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ORIGINAL PAPER

Inhibition of quorum sensing mediated biofilm development and virulence in uropathogens by *Hyptis suaveolens*

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Abstract Bacterial urinary tract infections (UTIs) are the most common nosocomial infections, accounting for about 40 % of all hospital-acquired infections. The bacterial spectrum of nosocomial UTIs is broad and the treatment of UTIs is becoming difficult owing to the emergence of drug resistance. Therefore, it is reasonable to investigate novel and alternative therapeutic strategies to treat UTIs. Since UTIs are caused by uropathogens with quorum sensing (QS)-dependent biofilm forming abilities, interruption of QS systems may be a novel approach to combat drug resistance. In the present study, a methanol extract (and hexane extract derived from it) of the medicinal plant *Hyptis suaveolens* (L.) were shown to have anti-QS activity against the biosensor strain *Chromobacterium violaceum* (ATCC 12472). Furthermore, the hexane extract of *H. suaveolens* (HEHS) inhibited biofilm formation by uropathogens such as *Escherichia coli, Proteus vulgaris, Proteus mirabilis, Klebsiella pneumoniae* and *Serratia marcescens.* HEHS promotes the loosening of biofilm architecture and strongly inhibits in vitro biofilm formation by uropathogens, which was more apparent from microscopic images. In addition to this, HEHS reduces the production of QS-dependent virulence factors like protease and hemolysin, along with motility. The partial purification and GC–MS analysis of the active fraction revealed the presence of several therapeutically important compounds which may synergistically act on the uropathogens and possibly reduce the QS-dependent phenotypes. These findings suggest HEHS as potential phytotherapeutic agent which can be employed to formulate protective strategies against biofilm linked infections caused by uropathogens.

Keywords Urinary tract infections (UTIs) · Nosocomial · Uropathogens · *Hyptis suaveolens* · Quorum sensing · Biofilm

Introduction

Urinary tract infections (UTI) represent one of the most prevalent infectious diseases encountered in community medical practice and are the most common nosocomial infections, accounting for about 40 % of all hospital-acquired infections. The incidence of UTI is more common in elderly patients and females (Stamm and Norrby 2001). The microbial etiology of UTI has been considered as limited and predictable. *Escherichia coli, Klebsiella pneumonia*, *Proteus* spp., *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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are the principal pathogens responsible for complicated and uncomplicated UTI (Nicolle 2001). Usually these bacteria exist as surface-associated communities and these compact microbial consortia are called biofilms. According to National Institute of Health (NIH, USA), more than 60 % of all bacterial infections have been probably caused by biofilm forming organisms (Costerton et al. 1999; Lewis 2001). Pathogenic bacteria have the ability to colonize and form biofilms in almost all medical implants and these biofilms often serve as a root of persistent infections. Biofilm mediated infections are mainly problematic because biofilm-dwelling bacteria are more resistant to antibiotics, immune defenses, biocides and hydrodynamic shear forces compared to their planktonic counterparts (Costerton et al. 1995). Numerous studies have documented that biofilm formation in the urinary tract and catheters not only instigates particular infections but also cause several complications such as pyelonephritis, cystitis, generation of gravels and catheter encrustation (Hola et al. 2010).

Control of bacterial infections by hindering microbial growth has been a prime approach of antimicrobial chemotherapy. Selective pressure made by antimicrobial substances in a variety of environmental settings causes the emergence of antimicrobial resistant clones and the wide spread of such clones. As resistance is ever increasing, new strategies need to be employed to combat the pathogens. Research has indicated that functions such as motility, secretion of virulence factors, biofilm formation and acquiring competency play a significant role in pathogenesis of bacterial infections in living systems. Hence, these processes are common among bacterial pathogens and in several cases have been shown to be regulated by a cell density dependent phenomenon mediated through the synthesis and recognition of small signalling molecules called autoinducers. This process was termed as quorum sensing (QS) and has now been revealed to modulate the expression of genes responsible for survival, virulence and pathogenicity (Schauder and Bassler 2001; Lyon and Muir 2003; Raffa et al. 2005). As the pathogenicity traits of several significant pathogens are under the control of QS systems, recent research has been focused on the inhibition of QS to develop novel therapeutic agents as an alternative to antibiotics and to decrease the risk of development of resistance (Schauder and Bassler 2001; Lyon and Muir 2003; Raffa et al. 2005). It is well documented that many uropathogens possess a QS regulated biofilm mode of growth for the successful establishment of UTIs and hence targeting of the QS system by the use of anti-QS compounds would be a promising treatment system (Nashikkar et al. 2011; Jones 2004).

Since ancient times, plants have been used extensively as a source of phytotherapeutics for a variety of diseases (Clardy and Walsh 2004). Recently, dietary phytochemicals have been examined for the purpose of controlling biofilm related infections, due to their non-toxic nature (Rudrappa and Bais 2008; Brackman et al. 2009). Terrestrial plants such as *Terminalia chebula, Punica granatum, Delisea pulchra, Medicago sativa* can produce mimics of QS signal molecules which have the ability to control bacterial QS systems (Sarabhai et al. 2013; Bakkiyaraj et al. 2013; Hentzer et al. 2002; Keshavan et al. 2005).

