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Inhibition of biofilm development of uropathogens by curcumin – An anti-quorum sensing agent from *Curcuma longa*

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ABSTRACT

Urinary tract infection is caused primarily by the quorum sensing (QS)-dependent biofilm forming ability of uropathogens. In the present investigation, an anti-quorum sensing (anti-QS) agent curcumin from *Curcuma longa* (turmeric) was shown to inhibit the biofilm formation of uropathogens, such as *Escherichia coli, Pseudomonas aeruginosa* PAO1, *Proteus mirabilis* and *Serratia marcescens,* possibly by interfering with their QS systems. The antibiofilm potential of curcumin on uropathogens as well as its efficacy in disturbing the mature biofilms was examined under light microscope and confocal laser scanning microscope. The treatment with curcumin was also found to attenuate the QS-dependent factors, such as exopolysaccharide production, alginate production, swimming and swarming motility of uropathogens. Furthermore, it was documented that curcumin enhanced the susceptibility of a marker strain and uropathogens to conventional antibiotics.

1. Introduction

Urinary tract infections (UTIs) are one of the most common infectious diseases in humans. It is well known that the occurrence of these infections is much more frequent in females than in males (Hunjak, Pristas, & Stevanović, 2007). Several pathogens including *Escherichia coli, Proteus mirabilis*, *Pseudomonas aeruginosa* and *Serratia marcescens* are often implicated in UTIs (Ronald, 2002). It is well documented that all these uropathogens possess a quorum sensing (QS) controlled ability to form biofilms, which leads to severe infections (Jones, 2004). QS is termed as the ability of bacteria to perceive and respond rapidly to changes in cell density, with the help of small secreted signalling molecules known as auto-inducers (AI). Several QS-based factors, such as biosurfactant production, exopolysaccharide (EPS) production, swimming and swarming motility have been attributed to the biofilm formation and are associated with UTIs. Swarming, a flagellar driven motility, plays a crucial role in the establishment of urinary infections, which begins with the colonisation of the lower urinary tract, followed by ascending migration of these uropathogens (Jones, 2004).

Biosurfactants possess tensioactive properties which are capable of enhancing swarming motility and thereby influencing biofilm development in bacterial pathogens (Caizza, Shanks, & O'Toole, 2005). EPS is essential for the development of biofilm architecture and acts as a protective barrier by restricting the penetration of chemotherapeutic molecules. Those bacterial cells that grow inside the biofilm are resistant to phagocytes, antibodies and antibiotics, which in turn leads to the failure of host defence system and antimicrobial chemotherapy to clear biofilm inhabitant bacterial pathogens (Fux, Costerton, Stewart, & Stoodley, 2005). Therefore, there is a pressing need to find out an alternative strategy to overcome the resistance of the uropathogens. Since, the successful establishment of UTIs by several pathogens is mediated through the QS-dependent biofilm mode of growth, targeting this system by means of anti-QS compounds would be a possible means for healthier treatment measures. An anti-QS compound acts by causing an interruption of the QS system of uropathogens and therefore reducing the chance for the development of resistance towards antibiotics (Nashikkar et al., 2011).

Plant-derived compounds have long been used to treat microbial infections (Gibot, 2004) and have gained much interest as a source of anti-QS compounds for the treatment of biofilms (Musthafa, Ravi, Annapoorani, Packiavathy, & Pandian, 2010). Recently, dietary phytochemicals with a long history of medical use in humans are being investigated for the purpose of controlling biofilms, due to their non-toxic nature (Brackman et al., 2008; Rudrappa & Bais, 2008). One such dietary phytochemical, curcumin, a major constituent of turmeric (*Curcuma longa* L.) rhizomes, was selected in this study. Turmeric has traditionally been used as anti-inflammatory, antimicrobial and anti-fungal, but reports on its anti-QS properties are scarce (Rudrappa & Bais, 2008). The present study evaluated the antibiofilm and anti-QS activities of curcumin in the prevention of UTI-causing pathogens *E. coli*, PAO1, *P. mirabilis* and *S. marcescens.*

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2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study included the biomarker strains *Chromobacterium violaceum* (ATCC 12472), *Chromobacterium violaceum* CV026 and uropathogens, such as *E. coli* (ATCC 10536), *P. aeruginosa* PAO1, *P. mirabilis* (ATCC 7002) and clinical isolate *S. marcescens* (FJ584421). All bacterial strains were cultivated in Luria–Bertani (LB) medium (pH 7.0) and maintained at 37 °C, except *C. violaceum* and *S. marcescens*, for which the temperature was 30 °C. For experimental analysis, all target bacterial pathogens were subcultured until OD of 0.4 at 600 nm was reached.

2.2. Determination of minimal inhibitory concentration (MIC) of curcumin

Curcumin was purchased from Sigma (St. Louis, MO). Stock solutions were prepared by dissolving 10 mg of curcumin in 1 ml of 95% methanol. From the stock solutions, appropriate volume was added in sterile Milli-Q water to make a suspension and used for further assays. The MIC of curcumin against each test pathogen was determined as per the guidelines of Clinical and Laboratory Standards Institute, USA (2006). Briefly, 1% of test pathogens (0.4 OD at 600 nm) were added to appropriate growth medium supplemented with twofold serially diluted curcumin to attain final concentrations ranging from 0.125 to 600 μg/ml in a microtitre plate (MTP), and incubated for 24 h. The MIC was recorded as the lowest concentration which showed complete inhibition of visible growth of the bacterial pathogens. All further experiments in the present study were performed only at sub-MIC concentrations of curcumin.

