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Lagerstroemia speciosa fruit extract modulates quorum sensing-controlled virulence factor production and biofilm formation in *Pseudomonas aeruginosa*

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Lagerstroemia speciosa (*Lythraceae*) is a south-east Asian tree more commonly known as 'Jarul'. Research on health benefits suggests that the *L. speciosa* plant contains phytomolecules that may have antioxidant, anti-diabetic and anti-obesity properties. However, antimicrobial activities have not been reported for this plant. The ability of *L. speciosa* fruit extract (LSFE) to antagonize cell-to-cell communication, expression of virulence genes and factors, and biofilm formation was evaluated in *Pseudomonas aeruginosa* strain PAO1. Our results suggested that LSFE caused downregulation of quorum sensing (QS)-related genes (*las* and *rhl*) and their respective signalling molecules, *N*-acylhomoserine lactones, without affecting the growth of *P. aeruginosa* PAO1. Significant inhibition of virulence factors: LasA protease, LasB elastase, and pyoverdin production, was also recorded. Application of LSFE to *P. aeruginosa* PAO1 biofilms increased bacterial susceptibility to tobramycin. These data suggest a possible role for quorum-quenching mechanisms unrelated to static or cidal effects, and also suggest that *L. speciosa* could serve as a cost-effective source in the development of new QS-based antibacterial drugs.

INTRODUCTION

Bacteria are highly interactive and employ a range of cell-to-cell communication mechanisms, a process known as quorum sensing (QS), to promote collective behaviour within a population (Rasmussen *et al.*, 2005; Singh *et al.*, 2009a). QS is generally considered to facilitate gene expression only when the population is at threshold cell density. It also depends on the synthesis of small, diffusible signal molecules (sometimes called 'autoinducers' or 'pheromones') that travel in and out of bacterial cells (Singh *et al.*, 2009b). The *N*-acylhomoserine lactone (AHL) autoinducers are synthesized by the family of LuxI homologue proteins. AHLs, beyond a certain threshold concentration, bind to receptor molecules (LuxR), and this, in turn, activates the formation of dimers or multimers of the LuxR–AHL complex. These forms act as transcriptional regulators of target genes of the QS systems in a cascade of regulatory events (Parsek & Greenberg, 2000). Gram-positive and Gram-negative bacteria use QS communication circuits to regulate a diverse array of physiological activities, including the secretion of virulence factors (Mittal *et al.*, 2008), biofilm formation (Ren *et al.*, 2002), antibiotic production, competence, motility, swarming, sporulation (Grossman, 1995), bioluminescence (Sitnikov *et al.*, 1995) and the exchange of DNA (Fuqua & Greenberg, 2002), all of which facilitate bacterial pathogenesis. Thus, QS is a mechanism that allows bacteria to function as a multicellular organism.

Several autoinducers have been identified, the best characterized being the AHLs in Gram-negative bacteria (Eberhard *et al.*, 1981). The AHLs are highly conserved,

Abbreviations: AHL, *N*-acylhomoserine lactone; C4-AHL, *N*-butanoylhomoserine lactone; C12-AHL, *N*-dodecanoylhomoserine lactone; CV, crystal violet; LSFE, *L. speciosa* fruit extract; QS, quorum sensing; SCLM, scanning confocal laser microscopy.

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having the same homoserine lactone moiety but different acyl side chains and substitutions (carbonyl or hydroxyl) at the C3 carbon (Fuqua & Greenberg, 2002). The best-described AHL signalling system is that governing gene expression, which has been characterized in a large number of bacteria associated with human diseases, e.g. *Pseudomonas aeruginosa* (Glessner *et al.*, 1999), *Yersinia pseudotuberculosis* (Atkinson *et al.*, 1999), *Clostridium difficile* (Carter *et al.*, 2005), *Burkholderia cepacia* (Lutter *et al.*, 2001) and *Escherichia coli* (Surette *et al.*, 1999); as well as plant-associated bacteria, e.g. *Rhizobium leguminosarum* (Rodelas *et al.*, 1999), *Ralstonia solanacearum* and *Erwinia carotovora* (Von Bodman *et al.*, 2003). The discovery that many pathogenic bacteria employ QS to regulate their pathogenicity and virulence factor production makes the QS system a novel target for attenuation of bacterial pathogenicity (Dong *et al.*, 2001). It has been suggested that inactivating the QS system of a pathogen can result in a significant decrease in virulence factor production (Zhu & Sun, 2008). Thus, QS inhibitors or blockers may be of great interest in the treatment of bacterial infections. To date, the only known QS blockers of non-bacterial origin are halogenated furanone compounds from the Australian macroalga *Delisea pulchra* (Givskov *et al.*, 1996). The natural furanones, as well as many synthetic derivatives, have been shown effectively to inhibit QS both *in vitro* and *in vivo* (Rasmussen *et al.*, 2005). Unfortunately, most of these furanones contain halogens, making them unsuitable for human use. The furanones currently being investigated are too reactive and may be too toxic for treatment of bacterial infections in humans (Hentzer & Givskov, 2003). Compared with established treatments for infectious diseases based on antibiotic compounds that kill or inhibit the growth of bacteria, non-toxic QS blockers that do not kill or inhibit microbial growth are less likely to impose a selective pressure for the development of resistant strains. The successful control of antibiotic resistance will require both the continued development of new drugs and the judicious use of our current arsenal of antibiotics (Bergstrom *et al.*, 1998). Some furanones have also been shown to be lethal to rainbow trout (Rasch *et al.*, 2004). There is thus a need for the identification of novel non-toxic natural products that are able to efficiently inhibit QS. The identification of such blockers could present us with new opportunities for the development of novel non-antibiotic drugs for treating bacterial diseases in humans as well as in other animals and plants.