In the present study, *Hyptis suaveolens* (L.) Poit, belonging to the family Lamiaceae, was selected to assess its antibiofilm and anti-QS potential. *H. suaveolens* is a fast-growing, strongly aromatic perennial herb found in dense clumps beside roadsides. Originally an inhabitant of tropical America, it is now considered a weed worldwide. Traditionally *H. suaveolens* is used for the healing of pain, respiratory tract infections, colds, skin diseases, fever and cramps (Iwu 1993). The main purpose of this study was to evaluate the antibiofilm activity of *H. suaveolens* leaf extract against uropathogens, as well as its impact on production of virulence factors.

Materials and methods

Bacterial strains

The uropathogens used in this study, i.e. *E. coli* (ATCC10536), *Proteus mirabilis* (ATCC 7002), *K. pneumoniae* (ATCC 10031), *Proteus vulgaris* (ATCC 49132) and *Serratia marcescens* (a clinical isolate having GenBank accession no. FJ584421 for 16SrRNA gene sequence), and the QS biosensor strain *Chromobacterium violaceum* (ATCC 12472) were cultivated and maintained in Luria–Bertani (LB) medium (pH 7.0). All strains were maintained at 37 °C, except *C. violaceum and S. marcescens*, for which the temperature was 30 °C.

Plant material collection and extract preparation

Leaves of *H. suaveolens* were collected from Karaikudi, Tamil Nadu, India during March–April, 2013. The plant was identified as *H. suaveolens* (L) by Dr. John Britto, The Rapinat Herbarium, St. Joseph's College, Tiruchchirappalli, Tamil Nadu, India. The voucher number for *H. suaveolens* was RHT-3509. The leaves were washed in running water and air-dried. Five hundred grams of powdered leaf materials was exhaustively extracted with methanol. The solvent was drained by filtration using Whatman No. 1 filter paper and the filtrate was concentrated using rotary vacuum dryer to obtain the methanolic extract. A portion of the methanolic extract was used for assessment of its anti-QS properties qualitatively.

Extraction by solvent partitioning

Ten grams of the methanol extract was thoroughly partitioned with hexane and water (1:6 water to hexane). The hexane layer was collected and the ensuing aqueous layer was then thoroughly partitioned with ethyl acetate (1:6 water to ethyl acetate). The ethyl acetate layer was also recovered. The remaining aqueous portion, hexane and ethyl acetate fractions were dried under vacuum to obtain respective extracts.

Qualitative QS inhibition assay

The *H. suaveolens* methanol, hexane, ethyl acetate and aqueous extracts were subjected to qualitative analysis of QS inhibition to determine their anti-QS potential against *C. violaceum* (ATCC 12472). *C. violaceum* synthesizes the purple coloured pigment violacein in response to its QS signal molecule, *N*-hexanoyl-L-homoserine lactone (HHL). The loss of violacein production provides a reporter for anti-QS activity and can be visualized directly (Nithya et al. 2010). Overnight cultures (1 %; OD adjusted to 0.4 at 600 nm) of *C. violaceum* were used to inoculate individual wells of sterile 24 well microtitre plates containing 1 ml of LB broth with and without varying concentration of test extracts (6.25–50 µg/ml). The microtitre plates were incubated at 30 °C for 16 h and observed for the reduction in violacein pigment synthesis.

Antibacterial activity assay

The antibacterial activity of the hexane extract of *H. suaveolens* (HEHS) was assessed by standard broth dilution assay (Clinical and Laboratory Standards Institute 2006). Briefly, in sterile 24 well microtitre plates, 1 % v/v of test uropathogens (0.4 OD at 600 nm) were used to inoculate LB medium supplemented with and without twofold serially diluted HEHS (50–400 µg/ml) and incubated for 24 h. After incubation, the cell densities of the uropathogens were measured at OD 600 nm using a multiwell reader (SpectraMax M3, USA).

Biofilm susceptibility assay

The effect of HEHS against biofilm formation of uropathogens was studied using static crystal violet (CV) assay (Thenmozhi et al. 2009). Briefly, in sterile 24-well polystyrene plates 1 % v/v of test bacterial cultures (OD 0.4 at 600 nm) were added to LB broth in the presence and absence of different concentrations of HEHS (50, 100, 200 and 400 µg/ml) without agitation for 24 h. After incubation, the plates were washed with sterile water to remove the planktonic cells. The surface adhered cells were stained with 200 µl of 0.4 % aqueous solution of CV. Following staining at room temperature for 2 min, the excess dye was removed by washing with sterile water and the CV bound cells were solubilised by the addition of 1 ml of 95 % ethanol. The biofilm biomass was quantified by measuring the optical density at 570 nm using the multiwell reader.

This biofilm inhibition assay was also used to assay selected HEHS components (undecanoic acid, phenanthrene and tetradecanoic acid; Sigma-Aldrich).

Microscopic analyses of bacterial biofilm

For light microscopy analysis, 1 % v/v overnight culture of test uropathogens were used to inoculate the wells of 24- well microtitre plates containing 1 ml of fresh LB medium along with glass pieces (1 × 1 cm) with and without HEHS at biofilm inhibitory concentration (BIC) and incubated for 24 h. After incubation, the glass pieces were rinsed with sterile distilled water and the biofilms adherent on the glass pieces were stained with 0.4 % crystal violet. The stained glass pieces were inspected with a light microscope (Nikon

Eclipse Ti 100) at a magnification of × 400 (Thenmozhi et al. 2009).

