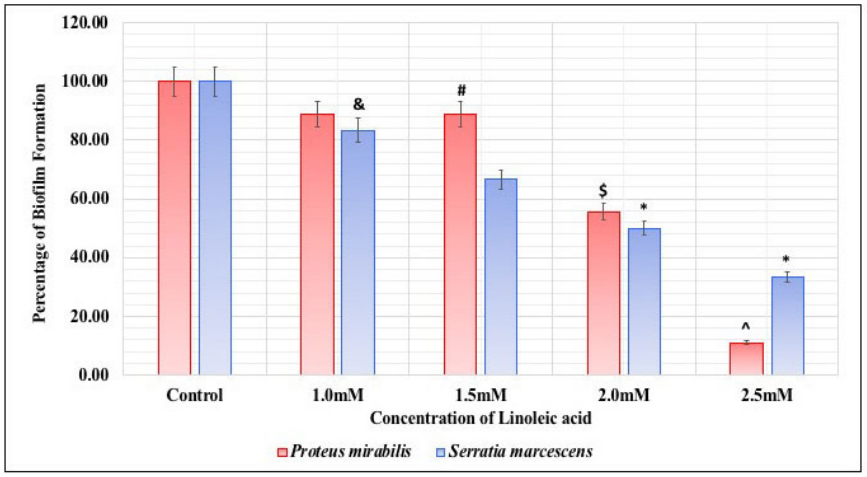

Fig. 4. Effect of Linoleic acid on Biofilm formation of *P. mirabilis* and *S. marcescens*. Error bars indicate that experiments were performed in triplicates; #p<0.02, $p<0.03, ^p<0.01, &p<0.006 and *p<0.001

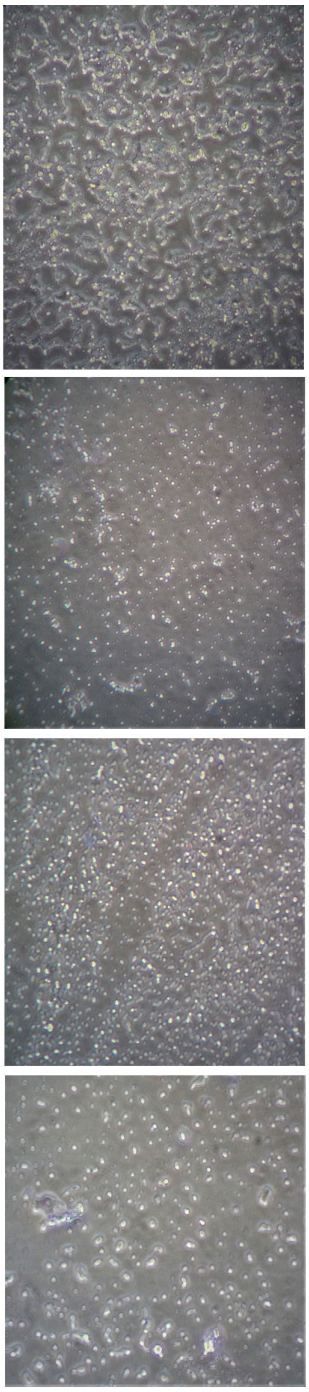

Fig. 5. Microscopic analysis of Biofilm
(a) Biofilm of *P. mirabilis* in presence of Control
(b) Biofilm of *P. mirabilis* in presence of 2.5mM linoleic acid
(c) Biofilm of *S. marcescens* in presence of Control
(d) Biofilm of *S. marcescens* in presence of 2.5mM linoleic acid

fluoroquinolones[23] have reported inhibition of urease activity in *P. mirabilis*. Urease enzyme containing nickel ions, a major virulence factor produced by *P. mirabilis*, hydrolyses urea to yield ammonia[24]. The products of urease action increase pH of urine and eventually initiate formation of struvites or apatites in bladder or kidney. Moreover, this increased pH is also toxic to host cells[25]. Thus, interfering with or reducing urease activity could possibly decrease virulence of pathogen in turn suggesting a possible role for linoleic acid in clinical applications.

Prodigiosin synthesis in *S. marcescens* was drastically reduced in presence of linoleic acid. Synthesis of prodigiosin was observed to be nil at 2.0mM and 2.5mM of linoleic acid as perceived from Table 2 and Fig 2. 1.0mM and 1.5mM of linoleic acid decreased prodigiosin synthesis to 67.98% and 21.91% in *S. marcescens*. Earlier studies made using petroselinic acid[26] and ambroxol[6] have demonstrated significant reduction of prodigiosin synthesis in *S. marcescens*. However, linoleic acid used in the present study was successfully able to inhibit prodigiosin synthesis. Synthesis of prodigiosin in *S. marcescens* is a QS controlled function and restraining its synthesis completely by linoleic acid highlights the effectivity of molecule in disrupting QS system of bacterium.

Protease synthesis in *S. marcescens* was affected in a dose dependent manner in presence of linoleic acid. The protease synthesis was gradually reduced from 77.27% to 65.91% in presence of 1.0mM and 2.5mM linoleic acid respectively. Effect of linoleic acid on protease activity of *S. marcescens* is depicted in Table 2 and Fig 3. Inhibition of protease activity in *S. marcescens*, an important virulence factor regulated by QS, has also been described in previous analysis carried out using phenol, 2,4-bis(1,1-dimethylethyl)[27], phytol from *Piper betle*[28], á-Bisabolol from *Padina gymnospora*[29] and vanillic acid from *Actinidia deliciosa*[30]. Proteases secreted by *S. marcescens* influence its pathogenicity by degrading various secreted proteins of host like immunoglobulins. Thus, protease synthesis benefits the bacterium by evading defence mechanism and hence establishing infection[31]. Consequently, decreased protease synthesis by *S. marcescens* in presence of linoleic

acid might serve as a useful tool for controlling pathogenicity and curbing the infection.