2.3. Violacein inhibition assay

Curcumin was subjected to qualitative analysis to find out its anti-QS potential against *C. violaceum* (ATCC 12472). *C. violaceum* synthesises the violet-coloured pigment violacein by responding to its QS signal molecule *N*-hexanoyl-L-homoserine lactone (HHL) produced by its autoinducer synthase CviI. This HHL binds to its receptor CviR and this complex triggers the expression of violacein production (Choo, Rukayadi, & Hwang, 2006). Overnight culture (10 μl) of *C. violaceum* (adjusted to 0.4 OD at 600 nm) was added into wells of sterile MTP containing 1 ml of LB broth and incubated in the presence and absence of varying concentrations of curcumin (25–100 μg/ml). The MTPs were incubated at 30 °C for 16 h and observed for the reduction in violacein pigment production. In addition, quantitative analysis was performed with *C. violaceum* CV026. It is a violacein-negative, double mini-Tn5 mutant of *C. violaceum* (ATCC 31532), deficient in the autoinducer synthase CviI and, therefore, requires exogenous addition of HHL to induce violacein production (Choo et al., 2006). In quantitative analysis, CV026 was supplemented with 5 μM of HHL (Sigma) and cultivated in the presence and absence of curcumin (25–100 μg/ml), following the method of Choo et al. (2006).

2.4. Microscopic analyses of bacterial biofilm

The light microscopy analysis of bacterial biofilm was performed following the method of Musthafa et al. (2010). Briefly, 1% of overnight cultures of the uropathogens were added into 1 ml of fresh growth medium containing cover glass of 1 × 1 cm in 24-well MTP along with curcumin (25–100 μg/ml) and incubated for 16 h. After incubation, the cover glasses were rinsed with distilled water to remove the planktonic cells. The biofilms adhered on the cover glasses were stained with 0.4% crystal violet (CV, Hi-Media, Mumbai, India) solution and then visualised under a light microscope at a magnification of 40× (Nikon Eclipse Ti 100, Tokyo, Japan).

The confocal laser scanning microscopic (CLSM) analysis of bacterial biofilm was performed as described by Nithya, Aravindraja, and Pandian (2010). In CLSM analysis, the uropathogens were allowed to form biofilm on the cover glasses in the absence and presence of test compound, as described in light microscopy analysis. After 16 h, biofilms formed on the cover glasses were stained with 20 μl of 0.1% acridine orange (Sigma) for 1 min. The excess stain was washed off and the stained cover glasses were visualised under CLSM (Model LSM710, Carl Zeiss, Jena, Germany) equipped with an excitation filter 515–560 and magnification at 20×.

2.5. Effect of curcumin on biofilm development

The effect of curcumin on uropathogenic bacterial biofilm development was determined by quantifying the biofilm biomass through MTP assay (Musthafa et al., 2010). Briefly, 1% of target bacterial cultures (OD adjusted to 0.4 at 600 nm) were added into 1 ml of respective growth medium and cultivated in the presence and absence of varying concentrations of curcumin (25–100 μg/ml) without agitation for 16 h. After incubation, the planktonic cells in the wells of MTPs were removed and the wells rinsed twice with sterile water. The surface-adhered cells in the MTP wells were stained with 200 μl of 0.4% CV solution. After 2 min, excess solution was removed and CV in the stained cells was solubilised with 1 ml of 95% ethanol. The biofilm biomass was quantified by measuring the intensity of CV in ethanol at OD_{650} using a UV–visible spectrophotometer (Hitachi U-2800, Tokyo, Japan).

Consequently, the effect of curcumin on EPS production, which occurs in biofilm maturation, was examined. Briefly, the test uropathogens were grown in the absence and presence of curcumin (25–100 μg/ml) at appropriate temperature for 18 h. At the end of incubation, the cultures were centrifuged at 10,000 rpm for 15 min. The cell pellets were resuspended in 50 ml of high-salt buffer (10 mM K_3PO_4 (pH 7), 5 mM NaCl, 2.5 mM $MgSO_4$) and cells were removed by centrifugation at 10,500 rpm for 30 min. The dislodged EPS was precipitated from the supernatant with three volumes of ethanol. The resulting precipitate was resuspended in an appropriate volume of Milli-Q water and stored at −20 °C. The extracted EPS was quantified as follows: 1 ml of the EPS solution was mixed with 1 ml of cold 5% phenol and 5 ml of concentrated sulphuric acid (H_2SO_4), to develop red colour. The intensity of the colour was measured spectrophotometrically at 490 nm.

Finally, to ensure the ability of curcumin in disrupting the mature biofilm of test uropathogens, the biofilm disruption assay was performed by following the method described by Abraham, Palani, Ramaswamy, Shunmugiah, and Arumugam (2011). Briefly, biofilms of uropathogens were allowed to develop on cover glasses for 12 h as mentioned in microscopic analyses of biofilm. The mature biofilms were then treated with different concentrations (25–100 μg/ml) of curcumin for 5 h and observed under CLSM.

2.6. Swimming and swarming assays

The swimming and swarming motility assays were performed by following the method of Packiavathy, Agilandeswari, Musthafa, Pandian, and Ravi (2012). In swimming assay, 3 μl overnight cultures of the uropathogens (0.4 OD at 600 nm) were point inoculated at the centre of the swimming agar medium consisting of 1% tryptone, 0.5% NaCl and 0.3% agar with increasing concentrations of curcumin (50, 75 and 100 μg/ml). For swarming assays, 5 μl (0.4 OD at 600 nm) overnight cultures of uropathogens were

inoculated at the centre of the swarming agar medium consisting of 1% peptone, 0.5% NaCl, 0.5% agar and 0.5% of filter-sterilised D-glucose with increasing concentrations of curcumin (50, 75 and 100 μg/ml). The plates were then incubated at 30 °C in upright position for 16 h. The reduction in swimming and swarming migration was recorded by measuring the swim and swarm zones of the bacterial cells after 16 h.

