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Quorum quelling efficacy of marine cyclic dipeptide -cyclo(L-leucyl-L-prolyl) against the uropathogen *Serratia marcescens*

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ABSTRACT

In the current study, the anti-quorum sensing (QS) efficacy of cyclic dipeptide -cyclo(L-leucyl-L-prolyl) (CLP) of marine origin was explored against *Serratia marcescens*. Minimal -inhibitory (MIC) and –bactericidal concentrations (MBC) of CLP against both reference as well as a clinical isolate of *S. marcescens* was identified to be 200 and 400 μg/mL, respectively. CLP proficiently inhibited the QS controlled prodigiosin production in *S. marcescens*, which affirm its anti-QS efficacy towards *S. marcescens*. At sub-MIC (100 μg/mL), CLP exhibited a phenomenal inhibitory propensity towards the production of virulence traits viz. biofilm, exopolymeric substance, protease and lipase to the level of 81, 77, 71 and 92%, respectively. Further, the confocal and scanning electron microscopic analyses validated the antibiofilm efficacy of CLP. Besides, CLP effectively modified the hydrophobic and motility characteristics of *S. marcescens*. Furthermore, the *in vivo* assay using *C. elegans* revealed the non-toxic and anti-adherence propensity of CLP. Concomitantly, the down regulation of QS controlled virulence genes (unveiled through qPCR analysis) are in accordance with the data of phenotypic and *in vivo* assays. Therefore, this study exemplifies that CLP could plausibly be a convincing alternative over conventional antibiotics in preventing the QS associated pathogenesis of uropathogens.

1. Introduction

Globally, urinary tract infections (UTIs) have long been recognized as one of the most frequently encountered human diseases in hospital settings, as it attributes nearly 40% of total nosocomial infections (Derbie et al., 2017; Hvidberg et al., 2000). The incidence of UTI is more predominant in women rather than men, plausibly due to the anatomical reasons (Stamm and Norrby, 2001). Etiological agents responsible for causing severe UTI includes *Escherichia coli*, *Proteus* spp., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Serratia marcescens* (Srinivasan et al., 2017). Unlike the other UTI causing agents, *S. marcescens* with its distinctive quorum sensing (QS) regulated phenotypic characteristics (such as virulence secretion, motility, biofilm formation and pigment production) poses severe distress to public health. Besides causing UTI, this Gram-negative opportunistic pathogen also causes a diverse array of nosocomial infections ranging from surgical wound infections to septicemia, central nervous system infections and bloodstream infections (Fedrigo et al., 2011; Guler et al., 2009).

The infectious nature of *S. marcescens* is coupled by its inherent competence to resist a massive stretch of antibiotics (González-Juarbe et al., 2015; Liou et al., 2014), specifically against broad spectrum beta-lactam antibiotics through secreting the enzyme-betalactamase (Yoon et al., 2005). Moreover, the occurrence of plasmids and efflux pumps in *S. marcescens* has substantially fuelled the unfortunate emergence of multiple drug resistance strains, which eventually makes the treatment process more complex (Morohoshi et al., 2007). As a consequence, the World Health Organization has acknowledged antimicrobial resistance as the third most significant threat to human health. Alarmingly, the recent emergence of New Delhi metallo-β-lactamase (NDM)-1 producing *S. marcescens* strains (Gruber et al., 2014) emphasizes the pressing need for the development of alternative treatment strategies with proficiency to nullify the phenomenon of antibiotic resistance.

Although the free floating *S. marcescens* cells are more vulnerable to antimicrobials, upon agglomeration they reorganize themselves as architecturally complex clusters made of self-synthesized extracellular polymeric matrix called biofilms, which resist most of the antimicrobials' action (Sethupathy et al., 2017; Srinivasan et al., 2017).

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Such ability of *S. marcescens* to develop as recalcitrant biofilm on indwelling medical implants (viz. urinary catheter, prosthetic heart valve, artificial hip prosthesis and intrauterine devices) makes the infection difficult-to-treat. Notably, an estimate by the National Institute of Health (NIH, USA) implies that the pathogenic bacterial biofilms attributes nearly 60% of bacterial infections in humans (Costerton et al., 1999; Lewis, 2001).

Despite the metabolic versatility and genome plasticity in *S. marcescens*, the occurrence of QS controlled pathogenic traits including biofilm formation has augmented a substantial clinical significance in recent times (Sethupathy et al., 2016). An optimal number (quorum) of *S. marcescens* population senses the self-secreted autoinducer (AI) molecule, chiefly acylated homoserine lactone (AHL) to regulate the production of various virulence factors (Sethupathy et al., 2017). Since, QS being a global regulon in controlling the comprehensive pathogenic traits of *S. marcescens*, targeting the QS system could plausibly be a promising alternative strategy to subvert the subsequent virulence pathways Thereby, the pathogen becomes disarmed inside the host system, which in turn facilities the host defence mechanism for successive clearance.

In this milieu, cyclic dipeptides (CDPs) from inexhaustible natural resources, for instance mangrove rhizosphere microbes are getting massive attention of many researchers working in the arena of QS, for unearthing novel QS inhibitors (QSI) against multidrug resistant pathogens (Gowrishankar et al., 2015). Beyond having enormous ecological values (Holguin et al., 2001), mangroves are the rich source of significant pharmaceutical thrust. Being a cluster of hormone-like molecules, CDPs are hierarchically conserved from bacteria to humans. Earlier reports have exemplified the therapeutic significance of CDPs for having proficiency in not only mitigating biofilm formation but also in thwarting virulence, which eventually tends the targeted bacterium to shed its pathogenic character (Borthwick, 2012; Gowrishankar et al., 2014; Li et al., 2011; Rhee, 2004; Scopel et al., 2013; Yan et al., 2004). In a recent review by Bellezza et al. (2014), CDPs were recorded to behave as QS signalling molecules during bacterial cross-talk and its impeding effect on the QS mechanism of certain Gram-negative pathogens have recently been explored (Campbell et al., 2009). Therefore, the present study was deliberately focused on exploring the *in vitro* and *in vivo* (using the animal model *Caenorhabditis elegans*) QSI potential of a marine cyclic dipeptide-cyclo(L-luecyl- L-prolyl) (Gowrishankar et al., 2014)against the uropathogen −*S. marcescens*, whose pathogenicity was entirely under QS control.

2. Materials and methods

2.1. Bacterial strains used and their growth conditions

In the current study, *S. marcescens* reference strain was obtained from American Type Culture Collection (ATCC: 14756), USA and the clinical isolate was acquired from Alagappa University Biobank repository with the GenBank accession no. FJ584421. Both the bacterial strains were grown in Luria-Bertani (LB) medium (Himedia) at 28 °C with aeration. The cells of *S. marcescens* were sub-cultured in LB medium and incubated until the OD_{600} nm reaches 0.4 (i.e., 1×10^8 CFU/mL) for experimental purpose. The test compound, CLP (purity > 98% based on high-performance liquid chromatograph) reported in our previous study (Gowrishankar et al., 2014) was used in the current study. CLP was dissolved in sterile Milli-Q water at final concentrations of 12.5, 25, 50, 100 μg/mL for biological assays. Phytol, a well known quorum sensing inhibitor of *S. marcescens* (Srinivasan et al., 2017) was used as the positive control in all the virulence assays.

2.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

According to the Clinical Laboratory Standards Institute guidelines (Wayne, 2010), the minimal concentration of CLP that effectively kill *S. marcescens* was determined by employing the microdilution susceptibility test. wherein the antibiotic oxacillin was used as the positive control. Concisely, the bacterial suspension of *S. marcescens* containing 10^8 cells/mL (0.4 OD at 600 nm) was used to inoculate LB medium in sterile 24-well microtiter plate (MtP) manifested with the serial two fold dilutions of CLP and incubated the experimental set-up at 28 °C for 24 h. For the determination of MBC and sub-MICs, 10 μL from MIC assay MtP was taken and spread cultured onto LB agar plate and incubated at 28 °C for 24 h (Gowrishankar and Pandian, 2017). The lowest concentration of CLP that allows growing not more than five colonies on each plate was considered as MBC. Whereas the maximum concentrations required below MIC that did not impede growth even after incubation for 24 h when compared to untreated control samples was measured as the sub-MIC.