Recently, anti-QS blockers have been characterized in various species of plants, including pea seedlings, garlic (Rasmussen *et al.*, 2005), vanilla (Choo *et al.*, 2006), seaweeds from southern Florida, medicinal plants (Adonizio *et al.*, 2006) and a few terrestrial plants (Teplitski *et al.*, 2000). L-Canavanine, purified from *Medicago sativa*, has been found to interfere with bacterial QS (Keshavan *et al.*, 2005). Moreover, several medicinal plants have been reported to interfere with cell-to-cell signalling (Manefield *et al.*, 2002) and biofilm formation (Adonizio *et al.*, 2008). It has been shown that some medicinal plants not only produce autoinducer mimics to disrupt the bacterial QS system, but also receive and respond to microbial signals (Adonizio *et al.*, 2006). In previous studies we confirmed Indian medicinal plants such as *Acacia nilotica* (Singh *et al.*, 2009a) and *Moringa oleifera* to have anti-QS activity (Singh *et al.*, 2009b).

Here we report the effects of *Lagerstroemia speciosa* (*Lythraceae*), a south-east Asian tree more commonly known as 'Jarul', on the production of virulence factors and biofilms, AHL levels, and QS gene transcription in *P. aeruginosa* PAO1. In the present investigation, we demonstrated that *L. speciosa* fruit extract (LSFE) significantly decreased virulence factor production (LasA protease, LasB elastase and pyoverdin) and biofilm formation. Decreased expression of QS-related genes (*lasI*/*R* and *rhlI*/*R*) and production of their respective autoinducer AHL molecules were also recorded in the presence of LSFE.

METHODS

Plant material and preparation of plant extracts. Nine medicinal plants, namely *Bixa orellana* (bark), *Boerhavia diffusa* (roots), *Cinnamomum tamala* (leaves), *Ficus bengalensis* (fruits), *L. speciosa* (fruits), *Polyalthia longifolia* (bark), *Santalabum album* (bark) and *Terminalia arjuna* (fruits), were collected from the medicinal plant garden of Banaras Hindu University, Varanasi, India. Plants were air-dried in the shade at 25 °C for 7–8 days to obtain a moisture level of <2 %, and were ground to powder (40–60 mesh size). The dried powder (5 g) was extracted for 24 h using 100 ml 80 % ethanol. The solvent was removed under reduced pressure by using a rotary evaporator (Eyela N series). The crude extracts were reconstituted in 80 % ethanol, passed through a 0.22 μm pore-size membrane filter and stored at −20 °C until use. Extracts were tested for microbial contamination at every step of processing by streaking to Luria–Bertani (LB) agar plates (Bosgelmez-Tinaz *et al.*, 2007).