2.7. QS inhibition assays in PAO1 and S. marcescens

2.7.1. Alginate inhibition in PAO1

Alginate was extracted from cultures of PAO1 treated and untreated with curcumin (25–100 μg/ml), and subsequently estimated as described by Owlia, Rasoolib, Saderia, and Aliahmadi (2007). Briefly, 70 μl of the sample were slowly added to 600 μl of boric acid/H_2SO_4 (4:1) solution in a test tube placed in an ice bath. The mixture was vortexed for 10 s and placed back in the ice bath. Twenty microlitres of 0.2% carbazole solution in ethanol were added to the mixture, which was then vortexed for 10 s. The mixture was placed in a water bath at 55 °C for 30 min. After incubation, the absorbance was measured spectrophotometrically at 530 nm. The percentage inhibition of alginate production was calculated by the following formula:

$$(\text{Control OD}_{530} - \text{Test OD}_{530}/\text{Control OD}_{530}) \times 100$$

2.7.2. Rhamnolipid inhibition in PAO1 by plate detection assay

Quantitative analysis of rhamnolipid production was assessed by following the method of Caizza, Shanks, and O'Toole (2005). Briefly, 50 μl of overnight culture from both curcumin treated (25, 50, 75 and 100 μg) and/or untreated PAO1 cells were inoculated into M8 medium supplemented with $MgSO_4$ (1 mM), glucose (0.2%), tryptone (0.5%), cetyltrimethylammonium bromide (CTAB) (0.02%) methylene blue (0.0005%) and agar (1.5%). Plates were incubated at 37 °C for 48 h and diameter of dark blue zone around the wells was measured to quantify the rhamnolipid production.

2.7.3. Rhamnolipid detection by TLC in PAO1

Rhamnolipids were extracted as described previously by Caizza, Shanks, and O'Toole (2005). Briefly, cultures of PAO1 in the absence and presence (25–100 μg/ml) of curcumin were grown at appropriate temperature for 24 h and cells were removed by centrifugation (10 min at 10,000 rpm). The cell-free culture supernatants were acidified to pH 2 with concentrated hydrochloric acid (HCl). Equal volumes (750 μl) of acidified supernatant and mixture of chloroform:methanol (2:1) were added and vortexed for 1 min. The lower organic phase was collected after centrifugation (10 min at 10,000 rpm). The extraction was repeated, the pooled organic phases were evaporated to dryness, resuspended in 1 ml methanol, filtered through a 0.45-μm membrane, evaporated to dryness and resuspended in 20 μl of methanol. The samples were analysed by TLC (TLC silica gel 60 F254; Merck, Darmstadt, Germany) with a mobile phase consisting of 80% chloroform, 18% methanol, and 2% acetic acid. The plates were dried and the rhamnolipid spots were visualised by iodine vapour.

2.7.4. Effect of curcumin on prodigiosin production in S. marcescens

Prodigiosin assay in *S. marcescens* was performed following the method of Morohoshi et al. (2007). Briefly, 1% of *S. marcescens* cells (0.4 OD at 600 nm) were inoculated into 2 ml of fresh LB medium and cultivated with and without curcumin at final concentrations of 25–100 μg/ml. The experimental set up was incubated for 18 h at 30 °C. During late stationary phase the cultures from each tube was collected in a 2-ml sterile tube and centrifuged at 10,000 rpm for 10 min to pelletise bacterial cells along with prodigiosin. Prodigiosin from the cell pellet was extracted with acidified ethanol solution (4% 1 M HCl in ethanol) and absorbance of the extracted prodigiosin in ethanol was measured at 534 nm using a UV–visible spectrophotometer.

2.8. Synergistic effect of curcumin with antibiotics

In order to examine the synergistic activity of curcumin with antibiotics the synergistic assay was performed as described by Rogers, Huigens, Cavanagh, and Melander (2010). Briefly, 1% cultures of *C. violaceum*, *E. coli,* PAO1, *P. mirabilis* and *S. marcescens* (0.4 OD at 600 nm) were added to 1 ml of LB broth in 24-well MTP containing antibiotic discs bacitracin (10 μg/ml), clindamycin (10 μg/ml), azithromycin (10 μg/ml), erythromycin (10 μg/ml) and erythromycin (10 μg/ml), respectively, along with curcumin at concentrations ranging from 25 to 100 μg/ml. The controls were maintained with respective antibiotic discs and without curcumin. The plates were incubated overnight at 30 °C and growth was measured after 24 h of incubation at 600 nm using a UV–visible spectrophotometer.

2.9. Growth curve analysis

Overnight cultures from test pathogens (1%; 0.4 OD at 600 nm) were inoculated in a 250-ml Erlenmeyer flask containing 50 ml of LB broth supplemented with various concentrations (25–100 μg/ml) of curcumin. The flasks were incubated at the optimum temperature of respective pathogens under 180 rpm in a rotatory shaker. The cell density was measured by UV–visible spectrophotometry at 1-h intervals up to 18 h.

2.10. Statistical analysis

All experiments were performed in triplicates and the data obtained from the experiments were presented as mean values; the differences between control and test were analysed using Student's *t*-test.

3. Results

3.1. Determination of MIC of curcumin

MIC of curcumin was assessed for all test pathogens using doubling dilution method with the concentrations varying from 0.125 to 600 μg/ml. The MIC of curcumin suspension was 192 μg/ml for *E. coli*, PAO1 and *P. mirabilis,* and 384 μg/ml for *C. violaceum* and *S. marcescens*.

3.2. Violacein inhibition assay in C. violaceum

In qualitative analysis, curcumin exhibited a concentration-dependent inhibition in HHL-mediated violacein production in *C. violaceum* (ATCC 12472). Further, in quantitative inhibition assay, violacein inhibition was observed to a maximum of 89% in CV026 when treated with curcumin at 100 μg/ml (Fig. 1).