2.3. In situ microscopic visualization of S. marcescens biofilm formation on glass and catheter surfaces

To evaluate the antibiofilm potential of CLP, the microscopic techniques such as light scanning electron (SEM), and confocal microscopy (CLSM) were employed. Initially, the *S. marcescens* was allowed to form biofilm on glass slides (1 × 1 cm) or catheter and kept in 24-well microtiter plates (MTP) with or without CLP and incubated at 28 °C for 24 h. After incubation, the glass slides or catheter were washed thrice with sterile distilled water to remove the unbound or loosely bound cells and air-dried. Either the glass slides or catheter coupons were processed according to the microscopic method adopted.

The glass slides were stained with 0.4% crystal violet for 5 min for light microscopic analysis, and washed gently with sterile distilled water to remove the excess dye. Finally, the slides after air dry were observed and imaged at 400 x magnifications under light microscope (Nikon Eclipse Ti 100, Tokyo, Japan).

For CLSM, the catheter lodging biofilm were stained with 0.1% acridine orange and kept aside in dark for 5 min. The excess dye was removed by washing the catheter thrice using sterile distilled water and air-dried. Successively, the dried catheter treated or untreated with CLP were observed and imaged under CLSM (LSM 710, Carl Zeiss, Germany), equipped with an excitation filter 515–560 nm and the Z-stack analysis was performed using the software Zen 2009 (Carl Zeiss, Germany) (Gowrishankar and Pandian, 2017). The bactericidal impact of CLP at MIC and 2 X MIC on the *S. marcescens* mature biofilms formed on catheter surface was examined using Live/Dead staining (BacLight, Invitrogen) and micrographs were documented using CLSM following the previously described protocol (Gowrishankar and Pandian, 2017).

In the process of samples for SEM analysis, the biofilms developed on the catheter surface were fixed before dehydration using gradient percentage of ethanol (Gowrishankar and Pandian, 2017). Briefly, the catheter was gently washed using sterile PBS and the adhered biofilms were fixed for 2 h with 2.5% glutaraldehyde diluted with PBS. Afterwards, catheter surfaces washed gently with 0.1 M sodium acetate buffer (pH 7.3). Yet again the catheter surfaces were rinsed with sterile distilled water and dehydrated with increasing concentrations of ethanol (20, 40, 60, 80, 100%) for 5 min each. Soon after the process of dehydration, the catheters were let to air-dry at ambient temperature. Finally, these fixed biofilm adhered catheters were gold sputtered and examined under SEM (Hitachi, S−300H, Japan).

2.4. Prodigiosin assay

The effect of CLP in inhibiting the production of prodigiosin was determined by the method of Morohoshi et al. (2007). Briefly, 10 μL of inoculum containing 1×10^8 CFU/mL of *S. marcescens* were inoculated in LB medium supplemented with or without CLP and incubated at 28 °C for 18 h. Once the bacterial growth reached the late stationary phase, the cells were collected by centrifugation (16,770 × *g* for

10 min). The cell pellet was extracted with 1 mL acidified ethanol solution (4% 1 M HCl in ethanol) to collect the prodigiosin. The resultant prodigiosin found in the acidified ethanol was quantified spectroscopically at 534 nm.

2.5. Protease assay

Proteolytic activity of *S. marcescens* being an important virulence factor, it was quantitatively estimated using azocasein as substrate. Briefly, 1% of bacterial suspension was used to inoculate 1 mL of LB broth with or without CLP for 18 h. Then the content was centrifuged at 7227 × *g* for 10 min and the cell free supernatant was collected. Finally, 125 μL of 2% azocasein in 0.25 M Tris (pH 8.0) was added to the 75 μL of CFCS and incubated at 37 °C for 30 min. The reaction was terminated by adding 100% trichloroacetic acid and subsequently centrifuge the mixture at 7227 × *g* for 10 min. The resultant supernatant was spectroscopically measured at 440 nm to quantify the protease (Musthafa et al., 2011).

2.6. Swimming and swarming motility assay

The method by Abraham et al. (2011) was followed to determine the potential of the CLP in inhibiting the swimming and swarming motility of *S. marcescens*. To determine the swimming of *S. marcescens*, 3 μL of bacteria cell suspension (OD adjusted to 0.4 at 600 nm) was stab inoculated at the centre of the petriplate containing swimming agar medium (1% peptone, 0.5% NaCl, and 0.3% agar supplemented with or without CLP). Then the plates were incubated at optimum temperature for 16 h and observed for the reduction in swimming zone of *S. marcescens*. For swarming assay, 5 μL of bacterial cell suspension (OD adjusted to 0.4 at 600 nm) of *S. marcescens* was spot inoculated at the centre of soft agar plate (containing 1% peptone, 0.5% NaCl, 0.5% agar, and 0.5% of filter sterilized D-glucose) with or without the test compound CLP. After incubation at 30 °C for 16 h, the plates were observed for the reduction in swarming zone of *S. marcescens* treated with CLP.

2.7. Lipase assay

To quantify the lipase production, the *S. marcescens* treated with or without CLP was centrifuged at 10,000 × *g* for 10 min. To the 100 μL of collected CFCS, 900 μL of buffered substrate containing 9 vol of 0.1% gummiarabicum and 0.2% sodium deoxycholate in 50 mM Na_2PO_4 buffer (pH 8.0) and one volume of 0.3% *p*-nitrophenyl palmitate in isopropanol and incubated for 1 h at room temperature. Then the reaction was terminated by adding 1 mL of 1M Na_2CO_3 and subsequently centrifuged the mixture at 10,000 × *g* for 10 min. Finally, the resultant supernatant was measured spectroscopically at 410 nm (Gupta et al., 2002).

2.8. MATH assay

Since, the cell surface hydrophobicity (CSH) of any microbe directly reflects their ability to adhere over any substratum, the CSH of *S. marcescens* alone and upon treatement with CLP were quantified through performing microbial adhesion to hydrocarbons (MATH) assay (Gowrishankar et al., 2016). Concisely, one percentage of the bacterial cell (10^8 cells/ml) was used to inoculate 3 mL of LB broth manifested with or without CLP at sub-MICs (12.5, 25, 50,100 μg/mL) and incubated at 28 °C for 24 h. The hydrocarbon used to evaluate the effect of CLP to modify the CSH of *S. marcescens* was toluene. Five hundred microliters of toluene was added to the experimental tubes and vortexed vigorously for 3 min. The tubes were kept undisturbed for 10 min to get the phase separated. Finally, the lower aqueous phase was aspirated carefully and spectroscopically measured at 600 nm before and after vortexing. The percentage inhibition of hydrophobicity was calculated using the formula:

Percentage hydrophobicity = [1 – (OD_{600nm} after vortexing/OD_{600nm} before vortexing)] X100

2.9. Extraction and quantification of exopolysaccharide (EPS)

The influence of CLP in inhibiting the synthesis of EPS was assessed by quantifying the total carbohydrate content of the bacterial cells (Kannappan et al., 2017). The LB medium (1 mL) with 1% overnight culture of *S. marcescens* was incubated for 18 h in the presence or absence of CLP. Then, the microtiter plates (MTP) containing the assay setup was washed with 0.9% NaCl. Afterwards, equal volume of 5% phenol and five volumes of H_2SO_4 was added to the MTP. The resultant mixture was incubated for 1 h in dark and subjected to centrifugation at 16,770 × *g* for 10 min. Finally, the collected supernatant was subjected spectroscopic quantification at OD 490 nm.

2.10. Assessing the in vivo therapeutic potential of CLP against S. marcescens in C. elegans

2.10.1. Maintenance and synchronization of C. elegans for bioassays

The Bristol N2 wild type nematodes were obtained from *Caenorhabditis* Genetics Centre (CGC), Minnesota, USA and consistently maintained at 20 °C on the *Escherichia coli* OP50 (laboratory food source) seeded Nematode Growth Medium (NGM). For assay purpose, the nematodes were age-synchronized by treating the healthy adult nematodes with commercial bleach (Sodium hypochlorite) and 5 M potassium hydroxide in an equal ratio and then the eggs were transferred to freshly prepared NGM plate seeded with OP50 followed by incubation at 20 °C. Subsequently, these age-synchronized (L4 stage) nematodes were washed from the NGM plates and used in the following *in vivo* assays.