Bacterial biofilms are exemplary structures of group behaviour formed under the regulation of QS [32]. Biofilm formation imparts bacteria with advantages like improved resistance to antibiotics and increased tolerance to environmental stress [33]. Linoleic acid affected biofilm formation in both *P. mirabilis* and *S. marcescens*. Biofilm formation was also reduced in studies carried out using eucalyptus oil [34] and *Capparis spinosa* [35]. The effect of linoleic acid Table 3 and fig 4

on biofilm formation of both bacteria has been depicted in Table 3 and illustrated in Fig 4. Biofilm formation was 11.11% and 33.33% in *P. mirabilis* and *S. marcescens* respectively at 2.5mM linoleic acid. Thus, biofilm inhibiting potential of linoleic acid was higher against *P. mirabilis* than *S. marcescens*. This apparent difference of biofilm inhibition by linoleic acid might be a result of type of biofilms formed by these bacteria. *P. mirabilis* forms crystalline biofilms which gradually flatten out, followed by swarming of cells for spreading the infection to new sites. Crystalline biofilms result from urease activity exhibited by the bacterium[36]. Conversely, *S. marcescens* biofilm are highly organised, porous and filamentous structures with chains and clusters of cells[37]. Accordingly, *P. mirabilis* biofilm formation was inhibited more than *S. marcescens* because of reduced urease activity.

Microscopic analysis of biofilm formation by *P. mirabilis* and *S. marcescens* in presence of 2.5mM linoleic acid indicated diminished biofilm formed when compared to control as seen from Fig 5. Previous work done with palmitoleic acid and myristoleic acid also depicted similar inhibitory effects [38]. Hence, this observation fortifies biofilm inhibitory potential of linoleic acid.

Growth curve assay of *P. mirabilis* and *S. marcescens* was accomplished to evaluate whether the changes seen in QS regulated properties are

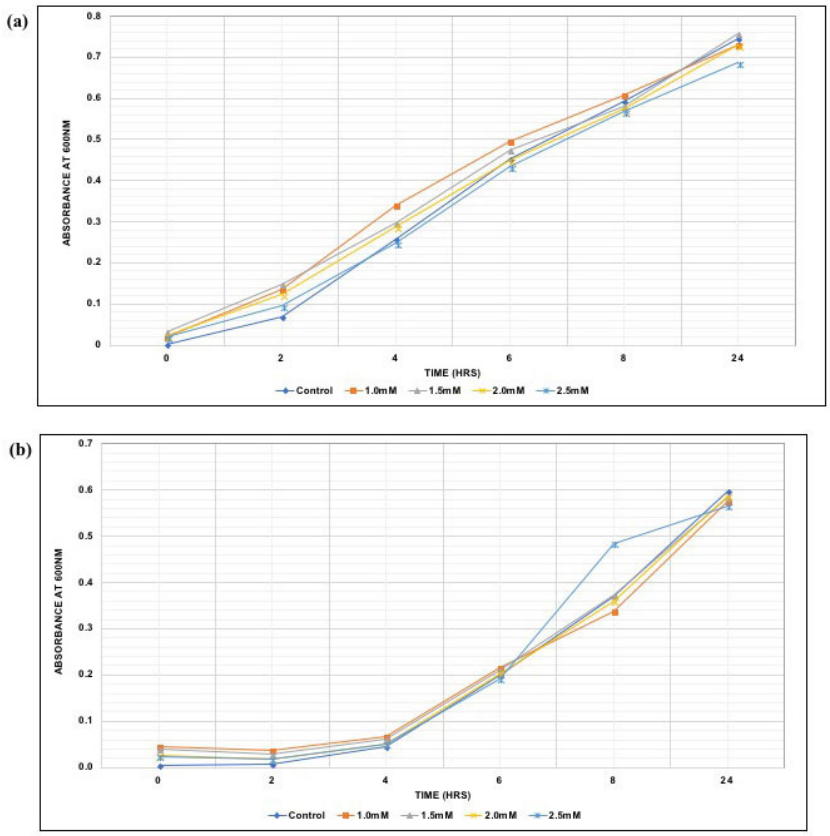

Fig. 6. Growth curve analysis of linoleic acid against (a) *P. mirabilis* and (b) *S. marcescens*

result of quorum inhibitory activity of linoleic acid and not its anti-bacterial activity. Analysis confirmed that there was no significant change observed in cell densities of *P. mirabilis* and *S. marcescens* at 24 hours when compared to control as seen from Fig 6. Similar results were perceived in prior investigation performed using cinnamon oil[16] and proanthocyanidins derived from cranberry [39]. Therefore, linoleic acid was able to efficaciously reduce virulence and quench quorum of *P. mirabilis* and *S. marcescens* without inflicting selective pressure.

CONCLUSION

Fatty acids including linoleic acid are known to be antimicrobial in nature [8]. But, the results of present study explained virulence inhibitory properties of linoleic acid and subsequently its QS inhibitory potential. Linoleic acid effectively reduced urease synthesis and biofilm formation in *P. mirabilis*. Moreover, it decreased QS controlled protease and prodigiosin synthesis in *S. marcescens* besides diminishing formation of biofilm.

Though, the exact route of this QS inhibition by linoleic acid is not known, this work provides an alternative approach of anti-virulence therapy from nutritional source for combatting bacterial infections in a milieu of increasing antibiotic resistance.

ACKNOWLEDGEMENT

The authors would like to express our gratitude towards CSIR for providing funding in the form fellowship [File no. 08/611(0001)2013-EMR-I].

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