3.3. Microscopic analyses of biofilm formation

Direct microscopic observations of biofilms after exposure to curcumin is known to provide valuable information of the action of curcumin on biofilms, therefore light microscopy and CLSM analyses were performed. A thick coating of biofilms was observed in controls, whereas a visible reduction in numbers of microcolonies was observed in the biofilms of curcumin-treated uropathogens (Fig 2A). In addition, curcumin deteriorated the architecture of the biofilm too, as was more evident from CLSM analysis (Fig 2B).

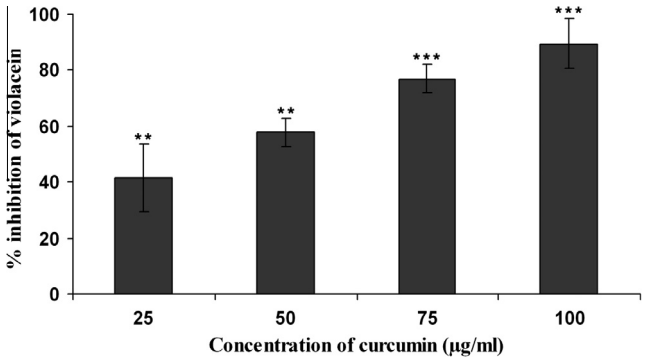

Fig. 1. Quantitative analysis of violacein inhibition in CV026 by curcumin. Data are represented as percentage of violacein inhibition. Mean values of triplicate independent experiments and SD are shown. **significant at $p < 0.05$ and ***significant at $p < 0.005$.

3.4. Effect of curcumin on biofilm development

The antibiofilm activity of curcumin against uropathogens using a standard quantitative biofilm assay method revealed a concentration-dependent reduction in biofilm biomass of uropathogens when treated with curcumin. Curcumin at 100 µg/ml efficiently dislodged the biofilm biomass by 52%, 89%, 52% and 76% in *E. coli*, PAO1, *P. mirabilis* and *S. marcescens*, respectively (Fig. 3A).

Since the development of biofilms by the uropathogens has been positively correlated with EPS production, an attempt was made to determine the effect of curcumin on EPS production of test pathogens. Curcumin at 100 µg/ml inhibited EPS production in *E. coli*, PAO1, *P. mirabilis* and *S. marcescens* by 50%, 97%, 96% and 65%, respectively (Fig. 3B).

In addition, curcumin was found to be very effective in disrupting the mature (preformed) biofilms of uropathogens. The CLSM images indicated major disruption in the biofilm architecture as well as reduced thickness in curcumin-treated mature biofilms of uropathogens. In *E. coli*, the thickness of the biofilm was reduced from 16 to 10 µm, whereas in *P. mirabilis* it was 11 µm in the control and 6.36 µm in curcumin-treated biofilm. Likewise, *S. marcescens* also displayed a higher reduction from 12 to 3.78 µm in biofilm thickness. However, curcumin was not found to be effective in disrupting the mature biofilm of PAO1 (Fig. 3C).

3.5. Swimming and swarming inhibition assays

As swimming and swarming migrations play an important role in QS-mediated biofilm formation in uropathogens, an effort was made to examine the anti-QS potential of curcumin against QS-dependent swimming and swarming motility in *E. coli*, PAO1,