For visualization by confocal laser scanning microscope (CLSM), biofilms were allowed to grow on glass pieces with and without HEHS at BIC, as described above. The biofilms formed on the glass pieces were rinsed with sterile distilled water and stained with acridine orange (0.1 %) for 1 min. The excess stain was washed off and the biofilms on stained glass pieces were visualized under CLSM at a magnification of × 200 (Zeiss LSM 710, Carl Zeiss, Germany). Further, the images were analyzed using COMSTAT2 software.

QS inhibition assays in uropathogens

Total protease assay

Proteolytic activities of cell-free culture supernatants of uropathogens (except *K. pneumoniae*) cultivated in the presence and absence of HEHS (50, 100, 200 and 400 µg/ml) were determined by azocasein assay. Briefly, 75 µl of both treated and untreated culture supernatants of uropathogens were added to 125 µl of buffer (0.05 M Tris- HC1 and 0.5 mM $CaCl_2$ (pH 7.5)) containing 2 % azocasein substrate (Sigma, St. Louis, USA), and incubated at 37 °C for 15 min. The reaction was terminated by the addition of trichloroacetic acid (10 %, 0.6 ml) and incubated at −20 °C for 20 min. After incubation, the tubes were spun at 8000 g for 5 min and to the clear supernatant, 700 µl of 1 M NaOH was added and the absorbance was measured at 440 nm using the multiwell reader (Nithya et al. 2010).

Hemolysis assay

Hemolytic activity in liquid assay was performed as described previously (Hertle et al. 1999). Briefly, 100 µl of cell free supernatants of *E. coli, S. marcescens* and *P. mirabilis* overnight cultures treated with and without HEHS (50, 100, 200 and 400 µg/ml) were added to 900 µl erythrocyte suspension containing 2 % washed sheep erythrocytes in phosphate-buffered saline. The reaction mixture was incubated at 37 °C for 1 h and centrifuged. The amount of lysis was determined by measuring the released hemoglobin spectrophotometrically at $A_{405\ nm}$. The total lysis was attained by the incubation of erythrocytes with distilled water and background lysis was determined with erythrocyte suspension incubated in phosphate-buffered saline. The percent hemolysis was defined as [(A_{405} of sample–A_{405} of background)/(A_{405} of total–A_{405} of background)] × 100.

Swimming and swarming motility assays

The effects of HEHS on swimming and swarming motility were analyzed as described previously (Packiavathy et al. 2012). Briefly, overnight cultures of the test uropathogens (except *K. pneumoniae*) (0.4 OD at 600 nm) were used to inoculate at the centre point of the swimming (1 % tryptone, 0.5 % NaCl and 0.3 % agar) and swarming (1 % peptone, 0.5 % NaCl, 0.5 % agar and 0.5 % of filter-sterilised D-glucose) agar medium, with and without HEHS at their BIC. The plates were then incubated at appropriate temperatures for 16 h in upright position. The swimming and swarming migration was recorded after 16 h by measuring the swim and swarm zones of bacterial cells.

Effect of HEHS on prodigiosin production in S. marcescens

S. marcescens cells were pelleted from 1 ml of HEHS untreated and treated (50, 100, 200 and 400 µg/ml) cultures by centrifugation at 8,000 g for 10 min. The prodigiosin pigment from the cell pellets was solubilized by the addition of 1 ml acidified ethanol (4 ml of 1 M HCl in 96 ml ethanol). The amount of prodigiosin production was determined by measuring the OD at 534 nm spectrophotometrically (Packiavathy et al. 2012).

Separation of bioactive components from HEHS

The HEHS was subjected to column chromatography using silica gel (60–120 mesh size, Merck) and eluted with a linear gradient of petroleum ether and ethyl acetate (100:0–0:100 v/v). Ten-milliliter fractions were collected up to entire elution of the loaded sample and subsequently all fractions were dried and assayed for anti-QS activity.

GC–MS analysis of bioactive fractions

Constituents of the bioactive fractions was analyzed by GC Clarus 500 (Perkin Elmer), using an Elite-5MS

capillary column [(5 % Diphenyl/95 % Dimethyl poly siloxane), 30 × 0.25 mm × 0.25 µm df]. The oven temperature was set at 110 °C with 2 min hold, which was increased to 200 °C at the rate of 10 °C/min and finally increased to 280 °C at the rate of 5 °C/min with 9 min hold. The injector temperature was 250 °C. The ionization voltage was 70 eV and helium was used as the carrier gas with a flow rate of 1 ml/min. The injection volume was 2 µl and the scan range was 45–450 atomic mass units (AMU). The total run time was 36 min. The major constituents of the bioactive fraction were identified by comparison with the MS reference database of NIST 2005 library.

Statistical analysis

All experiments were performed in triplicate and the data obtained from the experiments are presented as mean values. The difference between control and treated were analyzed by Student's *t* test using the SPSS (Chicago, IL, USA) statistical software package.

Results

Violacein inhibition assay

In the qualitative QS inhibition analysis, the methanol extract of *H. suaveolens* showed a concentration dependent inhibitory effect on violacein synthesis of *C. violaceum* (ATCC 12472) (Supplementary Fig. 1, 2, 3 and 4). The methanolic extract of *H. suaveolens* was subjected to extraction by solvent partitioning to yield hexane, ethyl acetate and aqueous extracts. Subsequent QS inhibition assays with these extracts indicated a higher relative activity of the hexane extract compared to the methanolic extract and ethyl acetate extract, while the aqueous extract did not show any activity (Supplementary Fig. 1). Therefore, the hexane extract was chosen for further experiments.