2.10.2. C. elegans liquid survival assay

The liquid survival assay has been performed to scrutinize the impact of CLP on the virulence of *S. marcescens* against *C. elegans*. The age-synchronized L4 stage nematodes from the NGM plates were washed with M9 buffer 4–5 times and accurately 10 nematodes were transferred to the 24-well MtP containing 20% inoculum of *S. marcescens* in the sterile M9 buffer along with the different concentrations of CLP (12.5, 25, 50, 100 μg/mL). The worms exposed with *E. coli* OP50 and *S. marcescens* and methanol were considered as negative, positive and vehicle controls correspondingly. The experimental MtP plates were kept for incubation at 20 °C and the nematodes were monitored for increase or decrease in their survival and recorded at every 6 h. Nematodes that did not respond to the gentle tap or touch by the platinum worm picker conjugated with ceased pharyngeal pumping was considered as dead.

2.10.3. Microscopic imaging of internal colonization in C. elegans

To examine the internal colonization of *S. marcescens* in *C. elegans,* the intestine of nematodes was visualized using bright field microscope. Batches of 20 nematodes were aseptically exposed with *S. marcescens* in the presence and absence of CLP. After an experimental infection, the control and treated nematodes from their respective wells were washed with M9 buffer and transferred to the agar pad containing 1 mM sodium azide (to anesthetize the nematodes) to avert the colonized bacterial expulsion from the intestine of nematodes and examined under the bright field microscope.

2.10.4. Quantification of internalized S. marcescens by CFU assay

In order to quantitatively determine the intestinal colonization of *S. marcescens* in infected control and CLP-treated *C. elegans,* colony forming unit (CFU) assay was carried out. In brief, batches of nematodes were transferred aseptically to the sterile 24-well MtP containing

pathogen or pathogen treated with CLP at sub-MICs. After experimental infection, the *C. elegans* from control and treated setups were washed 3–4 times carefully with sterile M9 buffer to eliminate the bacterial cells that are adhering on the surface of the *C. elegans* and subsequently anesthetized the nematodes by washing it with 1 mM sodium azide (to avert the internalized bacterial expulsion from the nematodes' intestine). Then, 100 mg of silicon carbide (1.0 mm mesh) was added to the anesthetized nematodes and vigorously vortexed for one minute at maximum speed to disrupt the body walls of nematode and to release the colonized bacterial cells. Further, the mixture was subjected for centrifugation at 1000 × *g* for one minute and the resultant supernatant was serially diluted aseptically in LB broth and spread plated on LB agar followed by the incubation at 28 °C for 12–16 h to determine the CFU.

2.11. Extraction of total RNA and qPCR analysis

In order to explore the effect of CLP on the *S. marcescens*' gene expression, it was grown in LB medium supplemented with 1/2 × MIC of CLP. After 12 h of incubation, the control and CLP treated cultures were harvested by centrifugation and were re-suspended in TRIzol reagent (Sigma-Aldrich, Switzerland) and transferred to a sterile RNase-free 1.5 mL microfuge tube. Following the guanidine thiocyanate/Phenol extraction method described previously by Salini and Pandian (2015), the total RNA was extracted and dissolved in 20 µL of 0.1% diethylpyrocarbonate (DEPC)-treated water. Further, the extracted RNA was reverse transcribed into cDNA using Superscript III kit (Invitrogen Inc., USA) according to the manufacturer's instructions.

Quantitative real time PCR (qRT-PCR) analysis was done in 10 µL total volume and contained Power SYBR Green PCR Master Mix (Applied Biosystems) with the primers listed in Table 1 using 7500 Sequence Detection System (Applied Biosystems Inc. Foster, CA, USA). RNA from control and treated samples extracted three times independently. Further, cDNA was converted and the regulations of candidate genes were normalized against *rplU*. Fold difference in gene expression was measured using the formula $2^{-\Delta\Delta ct}$ as described by Gowrishankar et al. (2015).

3. Results and discussion

With the intention to overcome the drawbacks of conventional antimicrobial therapy, an alternative treatment strategy that effectively target pathogens' virulence including, biofilm formation is being propelled among the researchers of recent days. Unlike antibiotic treatment, the menace of developing resistance with agents of antivirulence therapy is substantially low, as they specifically attenuate virulence pathway that aggravates pathogenicity, and not the basic metabolic activity of the pathogen. Given the prominence of QS in the virulence and pathogenesis of *S. marcescens*, targeting its QS mechanism would plausibly be a convincing strategy to combat drug resistance and associated biofilm infections.

Primarily, the efficacy of CLP on planktonic cells of *S. marcescens* ATCC and the clinical isolate was investigated through MIC and MBC determination. Data divulged the proficient antibacterial efficacy of CLP at 200 and 450 µg/mL concentrations, respectively.

Table 1
Nucleotide sequences of *S. marcescens* primers used in this study.

Gene	Primer sequence (5' - 3')	
	Forward	Reverse
rplU	GCTTGGAAAAGCTGGACATC	TACGGTGGTGTTTACGACGA
fimA	ACTACACCCTGCGTTTCGAC	GCGTTAGAGTTTGCCTGACC
fimC	AAGATCGCACCGTACAAACC	TTTGCACCGCATAGTTCAAG
flhC	AAGAAGCCAAGGACATTCAG	TTCCCAGGTCATAAACCAGT
flhD	TGTCGGGATGGGGAATATkGG	CGATAGCTCTTGCAGTAAATGG
bsmB	CCGCCTGCAAGAAAGAACTT	AGAGATCGACGGTCAGTTCC
pigP	GAACATGTTGGCAATGAAAA	ATGTAACCCAGGAATTGCAC

3.1. Dose-dependent quorum quelling efficacy of CLP at non-bactericidal concentrations

Since the pivotal criterion for any antivirulence agent is its non-interference towards the basic metabolic activity of targeted pathogen, much emphasis was given on the bactericidal effect of sub-MIC (100 µg/mL) of CLP, as these concentrations were employed in all further virulence assays. From spectrophotometric analysis at OD600 nm, the sub-MIC of CLP was evaluated for antibacterial activity. The results suggested that CLP did not exhibit bactericidal or bacteriostatic effect, since the growth of *S. marcescens* cells treated with CLP at 100 µg/mL remains the same as that of the control samples.

Further, to ascertain the null-antibacterial effect of CLP, the physiological characterization of *S. marcescens* was carried out under the effect of CLP at below sub-MICs (12.5, 25, 50 and 100 µg/mL) through Alamar Blue assay. The assay is based on the reduction of resazurin (-a non-fluorescent blue-colour molecule) to a highly fluorescent red-colored resorufin by metabolically active cells. Data of Alamar Blue assay validated the non-interference of sub-MICs of CLP towards the metabolic activity of *S. marcescens*, as there was no significant difference in the production of resorufin in both CLP-treated and untreated samples spectroscopically (Fig. 1). Hence, these sub-MICs of CLP (12.5, 25, 50, 100 µg/mL) were tested in all subsequent virulence assays to omit the CLP-induced growth-retardation effect. It has been well proved that the biosynthesis of prodigiosin, -a tripyrrole red pigment, is one of the QS synchronized factors in *S. marcescens*(Morohoshi et al., 2007). Hence, the likelihood of QS inhibitory efficacy of CLP was primarily investigated through quantification of secreted prodigiosin under sub-MICs of CLP exposure. Data of the assay unveiled the proficient quorum quelling efficacy of CLP, as it significantly hindered the synthesis of prodigiosin in *S. marcescens* compared to the control sample (Fig. 1). At the least (12.5 µg/mL) and maximum (100 µg/mL) concentrations, CLP prominently inhibited 22% and 86% of prodigiosin production ($p \leq 0.05$) (Fig. 2), and thereby it displayed a remarkable dose-dependent anti-QS activity against *S. marcescens*.