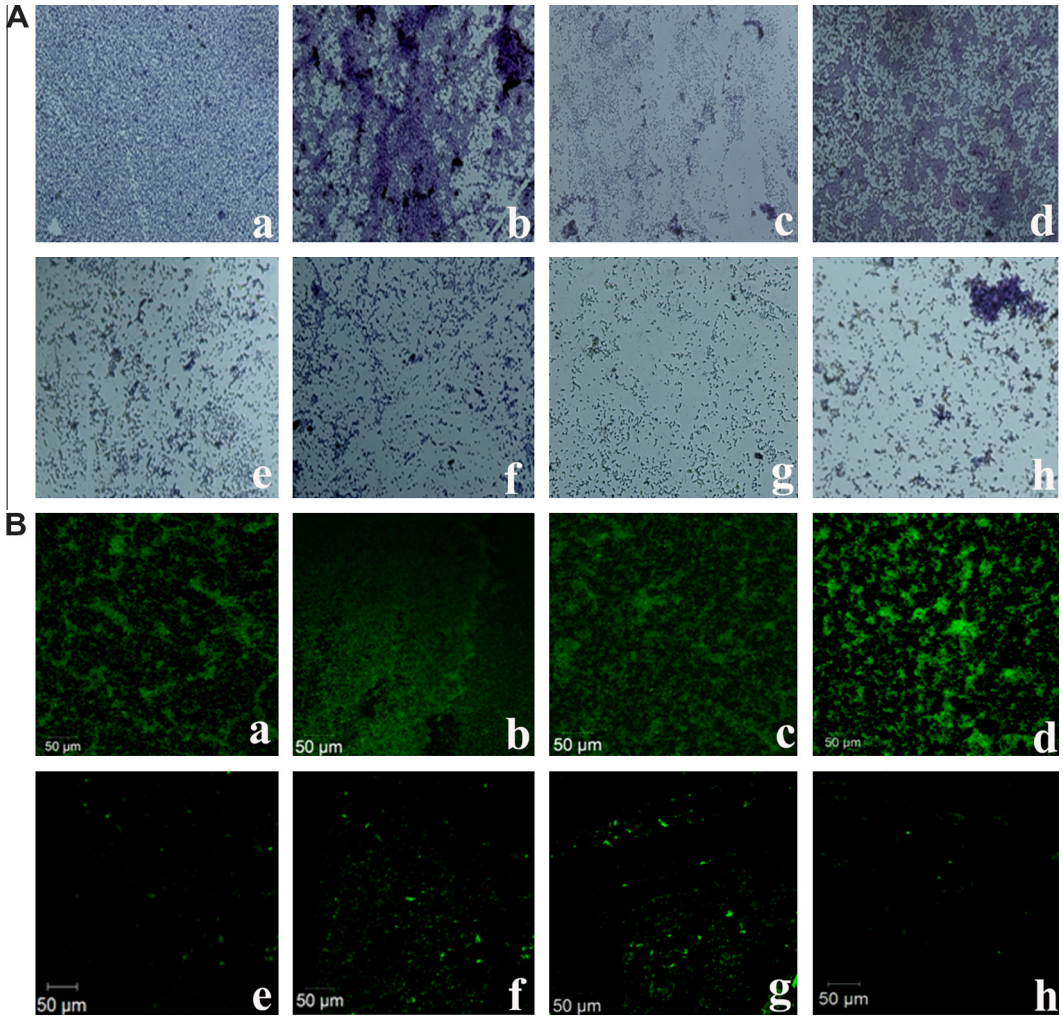

Fig. 2. Microscopic images of bacterial biofilms grown in the absence and presence of curcumin (100 µg/ml). (A) Light microscopic images and (B) confocal laser scanning microscopy (CLSM) images of (a–d) untreated controls and (e–h) curcumin-treated biofilms of *E. coli*, PAO1, *P. mirabilis* and *S. marcescens*, respectively.

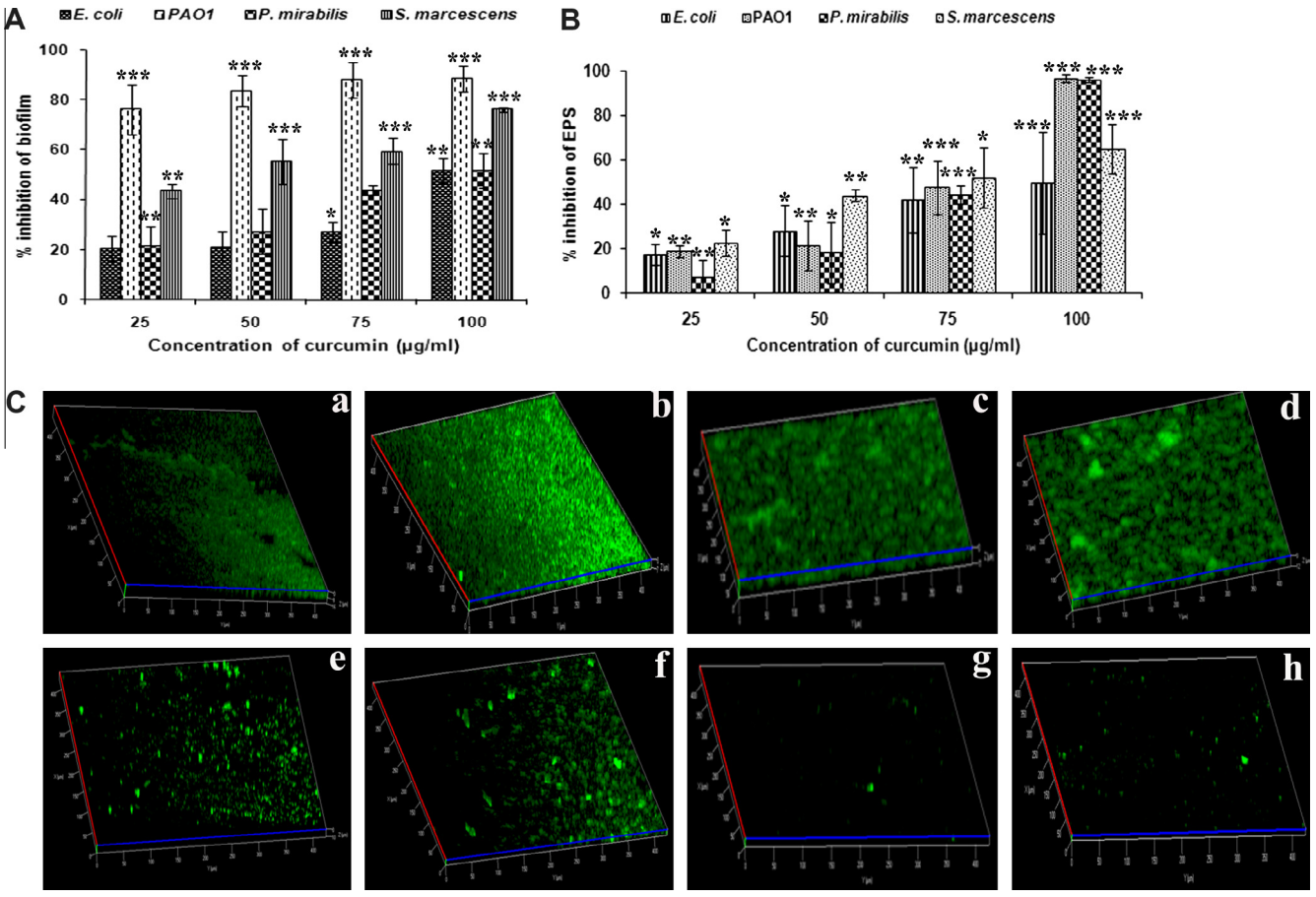

Fig. 3. Effect of curcumin on biofilm development. (A) Quantitative assessment of biofilm biomass inhibition; (B) Quantitative analysis of biofilm EPS. Mean values of triplicate independent experiments and SD are shown. *Significant at $p < 0.025$, **significant at $p < 0.05$ and ***significant at $p < 0.005$. (C) CLSM images showing inhibitory activity of curcumin on mature (preformed) biofilms of uropathogens. (a–d) untreated controls and (e–h) curcumin-treated biofilms of *E. coli*, PAO1, *P. mirabilis* and *S. marcescens*, respectively.