Growth inhibition assay

The effects of different concentrations of HEHS ranging from 50 to 400 µg/ml on the growth of uropathogens were studied by the broth dilution method. None of the tested concentrations of HEHS showed any notable growth inhibitory activity against the tested uropathogens (Supplementary Fig. 2).

Antibiofilm activity of HEHS

The effect of HEHS on uropathogen biofilm formation was assessed by the CV assay method. The results revealed a dose-dependent reduction in biofilm biomass of uropathogens after treatment with HEHS. At 200 µg/ml, HEHS effectively dislodged the biofilm by 42, 39, 59 and 90 % with *P. mirabilis, K. pneumoniae, P. vulgaris and S. marcescens* respectively (Fig. 1) and hence, for these uropathogen strains the biofilm inhibitory concentration (BIC) was taken to be 200 µg/ml. For *E. coli,* the BIC was determined to be 400 µg/ml (Fig. 1).

Microscopic analyses of biofilm formation

Direct microscopic observations of biofilms clearly revealed the effect of HEHS on uropathogens' biofilm development. The results of light microscopic analysis revealed that the control slides presented a thick coating of biofilm whereas a noticeable reduction in biofilm development and microcolony formation was observed in the treated samples at their BIC (Fig. 2a). To confirm the results obtained by light microscopy, the CLSM analysis was also performed to further reveal the antibiofilm potential of HEHS against uropathogens. The CLSM images also indicated a strong biofilm inhibitory activity of HEHS by disintegrating the biofilm architecture of uropathogens upon treatment (Fig. 2b). Apart from the disintegration of biofilm architecture, HEHS markedly reduced the surface area coverage and the thickness of the biofilms in all the uropathogens tested compared with their respective untreated controls, as supported by COMSTAT analysis (Table 1).

QS inhibition assays in uropathogens

Total protease assay

In the total protease assay, a concentration dependent inhibition in protease production was observed for all the tested pathogens when treated with HEHS. The HEHS treatments led to 30–67 %, 28–46 %, 11–41 % and 37–64 % reduction in protease production of *S. marcescens, E. coli, P. mirabilis* and *P. vulgaris*, respectively, at concentrations of 50–400 µg/ml (Fig. 3).

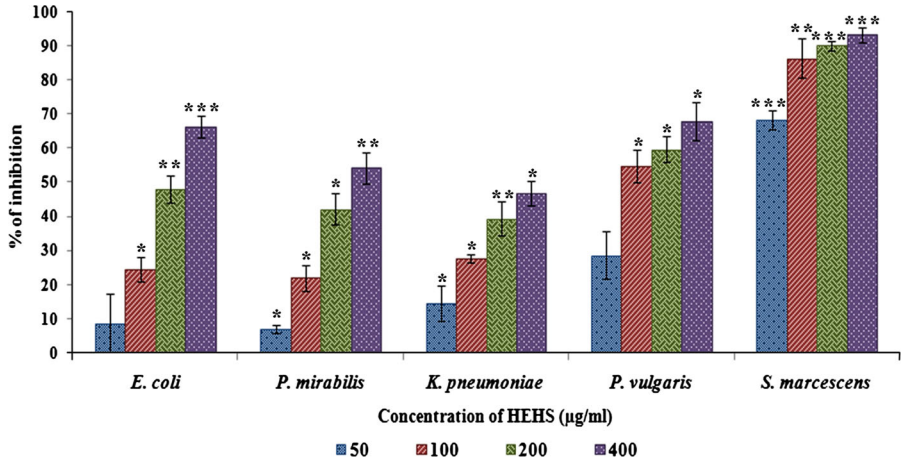

Fig. 1 Effect of HEHS on biofilm formation of test bacterial pathogens. *Data* are represented as the percentage inhibition of biofilm formation. Mean values of *triplicate* individual experiments and SDs are shown. *Indicates statistical significance ($p < 0.01$) **Indicates statistical significance ($p < 0.002$) and ***Indicates statistical significance ($p < 0.001$)