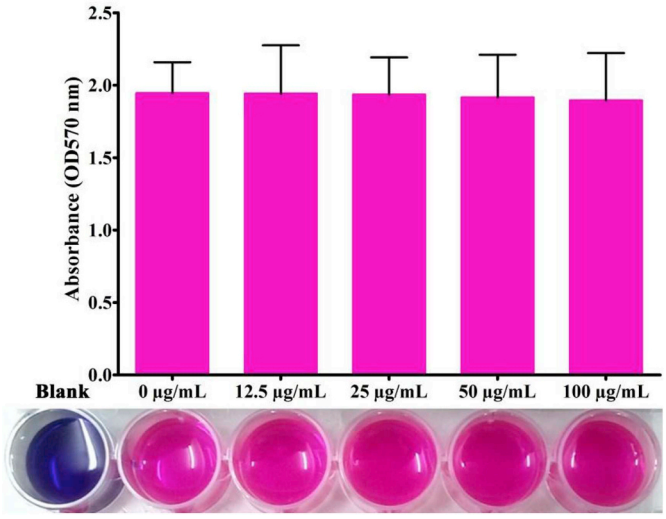

Fig. 1. The influence of CLP (12.5, 25, 50 and 100 µg/mL) on the basic metabolic activity of *S. marcescens* cells grown for 18 h was determined through Alamar Blue assay. The mean values of triplicate individual experiments and SDs are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

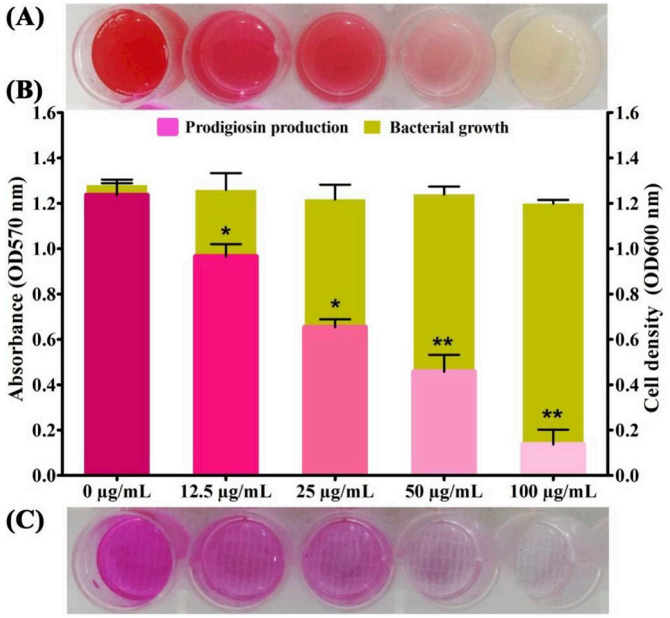

Fig. 2. Anti-QS efficacy of CLP at sub-MICs: Effect of CLP at null-bactericidal concentration on the QS regulated prodigiosin production of *S. marcescens*. (A) MTP wells portraying the uniform growth of *S. marcescens* irrespective of their inefficiency in synthesising prodigiosin; (B) Bar graph depicting the gradual dose dependent reduction in the production of prodigiosin, and the unchanged growth OD 600 in all the wells of CLP treatment; (C) MTPS wells signifying the dose dependent decrease in prodigiosin extracted from control and treated cells of *S. marcescens*.

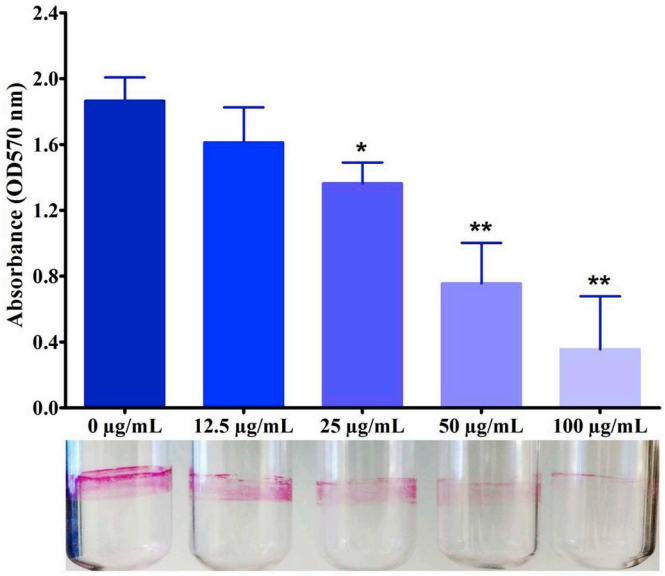

Fig. 3. Antibiofilm efficacy of CLP at sub-MICs: Effect of CLP at non-bactericidal concentration on the QS regulated biofilm assemblage in *S. marcescens*, wherein the lower panel portrays the biofilm rings of *S. marcescens* adhered to the walls of tube with and without of CLP at sub-MICs.

3.2. Antibiofilm efficacy of CLP at non-lethal concentration against S. marcescens

Although, a plethora of scientific reports have very well demonstrated the promising antibiofilm propensity of CLP alone or in combination with other molecules against an exclusive stretch of Gram-positive pathogens (Gowrishankar et al., 2014, 2015, 2016; Gowrishankar and Pandian, 2017; Scopel et al., 2013), none of the study have delineated its inhibitory efficacy against Gram-negative pathogen. Therefore, the efficacy of CLP towards the *S. marcescens* biofilm subdual was evaluated deliberately using ring biofilm assay in test tubes. *S. marcescens* cells were cultured for 24 h in 3 mL of LB containing test tubes with the absence (control) and presence of CLP at sub-MICs. After incubation, the formed biofilms were stained with crystal violet and spectrophotometrically quantified at OD570 nm, in which the level of dye absorbed is directly comparative to the adhered biofilm biomass. As anticipated, the presence of CLP at sub-MICs was potent enough to curtail the biofilm assemblage of *S. marcescens* in a concentration-dependent manner. At the least (12.5 µg/mL) and maximum (100 µg/mL) concentrations, CLP notably inhibited 13.6% and 81% of biofilms ($p \leq 0.05$) (Fig. 3) by lending no harm to the basic cellular metabolism of *S. marcescens*. Thus, the current study is the first of its kind to uncover the potent antibiofilm efficacy of the cyclic dipeptide -CLP against a Gram-negative pathogen, specifically *S. marcescens*.

3.3. CLP subvert early phases of biofilm development in S. marcescens

In the course of the early growth phase, the rapid attachment of *S. marcescens* on the surface of implanted medical devices turns to be the prime stage in biofilm cascade, and reversibility of this attachment process could be possible during initial few hours of growth (Moltz and Martin, 2005). Concomitantly, an adequate number of scientific reports have signified that the active molecules having the competence to mitigate bacterial adhesion at an early stage could considerably reduce the risk of further colonization (Sethupathy et al., 2017; Srinivasan et al., 2017). Therefore, to ensure certainty of the above notion, a microscopic investigation was accomplished to determine the impact of CLP on the different biofilm developmental stages of *S. marcescens*. The cells of *S. marcescens* were growth in 24-well MtPs containing glass pieces (1 × 1 mm), during which CLP (100 µg/mL) was added at two different time intervals (16 and 32 h).

The results of stage biofilm assay revealed that the early stage biofilm assemblage by *S. marcescens* was hugely intervened in CLP manifested wells. However, on the wells of CLP incorporation after 16 h have depicted no difference in the assemblage of biofilm with that of the control wells. It is therefore inferred that, CLP at sub-MIC has imposed a substantial hindrance on the initial attachment of *S. marcescens* cells. In this manner, CLP's action makes *S. marcescens* incompetent to develop recalcitrant biofilm architectures ($P \leq 0.01$) (Fig. 4 (upper panel). Further, the data was also affirmed with the light microscopic examinations of glasses adhered with biofilms (Fig. 4 (lower panel)). In the pathogenesis of uropathogens, the assemblage of biofilms plays a pivotal role in complicating the severity of implanted medical devices associated infections (Jones et al., 2000). On the biofilm mode of growth, pathogens can endure huge concentration of antibiotics and uncontrolled exploitation of such antimicrobial has culminated in the raise of multiple drug resistance in uropathogens.