P. mirabilis and *S. marcescens*. Interestingly, the obtained results indicated that curcumin inhibited the swimming and swarming behaviour of all test uropathogens. However, a major level of inhibition in the migration of all uropathogens was recorded at higher concentrations (Table 1).

3.6. QS inhibition assays in PAO1and S. marcescens

3.6.1. Inhibition of alginate production in PAO1

As alginate constitutes the major portion in the EPS of PAO1 biofilm, the effect of curcumin was examined for its efficacy to reduce the production of alginate. The attained results showed that the production of alginate was reduced significantly as the concentration of curcumin increases. At a concentration of 100 µg/ml, curcumin inhibited alginate production by 63% in PAO1 (Fig. 4A).

3.6.2. Rhamnolipid inhibition in PAO1

This assay was carried out to evaluate the ability of curcumin in inhibiting the production of rhamnolipid in PAO1. In CTAB plate assay, the interaction between CTAB with surfactants results in the formation of a dark blue halo around the culture-containing wells indicating the presence of rhamnolipid. From the obtained results, when compared to the control, curcumin-treated wells showed reduced level of dark blue halo, which indicated the reduced production of rhamnolipid. The diameter of the rhamnolipid zone was 18 mm in untreated control, whereas it was measured as 17, 12, 9 and 8 mm, respectively in samples treated with curcumin at 25, 50, 75 and 100 µg/ml (Fig. 4B). The reduction in rhamnolipid production was also confirmed by TLC assay. The obtained results revealed that the intensity of the rhamnolipid bands were reduced with increasing concentration of curcumin. Curcumin at 100 µg/ml displayed a very faint band when compared with control (Fig. 4C).

Table 1
Effect of curcumin at different concentrations (0, 50, 75 and 100 µg/ml) on swimming and swarming motility of bacterial pathogens.

Pathogen	Radius of migration zone after 16 h (mm)							
	Swimming motility				Swarming motility			
	0 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	0 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
E. coli	45.8 ± 2.8	23.3 ± 0.9	8.6 ± 1.8	4.3 ± 1.5	24.0 ± 2.6	9.2 ± 1.5	7.9 ± 1.3	5.9 ± 1.7
PAO1	46.1 ± 0.8	20.0 ± 1.4	14.2 ± 1.9	6.6 ± 0.7	22.3 ± 1.0	12.8 ± 1.3	9.7 ± 0.6	4.4 ± 0.7
P. mirabilis	43.4 ± 0.4	36.2 ± 2.8	25.6 ± 1.2	20.8 ± 1.5	43.7 ± 0.5	16.0 ± 1.8	15.1 ± 0.8	7.8 ± 2.3
S. marcescens	44.6 ± 1.1	26.7 ± 2.4	21 ± 1.3	15.5 ± 1.9	45.4 ± 0.8	18.8 ± 1.9	12.5 ± 0.8	9.0 ± 0.8

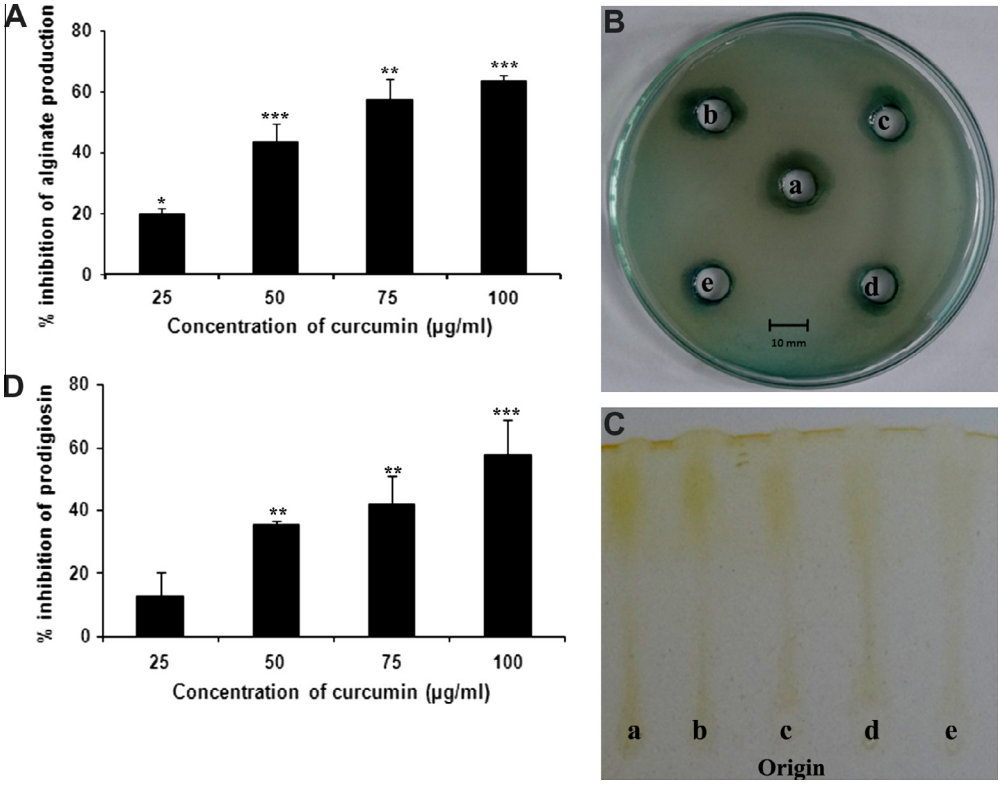

Fig. 4. Effect of curcumin on virulence factors production in PAO1 and *S. marcescens*. (A) Inhibition of alginate production in PAO1 in the presence of curcumin (B) Inhibition of rhamnolipid production by plate detection assay. (C) Inhibition of rhamnolipid production by TLC assay. In (B) and (C), (a) rhamnolipid from curcumin untreated culture of PAO1, (b-e) rhamnolipids from PAO1 treated with curcumin at concentrations of 25, 50, 75 and 100 µg/ml, respectively. (D) Quantitative analysis of prodigiosin inhibition in *S. marcescens* by curcumin. Data are represented as percentage of prodigiosin inhibition. Mean values of triplicate independent experiments and SD are shown. *Significant at $p < 0.025$, **significant at $p < 0.05$ and ***significant at $p < 0.005$.