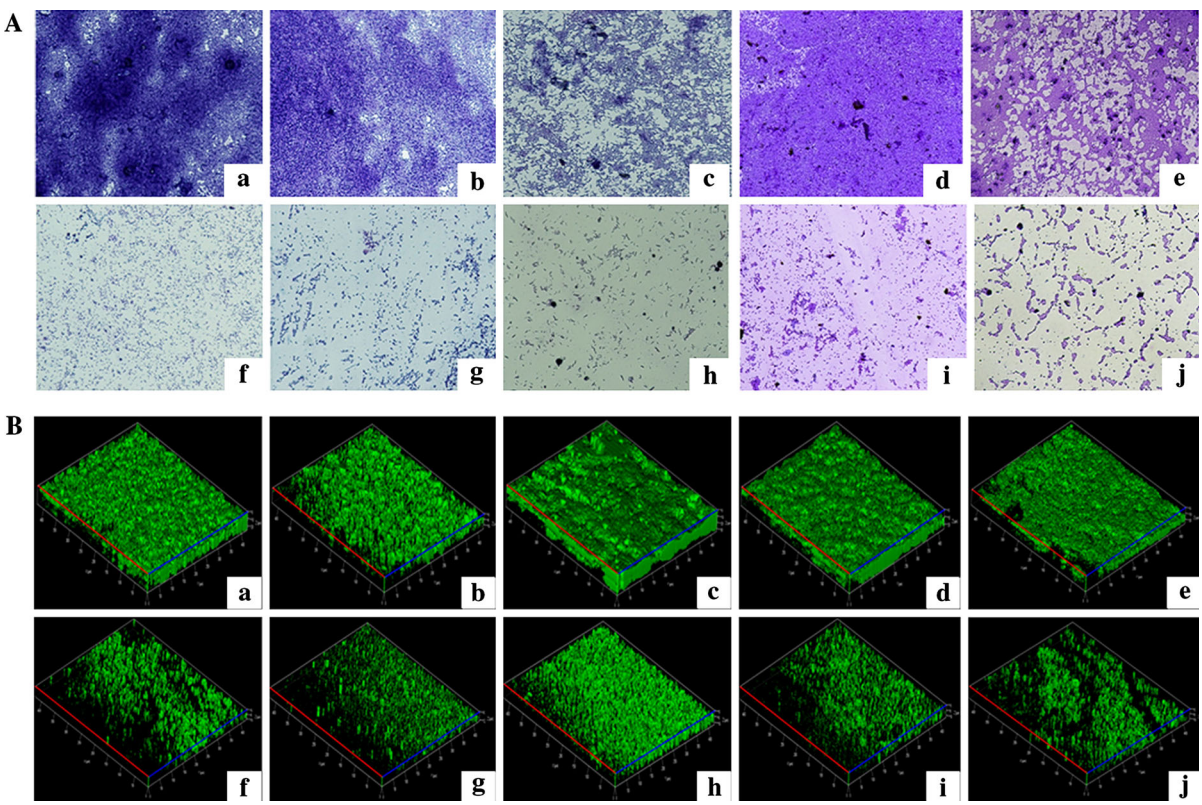

Fig. 2 Microscopic images of bacterial biofilms grown in the absence and presence of HEHS at BIC. **a** Light microscopic images and **b** confocal laser scanning microscopy (CLSM) images of (*a–e*) untreated controls and (*f–j*) HEHS-treated biofilms of *E. coli, K. pneumoniae, P. mirabilis, P. vulgaris* and *S. marcescens*, respectively

Table 1 COMSTAT analysis of the HEHS treated and untreated (control) uropathogens biofilms

Pathogen	Biomass ($\mu m^3/\mu m^2$)		Average thickness (μm)		Maximum thickness (μm)		Surface to volume ratio ($\mu m^2/\mu m^3$)	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
E. coli	27.5 ± 0.0	20.4 ± 3.1	25.7 ± 0.0	19.1 ± 2.9	25.7 ± 0.0	19.1 ± 2.9	0.04 ± 0.01	0.05 ± 0.007
P. mirabilis	32.8 ± 4.7	18.8 ± 1.3	30.2 ± 0.4	14.4 ± 4.8	30.2 ± 0.4	14.4 ± 4.8	0.04 ± 0.02	0.06 ± 0.02
P. vulgaris	26.2 ± 3.2	18.4 ± 2.1	24.0 ± 5	16.7 ± 2.7	24.0 ± 5.2	16.7 ± 2.7	0.04 ± 0.02	0.07 ± 0.02
S. marcescens	26.4 ± 3.2	19.6 ± 1.9	24.0 ± 3.8	14.8 ± 4.8	24.0 ± 3.88	14.8 ± 4.8	0.05 ± 0.04	0.07 ± 0.03
K. pneumoniae	23.5 ± 0.0	15.1 ± 0.3	21.6 ± 0.4	14.1 ± 0.2	21.6 ± 0.4	14.1 ± 0.2	0.04 ± 0.07	0.08 ± 0.01

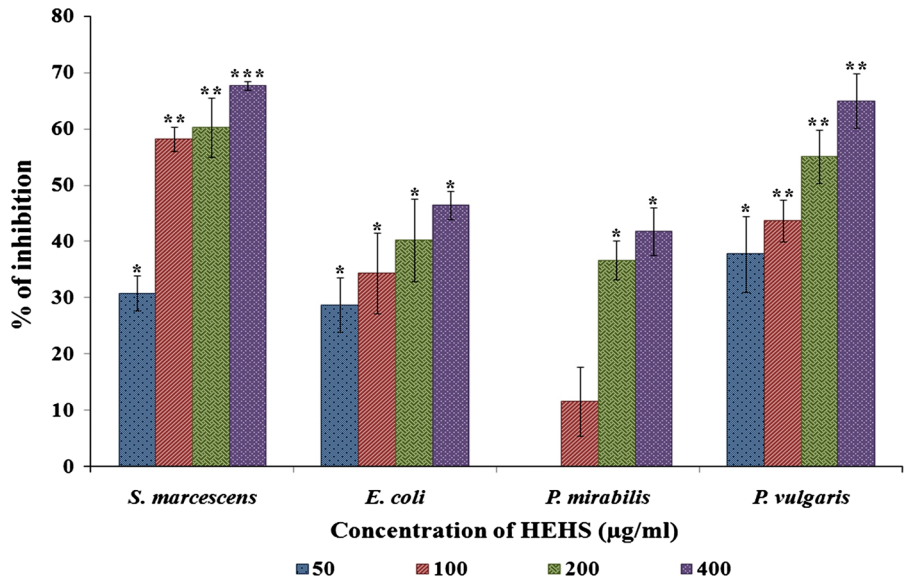

Fig. 3 Effect of HEHS on protease production of test bacterial pathogens. *Data* are represented as the percentage inhibition of protease production. Mean values of *triplicate* individual experiments and SDs are shown. *Indicates statistical significance ($p < 0.01$) **Indicates statistical significance ($p < 0.002$) and ***Indicates statistical significance ($p < 0.001$)

Hemolysis assay

In the hemolysis assay, a gradual increase in the viability of sheep erythrocytes was observed upon treatment with HEHS. The percentage of hemolysis was high (up to 52, 86 and 87 % for the untreated controls of *S. marcescens, E. coli* and *P. mirabilis*) whereas it was reduced to 10, 31 and 24 % after treatment with HEHS (Fig. 4).