Rapidly, after the establishment of biofilm, the cells residing inside become incredibly robust and make the antibiotic therapy unsuccessful. Therefore, the impact of CLP at its MICs to kill and disrupt 48 h old biofilms preformed over the catheter surface was assessed. For evaluating the influence of CLP, confocal microscopic analysis using Live/Dead bacterial staining was employed. Despite the phenomenal antibiofilm efficacy of CLP at its sub-MICs, the manifestation of CLP at 1 × MIC and 2 × MIC (200 and 400 µg/mL) was inefficient to disrupt the 48 h old preformed biofilm. However, the CLP treatment at 1 × MIC and 2 × MIC had imposed a convincing killing effect on the cells spread over the upper surface of 48 h performed by *S. marcescens* cells (Fig. 5).

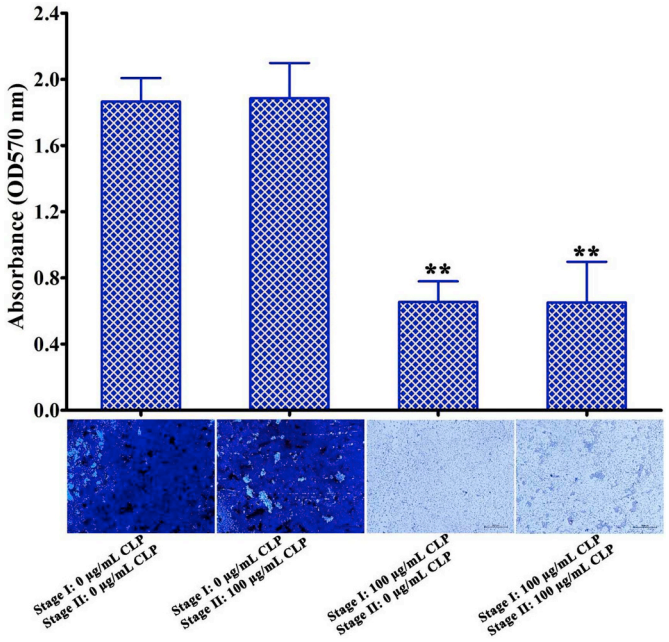

Fig. 4. (Upper panel) Influence of CLP at two different stages on biofilm development by *S. marcescens*. At the phase-I, the 3 h grown *S. marcescens* cells were used to inoculate MTP wells containing piece (1 × 1 cm) glass in sterile LB supplemented added with or without CLP (100 µg/mL) for 16 h of incubation (stage I). In the phase-II i.e. after 16 h, the spent media was aseptically discarded and replenished with fresh LB containing 100 µg/mL of CLP and incubated for 32 h (stage II). After required incubation of 48 h, the formed biofilms were quantified using crystal violet assay and (lower panel) the biofilms adhered over glass surface were subjected to microscopic examinations. '**' indicates the statistical significance ($P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

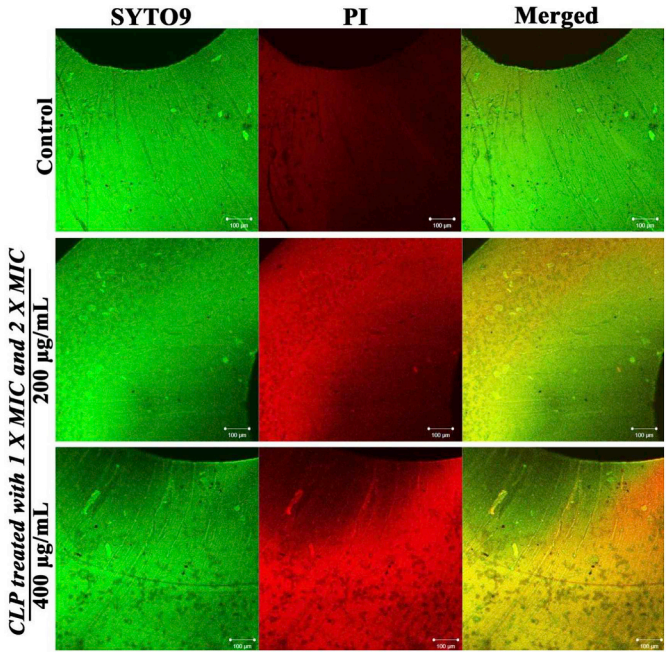

Fig. 5. Confocal micrographs of displaying the 48 h-preformed *S. marcescens* biofilms formed on catheter surface in the presence and absence of CLP at MIC (200 µg/mL) and 2x MIC (400 µg/mL). CLP was inefficient in disrupting or killing the mature biofilm cells of *S. marcescens*. Live/Dead BacLight viability staining (SYTO9/PI) was used to stain the preformed biofilm.

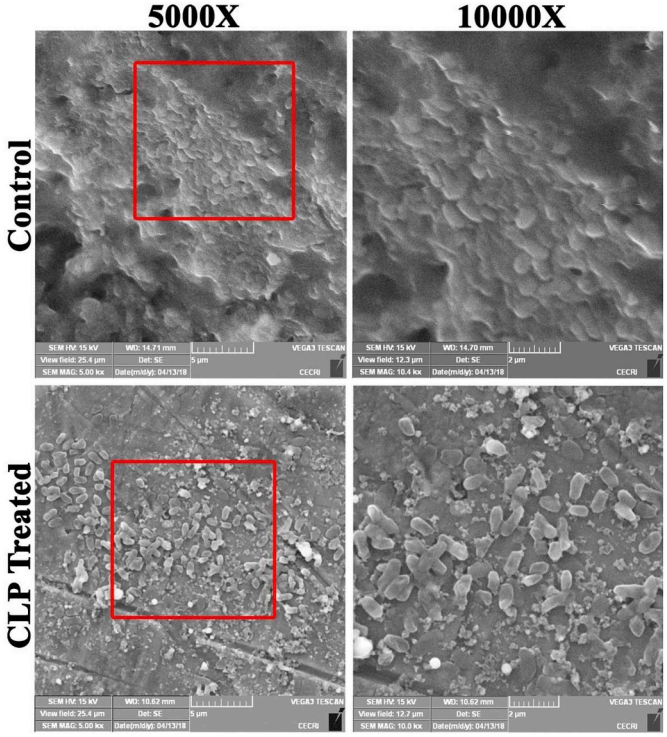

Fig. 6. The effect of CLP at 100 µg/mL concentration on the biofilm forming ability of *S. marcescens* through scanning electron microscopy.

3.4. *In situ* microscopic analysis of CLP induced biofilm deterioration on catheter surface

To intensively investigate the CLP-mediated modifications on the surface topology and 3D architecture of *S. marcescens* biofilms formed on catheter surface, an *in situ* microscopic examination was performed using SEM (Fig. 6) and CLSM (Fig. 7). Highly complex networking of multilayered cells with large biomass bound by exopolymeric matrix structure was well evident in the micrographs of *S. marcescens* biofilms grown on catheter without CLP treatment. On the other hand, the presence of CLP has made *S. marcescens* incompetent to produce its characteristic biofilm architecture, rather it could only form dispersed and disintegrated microcolonies. The CLP treated micrograph portrayed an apparent collapse rendered on the recalcitrant biofilm architecture of *S. marcescens*.

The observations of confocal micrographs have inevitably validated the results of anti-QS and biofilm biomass assays, as CLP's action was phenomenally dose dependent. In order to compare and infer the confocal microscopic observations (Fig. 7): (i) at 1/4 MIC (50 µg/mL), the complex multilayered biofilms were absolutely wrecked together with dramatic reduction in biomass; and (ii) at 1/2 MIC (100 µg/mL) treatment of CLP, the micrographs clearly portrayed the loosened microcolonies with scattered cells. Therefore, it is pertinent to state that CLP not only thwarts the synthesis of QS controlled prodigiosin production, but also impedes the QS regulated biofilm assembling ability of *S. marcescens*. Further, it is envisaged that the overwhelming efficiency of CLP in the breaking up architectural integrity and structural complexity of biofilm, could plausibly present the persister cells residing into the recalcitrant biofilms for the action of either antibiotic or host immune response.