3.6.3. Effect of curcumin on prodigiosin production of S. marcescens

Production of prodigiosin by *S. marcescens* is one of the QS-dependent behaviours. As expected, a concentration-dependent reduction in prodigiosin pigment production of *S. marcescens* was observed with curcumin treatment. A maximum of 58% inhibition in prodigiosin production was observed when treated with curcumin at 100 µg/ml (Fig. 4D).

3.7. Synergistic activity of curcumin with antibiotics

In synergistic assay, the enhanced susceptibility of *C. violaceum* to bacitracin, *E. coli* to clindamycin, PAO1 to azithromycin, and *P. mirabilis* and *S. marcescens* towards erythromycin were examined in the presence of curcumin. The results revealed that increasing concentration (25–100 µg/ml) of curcumin with antibiotics enhanced the susceptibility *of* test pathogens towards respective antibiotics in a dose-dependent manner (Table 2).

3.8. Growth curve assay

In order to confirm the non-antibacterial activity of curcumin at tested concentrations, the bacterial growth curve assay was performed for all test uropathogens. The obtained results revealed that the cell densities did not differ between untreated control and cultures treated with curcumin (25–100 µg/ml) (data are given in Supplementary file 1).

4. Discussion

In the present investigation the anti-QS potential of curcumin was assessed for its ability to inhibit signal-based biofilm formation and other production factors in uropathogens. Initially, the anti-QS potential of curcumin was studied using the biomarker strains *C. violaceum* (ATCC 12472) and CV026. The production of violacein pigment by these strains is mediated by signal-mediated QS system. The qualitative assessment of violacein pigment production in *C. violaceum* (ATCC 12472) clearly indicated that the anti-QS activity of curcumin was concentration dependent. Further, in quantitative assessment of violacein inhibition with CV026, curcumin showed about 89% reduction, as shown in Fig. 1. The results obtained in the present study are comparable with those of Khan, Zahin, Hasan, Husain, and Ahmad (2009), who reported the 92% inhibition of QS-mediated violacein production in CV026 by clove oil. Brackman, Hillaert, Calenbergh, Nelis, and Coenye (2009) reported 18% violacein inhibition by curcumin at a concentration of 184 µg/ml.

Formation of biofilm plays an important role in the pathogenesis of UTI pathogens and development of these biofilms are based on the signal-mediated QS system (Jones, 2004). Therefore, an interference with QS may prevent the development of uropathogenic bacterial biofilms. The result of biofilm biomass assay in the present study indicated a reduced production of biofilm biomass in uropathogens when treated with curcumin (Fig. 3A). This compound not only reduced the biofilm biomass but also reduced the microcolony formation, which was more apparent from the light microscopic images (Fig. 2A). Consistent with our findings, curcumin has been shown previously to inhibit the biofilm of PAO1 without affecting the planktonic growth (Rudrappa & Bais, 2008). In an another study, Nashikkar et al. (2011) found that the biofilm formation by the uropathogens such as *P. mirabilis* and PAO1 in the presence of latex extract of *Euphorbia trigona* (50 µg/ml) is meager when compared to that of untreated biofilms.

Table 2
Synergistic effect of curcumin with antibiotics.

Bacterial strain	Antibiotics (Concentration)	Growth reduction (%)	Growth reduction (%) with antibiotics in the presence of curcumin at 25–100 μg/ml.			
			25	50	75	100
C. violaceum	Bacitracin (10 μg)	–	24.7 ± 2.3	25.3 ± 3.6	30.3 ± 1.6	33.5 ± 1.5
E. coli	Clindamycin (10 μg)	60.14 ± 4.7	68.18 ± 2.0	75.0 ± 1.5	78.4 ± 4.4	81.9 ± 3.3
PAO1	Azithromycin (10 μg)	59.24 ± 2.5	60.8 ± 1.8	63.3 ± 1.9	64.2 ± 2.8	68.4 ± 4.2
P. mirabilis	Erythromycin (10 μg)	–	–	05.6 ± 2.4	07.0 ± 1.2	09.8 ± 3.7
S. marcescens	Erythromycin (10 μg)	58.32 ± 1.3	68.4 ± 4.1	69.4 ± 3.2	69.8 ± 3.7	74.9 ± 2.6

An additional interesting mechanism in the biofilm development is the characteristic biofilm architecture (You et al., 2007). In the present study, the results of CLSM analysis clearly revealed that the architecture of the curcumin-treated biofilms was looser and less thick when compared with that of untreated control biofilms (Fig. 2B). Our results are in accordance with previous reports of Hentzer and Givskov (2003) with furanone of *D. pulchra*, and Bjarnsholt, Jensen, and Rasmussen (2005) with garlic, wherein a considerable reduction in biofilm morphology and a reduction in thickness of *P. aeruginosa* was observed. There is increasing evidence that QS plays a crucial role in the maturation of bacterial biofilms (De Kievit, Gillis, Marx, Brown, & Iglewski, 2001). Consequently, the anti-biofilm activity of curcumin in disturbing the preformed biofilm was visibly observed through CLSM analysis (Fig. 3C). The loosening of architecture and morphology of the preformed biofilms by curcumin ultimately led to reduced antibiotic resistance of bacterial pathogens. Rogers et al. (2010) have also revealed the possible role of 2-aminoimidazoles to disperse the preformed PAO1 biofilms. Very recently, Abraham et al. (2012) reported the antibiofilm potential of the edible spice *Capparis spinosa* in disturbing the mature biofilms of *E. coli*, PAO1, *P. mirabilis* and *S. marcescens*. In the present study, the obtained results clearly showed the dual role of curcumin to inhibit the development of biofilm at the initiation as well as maturation stage.