Effect of HEHS on motility of uropathogens

Swimming and swarming motility play a key role in QS-mediated biofilm development by uropathogens. The addition of HEHS at BIC showed promising decrease in the swimming and swarming of all the tested uropathogens (Table 2).

Effect of HEHS on prodigiosin production in S. marcescens

Synthesis of the red coloured pigment prodigiosin by *S. marcescens* is a QS controlled behaviour. The treatment with HEHS decreased prodigiosin production in a concentration-dependent manner. Prodigiosin production was inhibited to the level of 68, 86, 90 and 93 %, respectively in the samples treated with HEHS at 50, 100, 200 and 400 μg/ml concentrations (Supplementary Fig. 3).

Purification and characterization of HEHS

The HEHS was subjected to bioassay-guided fractionation and 105 fractions were collected. Of these, fractions 21–25 showed promising anti-QS activity

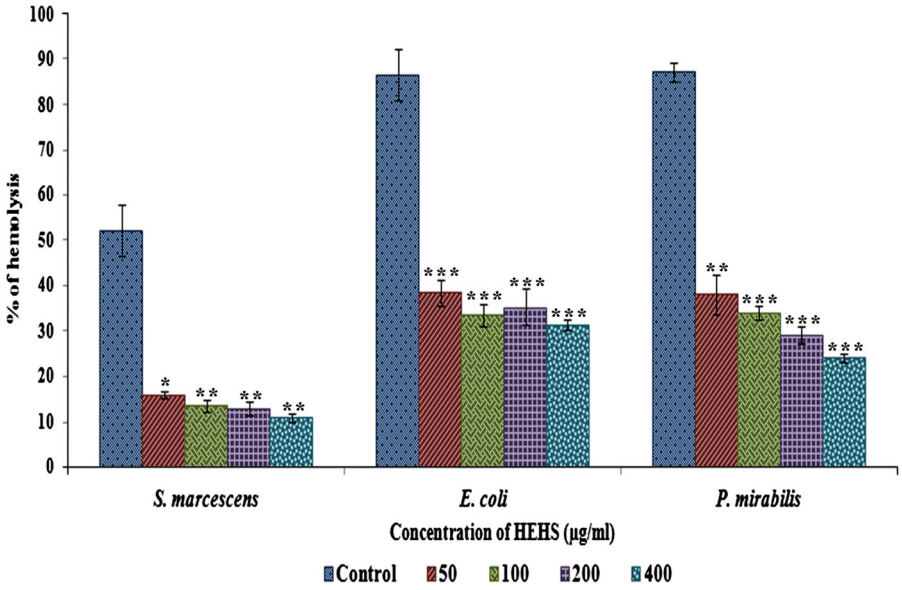

Fig. 4 Effect of HEHS on hemolysin production of test bacterial pathogens. Mean values of triplicate individual experiments and SDs are shown. *Indicates statistical significance ($p < 0.01$) **Indicates statistical significance ($p < 0.002$) and ***Indicates statistical significance ($p < 0.001$)

Table 2 Effect of HEHS at different concentrations (50, 100, 200 and 400 µg/ml) on swimming and swarming motility of uropathogens

Pathogen	Radius of migration zone after 16 h (mm)			
	Swimming		Swarming	
	Control	Treated (BIC)	Control	Treated (BIC)
E. coli	43.7 ± 0.9	25.1 ± 1.1**	26.5 ± 1.2	7.6 ± 0.7***
P. mirabilis	41.9 ± 0.3	19.0 ± 0.7***	42.9 ± 0.4	14.9 ± 0.7***
P. vulgaris	40.7 ± 0.8	15.1 ± 0.9***	43.0 ± 1.1	12.4 ± 0.9***
S. marcescens	43.5 ± 1.0	4.5 ± 0.6***	43.7 ± 0.7	5.1 ± 0.2***

Mean values of triplicate individual experiments and SDs are shown

** Indicates statistical significance ($p < 0.002$) and *** Indicates statistical significance ($p < 0.001$)

against *C. violaceum* (ATCC 12472). These five bioactive fractions were spotted onto thin layer chromatography (TLC) plates to monitor the component profiles (data not shown). The fractions 21–25 showed similar TLC profiles and hence these five fractions were pooled together and analyzed by GC–MS (Supplementary Fig. 4). The GC–MS analysis of this bioactive fraction revealed the presence of 15 compounds, of which undecanoic acid and phenanthrenemethanol accounted for about 41 and 24.7 % respectively (Table 3).

Antibiofilm activity of major compounds in HEHS

The antibiofilm activity of undecanoic acid, phenanthrene and tetradecanoic acid were tested individually. The results revealed that phenanthrene (50, 100 and 150 µg/ml) inhibited biofilm formation by uropathogens in a dose-dependent manner. At 150 µg/ml, phenanthrene effectively dislodged the biofilm by 75, 65, 33, 24 and 41 % with *E. coli, P. mirabilis, K. pneumoniae, P. vulgaris* and *S. marcescens* respectively (Fig. 5). Undecanoic acid showed antibacterial activity against *E. coli, P. mirabilis, K. pneumoniae* and *P. vulgaris* but showed antibiofilm activity against *S. marcescens* (Fig. 6). Tetradecanoic acid did not show any activity against the tested uropathogens (data not shown).