3.5. CLP thwarts QS regulated virulence traits of S. marcescens

Extracellular polymeric substance (EPS) plays an inevitable role in the maintenance of three dimensional biofilm architecture, which eventually lend resistance to the cells against antibiotics (Bomchil et al.,

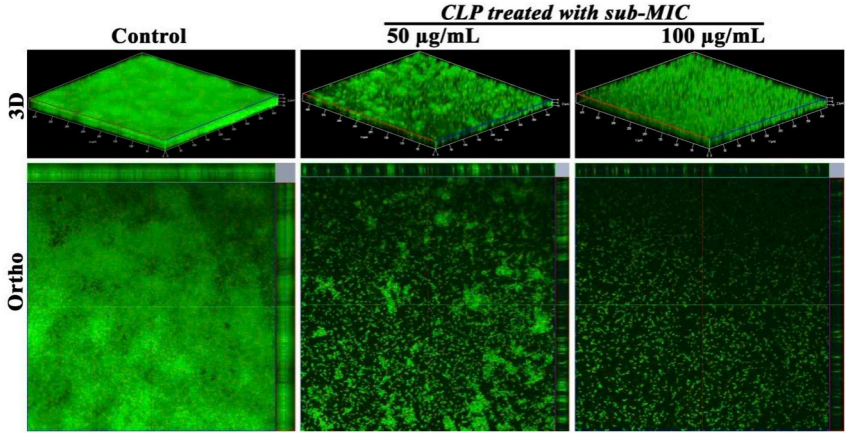

Fig. 7. The effect of CLP at 100 µg/mL concentration on the biofilm forming ability of *S. marcescens* through confocal laser scanning micrscopy.

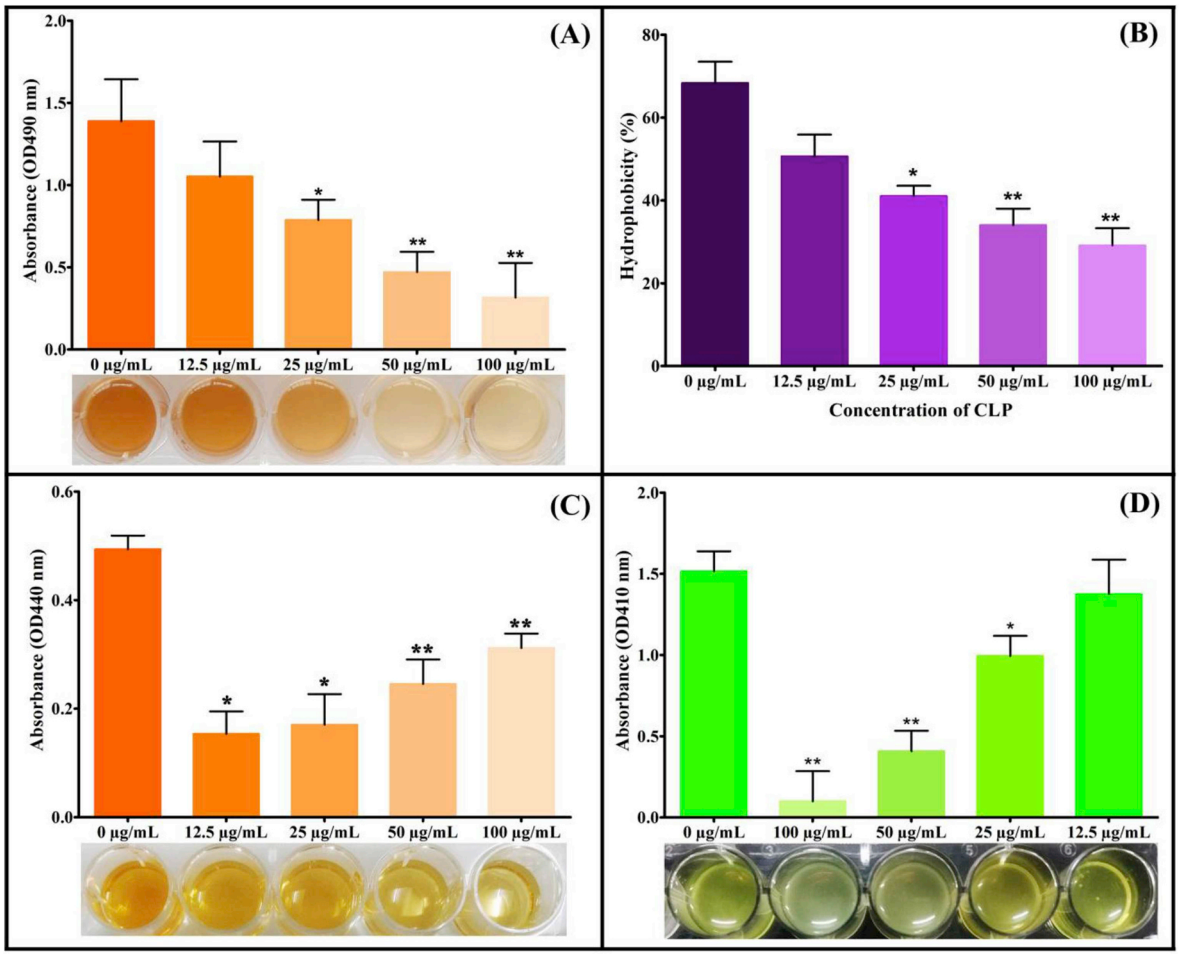

Fig. 8. Effect of CLP at non-bactericidal concentrations on the *S. marcescens* surface hydrophobicity (A); and the other QS controlled virulence traits such as EPS (B); protease (C); and lipase productions (D).

2003). In light of this, there is a mounting body of studies signifying that EPS subdual could slacken the biofilm architecture and conceivably mitigate the development of resistance towards antimicrobial (Santhakumari et al., 2016). It is envisaged that CLP has remarkably mitigated the synthesis of EPS by *S. marcescens* (Fig. 8A). Data unveiled that CLP at 100 µg/mL depicted 77% EPS subdual in *S. marcescens*. A study by Padmavathi et al. (2014) stands on par with our results, wherein they reported that culture supernatant of a marine bacterium (G16-T) has reduced the EPS production in *S. marcescens*.

The charge of the cell surface is denoted by hydrophobicity index, which is one of the crucial factors that aid cells in the process of accumulation. Therefore, reducing the hydrophobic index of any cell could possibly diminish their biofilm assemblage. In due response to this notion, the effect of CLP on the hydrophobic characteristics of *S. marcescens* has been evaluated. Data inferred that CLP was potent enough to modify the hydrophobic nature of *S. marcescens* up to one fold at 100 µg/mL, and the other concentrations remarkably portrayed a gradual dose dependent decrease in the hydrophobic index of *S.*

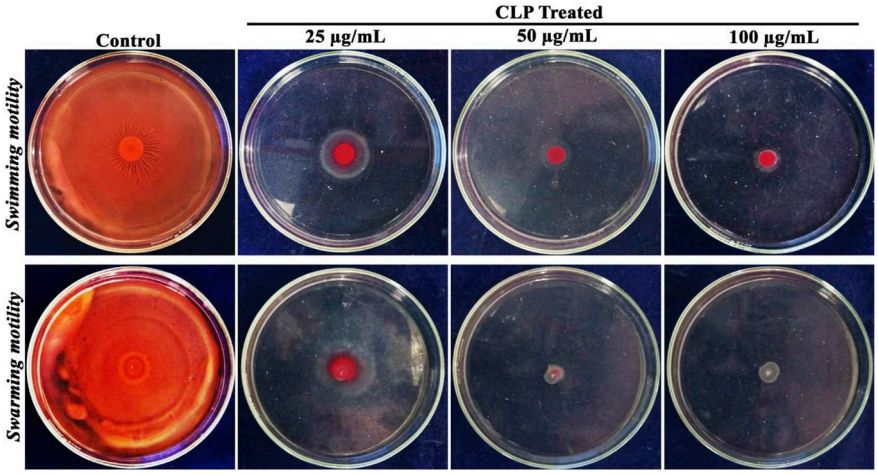

Fig. 9. The influence of CLP at sub-MICs (25, 50, 100 µg/mL) on both the swimming and swarming motility of *S. marcescens*.