QS-based EPS production is considered as an important factor for maintaining biofilm architecture and microcolony formation (Sauer & Camper, 2001). In addition, EPS confers antibiotic resistance to bacterial pathogens by acting as protective barrier and prevents the entry of antibiotics into bacterial cells (Fux et al., 2005). Besides, increased secretion of EPS would result in the alterations in biofilm architecture which are associated with an increased resistance to killing agents (Yildiz & Schoolnik, 1999). Hence, inhibition of EPS production will facilitate the direct exposure of urinary biofilms towards antibiotics and that in turn will facilitate the eradication of biofilm. In this present study a significant reduction in EPS production was observed in the uropathogens when treated with curcumin (Fig. 3B). Findings of Brackman et al. (2008) have observed the reduced production of EPS by *Vibrio* spp. upon treatment with cinnamaldehyde and its derivatives.

Flagellar-mediated swimming motility is associated with biofilm formation by instigating the cell-to-surface attachment (Pratt & Kolter, 1998), and plays a key role in the virulence of uropathogens. Therefore any interruption in swimming motility will possibly affect the formation of biofilm. From Table 1, it is evident that the swimming motility of tested pathogens was comparatively poor with that of control when treated with curcumin, and hence, curcumin appeared to reduce the biofilm formation in uropathogens by interfering with its ability to reach the substratum. The results are in accordance with the findings of Niu and Gilbert (2004) wherein cinnamaldehyde reduced the biofilm formation of *E. coli* by interfering with its swimming motility. In addition to swimming motility, swarming behaviour of bacteria is also considered to be an important virulence factor, since it is involved in biofilm formation. Here, a remarkable reduction in swarming motility of the uropathogens *E. coli*, *P. mirabilis*, *S. marcescens* including PAO1 by curcumin was observed with a marked change in the spacing of the radiating tendrils. The dendrites were closely spaced and thin in untreated control but broader and widely spaced in the presence of higher concentration of curcumin, which may in turn, lead to the development of immature or reduced biofilm and this may have important applications in treating urinary infections. It is also proven that the mass translocation of cells during swarming migration relies on expression of biosurfactants molecules, whose expression is controlled by QS (Daniels, Vanderleyden, & Michiels, 2004). This present study also revealed the ability of curcumin to inhibit the production of biosurfactant (rhamnolipid) in PAO1 (Fig. 4B and 4C). Previously, the dietary spice *Capparis spinosa* was reported to inhibit the rhamnolipid production of PAO1 at sub-MIC concentrations (Abraham et al., 2011). As depicted in Fig. 4A, alginate production of PAO1 was also decreased with increasing concentration of curcumin. Since, alginate confers increased resistance to antimicrobial agents; inhibition of alginate production would decrease the rate of bacterial resistance. Previously, essential oil of *Matricaria chamonilla* was shown to inhibit alginate production of *P. aeruginosa* (Owlia et al., 2007).

Since prodigiosin is considered as a major virulence factor (Liu & Nizet, 2012), the anti-QS activity of curcumin was assessed against prodigiosin production in *S. marcescens*. The two signal molecules *N*–butanoyl homoserine lactone and HHL are shown to regulate the production of prodigiosin (Morohoshi et al., 2007). Therefore, any interference with these QS systems leads to a reduction in prodigiosin production. As shown in Fig. 4D curcumin exhibited a concentration-dependent reduction in prodigiosin production without any hindrance on its growth. Recently, Nithya et al. (2010) also observed a similar effect, in which, crude extract obtained from marine *Bacillus* spp. inhibited the production of prodigiosin to a considerable degree without any reduction in the growth.

It is well documented by Bjarnsholt et al. (2005) that increased sensitivity towards antibiotics depends on the process of QS. The UTI pathogens are generally resistant or less sensitive towards antibiotics. It has been found that *E. coli*, PAO1, *P. mirabilis* and *S. marcescens* cells in biofilm are less sensitive to antibiotics, such as clindamycin, azithromycin and erythromycin. In the present study, curcumin was found to enhance the susceptibility of test pathogens towards respective antibiotics (Table 2). This was also supported by the reports of Rogers et al. (2010), stating that compounds which reduce bacterial biofilms without targeting bacterial growth may likely act synergistically with conventional antibiotics to overcome the resistance among bacterial pathogens. Our results are consistent with previous reports of Hentzer and Givskov (2003), wherein halogenated furanone compounds act synergistically with tobromycin to eradicate *P. aeruginosa* biofilms. Similarly, cinnamaldehyde increases the susceptibility of *V. vulnificus* towards doxycycline (Brackman et al., 2008). Given the widespread occurrence of signal-mediated QS systems, it has been revealed that interfering with the QS system may pave the way to prevent the development of signal-mediated biofilm formation and subsequent urinary infections.

The present work concluded that the *Curcuma longa*-derived phytochemical curcumin inhibited the formation of biofilm and its associated behaviours in uropathogens by interfering with the signal molecule-based QS system. Since this compound is used at its sub-MIC concentration, it is less expected to impose any selective pressure for the development of antibiotic resistance. The multifunctional activity of curcumin with promising antibiofilm activity could be used in the development of novel antipathogenic drugs to treat urinary infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.08.002.

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