Discussion

Development of biofilms plays an important role in the pathogenesis of uropathogens (Jones 2004). Furthermore,

Table 3 Compounds identified from the partially purified bioactive fraction of HEHS by GC-MS analysis

No.	RT	Name of the compound	Molecular formula	MW	Peak Area (%)
1	3.84	Octanoic acid	$C_8H_{16}O_2$	144	2.3
2	6.20	*n*-Decanoic acid	$C_{10}H_{20}O_2$	172	3.4
3	8.81	Undecanoic acid	$C_{11}H_{22}O_2$	186	41.0
4	10.76	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	9.8
5	11.76	Oxirane, [(tetradecyloxy)methyl]-	$C_{17}H_{34}O_2$	270	2.0
6	12.11	3-Tetradecene, (Z)-	$C_{14}H_{28}$	196	0.4
7	12.83	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	3.7
8	14.32	Diethylene glycol monolaurate	$C_{16}H_{32}O_4$	288	1.2
9	14.41	Phytol	$C_{20}H_{40}O$	296	1.5
10	17.02	1,3;2,5-Dimethylene-l-rhamnitol	$C_8H_{14}O_5$	190	1.7
11	18.04	1-Phenanthrenemethanol, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1*R*-(1à,4aá,10aà)]-	$C_{20}H_{30}O$	286	24.7
12	25.48	Hexaethylene glycol monododecyl ether	$C_{24}H_{50}O_7$	450	1.7
13	30.05	Diazoprogesterone	$C_{21}H_{30}N_4$	338	1.8
14	31.20	Spiro[androst-5-ene-17,1′-cyclobutan]-2′-one, 3-hydroxy-, (3á,17á)-	$C_{22}H_{32}O_2$	328	3.1
15	32.33	2*H*-Pyran, 2-(7-heptadecynyloxy) tetrahydro-	$C_{22}H_{40}O_2$	336	1.4

RT retention time, *MW* molecular weight

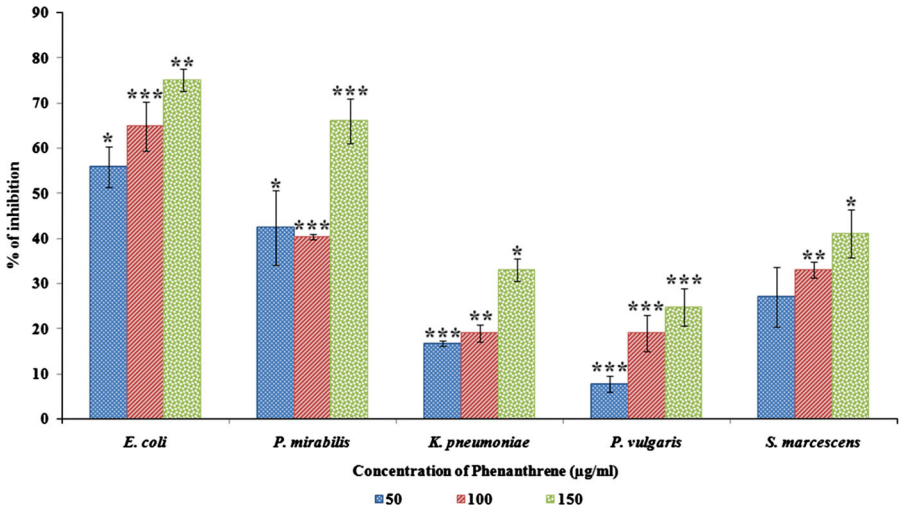

Fig. 5 Effect of phenanthrene on biofilm formation of test bacterial pathogens. *Data* are represented as the percentage inhibition of biofilm formation. Mean values of triplicate individual experiments and SDs are shown. *Indicates statistical significance ($p < 0.01$) **Indicates statistical significance ($p < 0.002$) and ***Indicates statistical significance ($p < 0.001$)

formation of biofilms on implanted medical devices leads to intractable infections. In biofilm mode, pathogens can tolerate very high concentrations of antimicrobial agents and excess use of these agents has resulted in the development of multidrug resistance in uropathogens. Recent studies have suggested that blocking of QS systems would be a novel approach to the control of biofilm mediated infections (Hentzer et al. 2002; March and Bentley 2004). In the present study, the anti-QS potential of *H. suaveolens* extract was evaluated for its ability to hinder the biofilm formation and virulence factor production in uropathogens. Initially, the anti-QS activity of a *H. suaveolens* methanol extract was tested using the QS biosensor strain *C. violaceum* (ATCC 12472). The methanol extract of *H. suaveolens* showed a concentration dependent inhibition of violacein