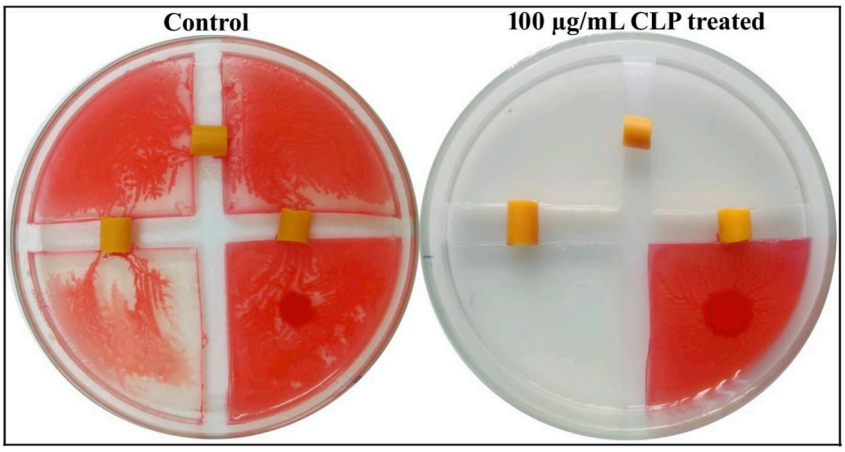

Fig. 10. Effect of CLP at different sub-MICs (100 µg/mL) on swarming motility of *S. marcescens* to pass across through catheter. The swarming plates were incubated upright position at 30 °C for 48 h.

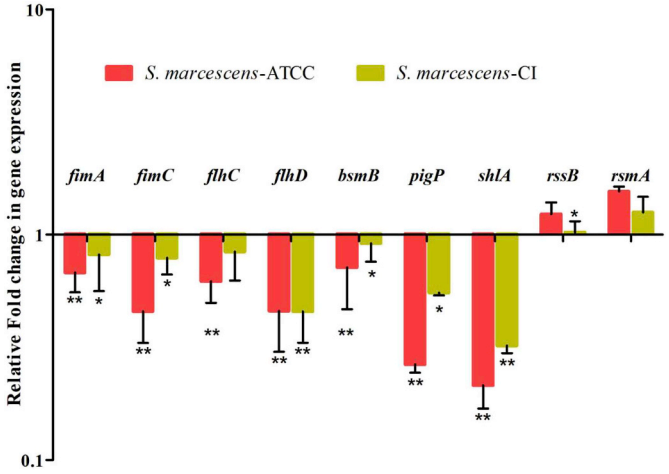

Fig. 11. Relative fold difference observed in the expression of specific genes (involved in biofilm assemblage, initial attachment and virulence genes involved in QS mechanisms) of *S. marcescens* under the presence and absence of CLP through real time-PCR assay. The experiment was carried out in triplicate. Data presented were generated from at least three independent sets of experiments.*and**indicate the statistical significance $p < 0.05$ and $p < 0.01$ respectively.

marcescens (Fig. 8B). Bioactive molecules with efficacy to inhibit the proteolytic activity of any pathogen could be employed to facilitate hosts' innate immune response as well as antimicrobial therapy (Sethupathy et al., 2017). In this milieu, a significant dose-dependent decrease in the secretion of protease by the cells of *S. marcescens* that were grown under CLP (sub-MICs) was observed quantitatively (Fig. 8C). To establish infection inside the host, most of the microbial pathogens exploit lipolytic enzymes for degrading the phospholipid bilayer, and thereby it influences the cell signalling pathways of host. CLP has significantly suppressed the lipase production in *S. marcescens* to a level of 92 and 78% upon 100 and 50 µg/mL treatments, respectively (Fig. 8D).

In the developmental stages of biofilm, the initial attachment to any substratum plays a pivotal role. For the effective initial adherence over biotic and abiotic surfaces, the cells' propensity to swarming and swimming motility using their flagella holds prime importance. In particular, *S. marcescens* is well renowned for its multicellular structural movement through its swarming nature (Shanks et al., 2013; Soo et al., 2014). Therefore, any intervention with the swarming character of this pathogen would pose an eventual negative impact on *S. marcescens* biofilm assemblage. Being a phenomenal antibiofilm agent, CLP was tested for its inhibitory efficacy towards the swarming and swimming nature of *S. marcescens*. The soft agar plates (that facilitate swimming and swarming motility) incorporated with CLP at varying sub-MICs displayed exceptional suppression in both the swimming and swarming motility (Fig. 9) when compared to untreated control plates. In line

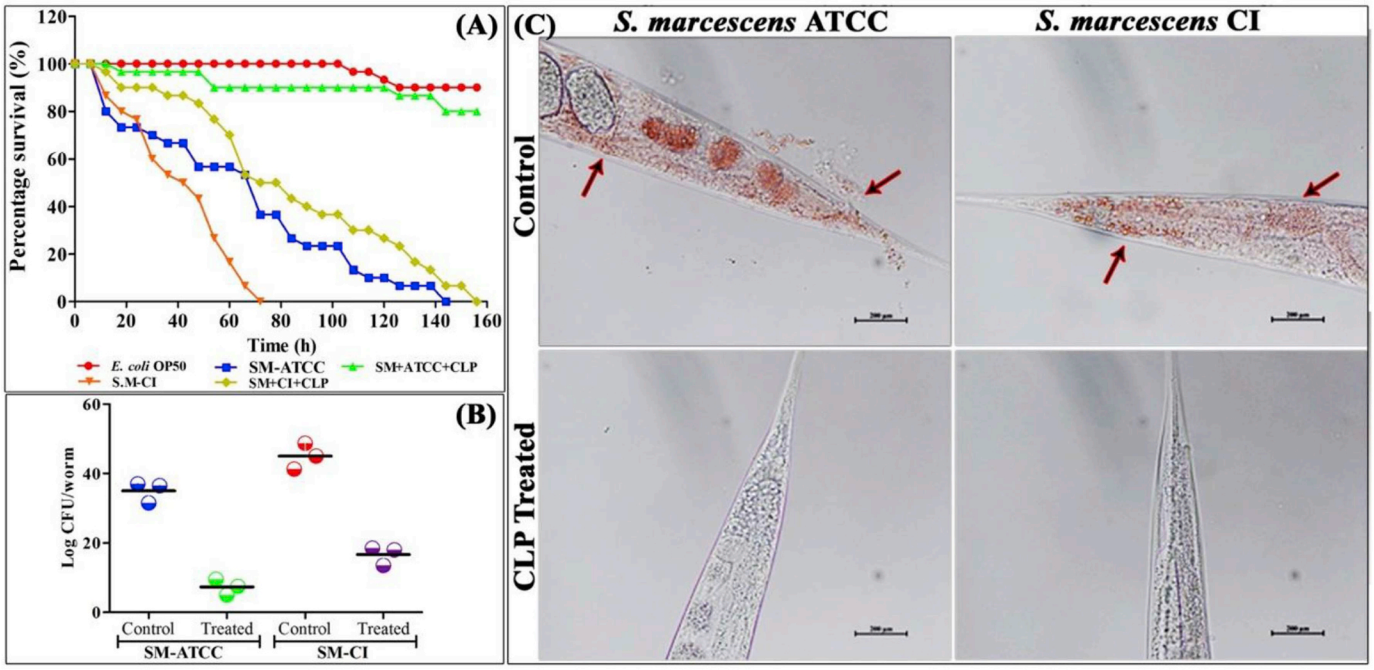

Fig. 12. An *in vivo* anti-adherence and rescuing efficacy of CLP against *S. marcescens* infection in *C. elegans*. (A) Survival graph displaying CLP –induced longevity of *S. marcescens*-ATCC and *S. marcescens*-CI infected nematodes. (B) CFU assay demonstrating the CLP-mediated reduction in the colonization of *S. marcescens* (both reference and clinical strains) in infected worms. (C) An *in situ* qualitative light microscopic observations portraying the dramatic diminish in the colonization of *S. marcescens*-ATCC and *S. marcescens*-CI in nematodes manifested with CLP than that of their respective controls. Arrows in the control panel denotes the typical colonization of *S. marcescens*, whereas arrows in CLP treated panel indicates the decreased bacterial load inside the anal region of nematodes.

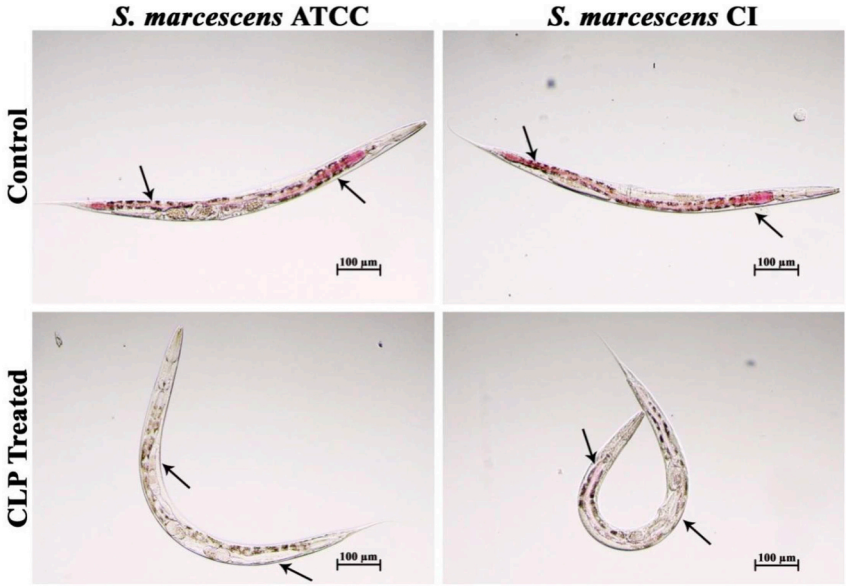

Fig. 13. Light micrographs displaying CLP-mediated reduction in colonization of *S. marcescens*-ATCC and *S. marcescens*-CI infected nematodes than their respective controls. Arrows in the control and treated panels signifies *S. marcescens*' huge colonization and de-colonization, respectively.

with the data of the present study, the findings of Salini and Pandian (2015), Srinivasan et al. (2016), Srinivasan et al. (2017) and Sethupathy et al. (2017) have very well demonstrated the ability of natural compounds and/or extracts to slow down the swarming and swimming motility in *S. marcescens*. Fig. 10 portrays the inefficiency of strong motile *S. marcescens* to swarm across the catheter in the presence of CLP at sub-MIC, which signifies the overwhelming efficacy of CLP in mitigating one of the important QS controlled virulence factors i.e. swarming motility in *S. marcescens*.

3.6. CLP down regulates QS regulated virulence genes' expression

As CLP has phenomenally mitigated most of the QS regulated virulence traits of *S. marcescens* phenotypically, the genes that are involved in early attachment, motility and virulence factor production were intentionally used in this current study to investigate the quorum quelling mechanism of CLP at the molecular level. Primarily, the adherence of *S. marcescens* cells over biotic or abiotic surfaces is being facilitated by fimbrial proteins encoding genes viz. *fimA* and *fimC* that are expressed under QS mechanism (Labbate et al., 2007). The gene

expression analysis done through real time PCR divulged that treatment with CLP has substantially reduced the expression of these *fimA* and *fimC* (Fig. 11). In line with this, earlier studies by Salini and Pandian (2015) and Srinivasan et al. (2016) have also reported a similar phenomenon, wherein, the extracts of *Anethum graveolens* and *Piper betle* with QSI propensity significantly down regulated the expression of *fimA* and *fimC*. Besides, in *S. marcescens*, f*lhDC* is a master operon producing two global gene regulators FlhD and FlhC that are responsible for the expression of few intrinsic determinants viz. cell division as well as differentiation and motility (Liu et al., 2000). CLP treatment has significantly down regulated the expression of f*lhD* and f*lhC* genes, which indeed, in total agreement with the outcome of motility and swarming across catheter assays, as CLP demonstrates a profound *in vitro* inhibitory efficacy against both the swimming and swarming motility of *S. marcescens*.

In par with the noted antibiofilm and antivirulence efficacies of CLP, its treatment significantly reduced the expression of *bsmB*, an essential virulence gene controlled by QS that attributes hugely to biofilm assemblage, secretion of lipase as well as protease, and S-layer protein productions in *S. marcescens* (Labbate et al., 2007). Prodigiosin, one of the unique virulence traits of *S. marcescens* that is under QS, has been a product of master transcriptional regulator-*pigP* (Gristwood et al., 2011). The level of expression of prodigiosin in the presence of CLP was profusely controlled, which could be attributed to the CLP-mediated subdual of prodigiosin secretion *in vitro*.

3.7. In vivo anti-adherence and anti-infective efficacies of CLP

Owing to the fact that the capability of pathogens to alter the host response plays a crucial role in establishing successful infections in the host (Youn et al., 1992), we further aimed to investigate whether CLP could attenuate the *in vivo* bacterial adherence using *C. elegans* as a model host. Globally, the free-living, economic invertebrate nematode *C. elegans* has been well recognized as a potential animal model to explore for high-throughput *in vivo* screening of natural and synthetic and anti-infectives (Moy et al., 2009). In particular, it was reported that *C. elegans* shares a natural habitat with *S. marcescens* (Schulenburg and Ewbank, 2004) and proved to be an excellent model organism to evaluate the virulence of *S. marcescens* as well as for exploring antibacterial potentials of wide a variety of small and chemical compounds (Breger et al., 2007). The anti-infective efficacy of CLP in protecting the nematodes against *S. marcescens* was quantitatively assessed by measuring the enhanced survival of CLP treated nematodes in liquid infection assay. Notably, CLP has significantly ($P \leq 0.05$) enhanced the survival of the nematodes to > 85% even after 6 days of infection when supplementing at a concentration of 100 μg/mL along with *S. marcescens*. On the other hand, nematodes infected with *S. marcescens* alone showed a significant ($P > 0.05$) reduction in worms survival (140 ± 5 h), and no apparent killing was observed in the worms treated with *E. coli* OP50 or in CLP alone control groups (Fig. 12).

To correlate the protective effect of CLP with its ability to attenuate the *S. marcescens* colonization in nematode, the light microscopic imaging was performed. The micrographs of nematodes manifested with CLP validated the anti-adherence efficacy by exhibiting a dramatically reduced bacterial colonization (Figs. 12 C and 13) than that of the CLP untreated nematodes (Fig. 12). Further, to ascertain the microscopic analysis, the CFU assay was done. *S. marcescens* displayed an increased bacterial load inside the infected nematode, whereas CLP significantly ($P \leq 0.05$) reduced the colonization in treated nematodes. The log CFU of CLP supplemented nematodes infected with *S. marcescens* was found to be 3.15 ± 0.92 and 2.20 ± 0.55, respectively, but it was increased up to 5.54 ± 0.61 in the infected groups. Furthermore, the toxicity was determined at 100 μg/mL concentration of CLP, and the results showed that CLP does not exert any toxic character as there was no significant killing and/or any signs of moribund in the nematodes recorded during experiment (Gowrishankar et al., 2016).

4. Conclusions

Despite the phenomenal antibiofilm and antivirulence efficacies of CLP against a stretch of Gram-positive pathogens, the present study provides more light on its anti-QS properties against a Gram-negative pathogen- *S. marcescens*. Data of this study portrays that CLP was exceptional in thwarting all the QS-regulated phenotypic characteristics of *S. marcescens*, which was further affirmed through qPCR analysis at the molecular level. Concomitantly, the results of *in vivo* assays convincingly pronounce CLP to be a potential antibiofilm and antivirulence agent, as it not only impedes *in vitro* initial adherence and biofilm assemblage, but also the *in vivo* replication as well as colonization of the notorious UTI causing pathogen- *S. marcescens*.

Conflicts of interest

The authors declare no conflict of interest.

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