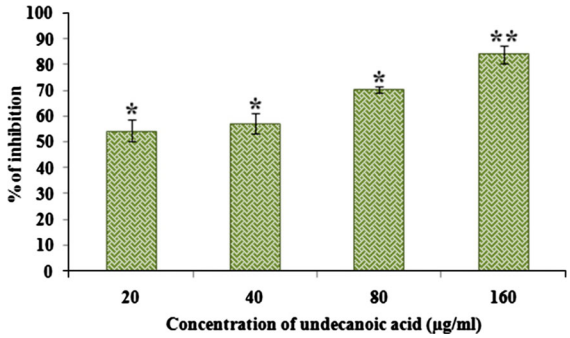

Fig. 6 Effect of undecanoic acid on biofilm formation of *S. marcescens*. *Data* are represented as the percentage inhibition of biofilm formation. Mean values of triplicate individual experiments and SDs are shown. *Indicates statistical significance ($p < 0.01$) and **Indicates statistical significance ($p < 0.002$)

production in *C. violaceum.* Subsequently, the methanolic extract of *H. suaveolens* was subjected to liquid–liquid partitioning to yield hexane, ethyl acetate and aqueous extracts. The hexane, ethyl acetate and aqueous extracts were also assayed for QS inhibitory activity using *C. violaceum* and the results revealed that the hexane extract exhibited promising anti-QS activity when compared to other extracts (Supplementary Fig. 1). Extracts from *D. pulchra*, *Terminalia catappa*, *Scorzonera sandrasica* and *Rhizophora* spp. have been shown to be effective in inhibiting violacein production in *C. violaceum* (Hentzer et al. 2002; Taganna et al. 2011; Tinaz et al. 2007; Annapoorani et al. 2013).

QS signalling molecules play an essential role in the development of the biofilm-specific physiology in uropathogens. Therefore, targeting the QS system may limit biofilm formation in uropathogens (Trautner et al. 2004). The result of biofilm inhibition assays in the present investigation indicated a decrease in biofilm biomass in uropathogens upon treatment with HEHS (Fig. 1). The HEHS not only reduced the biofilm biomass but also reduced the microcolony formation which was more apparent from the light microscopic images (Fig. 2a). Further, CLSM analysis also demonstrated the antibiofilm potential of HEHS (Fig. 2b). Previously, a latex extract of *Euphorbia trigona* was shown to inhibit biofilm formation by uropathogens (Nashikkar et al. 2011). Furocoumarins present in grapefruit juice inhibited biofilm formation by *E. coli* (GirennavarB et al. 2008). For *P. aeruginosa*, biofilm development was disrupted by rosmarinic acid present in the roots of *Ocimum basilicum* (Walker et al. 2004).

Conventional antimicrobial therapy mainly depends on the killing of bacteria and leads to the development of resistance (Warren et al. 1999). Hence, the ideal anti-QS agent should be non-bactericidal and it should not exert any selective pressure over the growth of the bacteria (Rasmussen and Givskov 2006). The assessment of the effect of HEHS on the growth of the test uropathogens revealed that the tested concentrations of HEHS did not show bactericidal activity (Supplementary Fig. 2).

The efficacy of HEHS on *S. marcescens* virulence factors such as protease and hemolysin production was evaluated. The results obtained revealed that the production of protease and hemolysis was inhibited in a dose-dependent manner (Figs. 3, 4). The prodigiosin pigment is considered as a major virulence factor of *S. marcescens* (Liu and Nizet 2009). In the present study, the production of prodigiosin pigment was highly reduced in the presence of HEHS (Supplementary Fig. 3). Previously, the synthetic analogues of acylhomoserine lactones were tested for their effects on prodigiosin production by *S. marcescens* and the analogue *N*-nonanoyl-cyclopentylamide (C9-CPA) showed promising prodigiosin inhibitory properties (Morohoshi et al. 2007).

Biofilm formation is necessarily preceded by attachment, which is mediated by flagellar driven motilities such as swimming and swarming (Klausen et al. 2003). Therefore any interruption in motility may possibly affect biofilm formation also. It was found that the swimming and swarming motility of the tested uropathogens were reasonably poor compared with that of controls when treated with HEHS at their BIC (Table 2). HEHS may diminish biofilm formation by the uropathogens tested through interfering with their ability to reach the substratum. In an earlier study, halogenated furanones from *D. pulchra* were shown to inhibit the swarming motility in *P. mirabilis* (Gram et al. 1996).

In the present study, GC–MS analysis of the bioactive fraction revealed the presence of several fatty acids such as undecanoic acid, caprylic acid, capric acid, myristic acid and palmitic acid along with other compounds. It has been reported that fatty acids such as myristic, palmitic and lauric acid have the ability to inhibit swarming motility and hemolysin production in *P. mirabilis* (Liaw et al. 2004). In another study, a branched-chain fatty acid (12-methyltetradecanoic acid; anteiso-C15:0) repressed the

swimming and swarming motility and also inhibited biofilm formation by *P. aeruginosa* (Inoue et al. 2008). Phenanthrene was found to affect biofilm by the tested uropathogens (Fig. 5), whilst undecanoic acid was shown to have antibiofilm activity against *S. marcescens* (Fig. 6). From these results it is suggested that the compounds present in the HEHS active fraction may synergistically act on the uropathogens and reduce the secretion of its virulence factors.

Conclusions

In conclusion, the present study demonstrates that HEHS could inhibit biofilm formation by uropathogens in a concentration-dependent manner. Further, it reduced the production of QS-dependent virulence factors such as protease, hemolysin, prodigiosin and motility. The partial purification and characterization of the bioactive fraction revealed the presence of several fatty acids and other therapeutically important compounds which may be responsible for the observed activities. These results suggest that HEHS may have potential therapeutic value and could prove to be a potent anti-QS and antibiofilm agent for formulating strategies against uropathogen biofilm associated infections.

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