Bacterial strains, media and growth conditions. Anti-QS activity was determined by employing *Chromobacterium violaceum* ATCC 12472 and *C. violaceum* CV026, purchased from the American Type Culture Collection (ATCC). In *C. violaceum* ATCC 12472, production of the natural antibiotic violacein, a water-insoluble purple pigment with antibacterial activity, is under the control of a QS system. This wild-type strain produces and responds to the cognate autoinducer molecules *N*-butanoylhomoserine lactone (C4-AHL) and *N*-hexanoylhomoserine lactone (C6-AHL). *C. violaceum* CV026 is a violacein-negative, double mini-Tn*5* mutant of *C. violaceum* ATCC 31532 (Singh *et al.*, 2009a, b). The bacterium is deficient in the autoinducer synthase CviI, and therefore requires exogenous addition of AHL to undergo QS and produce violacein. The synthesis of violacein is regulated by a QS system that uses AHL as its autoinducer (Hentzer & Givskov, 2003). Compounds that interfere with QS inhibit the production of violacein in both cases, making these strains ideal for screening. Biofilm studies were conducted using *P. aeruginosa* strain PAO1, obtained from the *Pseudomonas* Genetic Stock Center (http://www.ecu.edu/). Unless otherwise stated, the bacteria were routinely cultured aerobically in Luria–Bertani (LB) broth containing 0.5 % yeast extract, 1 % tryptone and 0.5 % NaCl at 27 °C with 180 r.p.m. agitation in a shaking incubator prior to experiments. The medium was solidified with 1.2 % agar when required, and supplemented with the appropriate antibiotic: kanamycin, 20 μg ml^{-1}, for both the *C. violaceum* strains; and potassium tellurite, 150 mg ml^{-1}, for PAO1. The solid medium was used to test the anti-QS activity of the extracts.

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Assessment of QS inhibition

Paper disk-diffusion assay. A standard paper disk-diffusion assay was used to detect the anti-QS activity of the extracts by using a biomonitor strain of *C. violaceum*, ATCC 12472, by the method described previously (Singh *et al.*, 2009a). *C. violaceum* ATCC 12472 was grown on LB agar and supplemented with the appropriate antibiotic. Five millilitres of molten LB agar (0.3 %, w/v) was inoculated with 50 ml of a culture of the bacterium. The agar/culture solution was immediately poured over the surface of pre-warmed LB agar plates. The extracts (5 mg ml^{-1}) to be tested were pipetted onto sterile paper disks placed on the solidified agar. The plates were incubated overnight at 37 °C and examined for violacein production. Anti-QS activity was detected by a colourless, opaque halo zone with viable bacterial cells around the disk.

Antibacterial versus anti-QS activity. The effect of LSFE on cell proliferation was determined by monitoring the *C. violaceum* CV026 and *P. aeruginosa* PAO1 growth curves (Adonizio *et al.*, 2006). Briefly, overnight cultures (LB) of *C. violaceum* CV026 and *P. aeruginosa* PAO1 were diluted 100-fold into 1 l autoinducer bioassay (AB) minimal medium or LB medium. OD_{600} was monitored at 45 min intervals until OD_{600} ~1.7 was reached (approx. 8 h). The cultures were then divided into 28 ml aliquots, to which 2 ml AB minimal or LB broth (controls) or 2 ml concentrated LSFE was added. The final extract concentration was 10 mg ml^{-1}. Cultures with added LSFE were normalized to the control OD_{600} of 1.7 at this time point to account for plant pigmentation. Optical density was monitored at 1.5 h intervals until a final time point of 24 h. All OD_{600} measurements were verified at a 1 : 10 dilution for greater accuracy.

Extraction and quantification of violacein. Violacein pigment extraction was carried out according to the method of Singh *et al.* (2009b). Briefly, bacterial cells were grown in the presence or absence of LSFE, lysed by 10 % SDS and incubated at room temperature for 10 min. Then, water-saturated n-butanol was added, vortexed and centrifuged at 13 000 r.p.m. (15 000 ***g***) for 5 min. The upper butanol phase containing the violacein was collected. The extracted violacein was quantified by A_{585}.

Assessment of virulence factors. PAO1 strains were grown in AB minimal medium in the absence or presence of LSFE and harvested as described by Hentzer *et al.* (2002). LasA protease activity was determined as described by Kong *et al.* (2005) and expressed as ΔOD_{600} h^{-1} (μg protein)$^{-1}$. The elastase activity of AB minimal medium supernatants was determined using Elastin Congo red (ECR) by the method of Ohman *et al.* (1980), with some modifications. Briefly, a 100 μl aliquot of the extract was added to 900 μl ECR buffer (100 mM Tris, 1.1 mM $CaCl_2$, pH 7.2) containing 20 mg ECR. This was then incubated with shaking for 3 h at 37 °C. Insoluble ECR was removed by centrifugation, and the absorbance of the supernatant was measured at 495 nm. Cell-free AB minimal medium alone and with LSFE was used as a negative control. Activity was expressed as ΔA_{495} h^{-1} (μg protein)$^{-1}$. The pyoverdin assay was adapted from the method of Cox & Adams (1985).

Extraction and quantification of AHL. The method of Shaw *et al.* (1997) was adopted for extraction of AHLs. Briefly, strain PAO1 was grown in 2.5 l AB minimal medium on a shaking incubator at 27 °C for 18 h. The culture was centrifuged at 12 000 ***g*** for 10 min, and the supernatant obtained was sterilized by membrane filtration (0.22 μm pore-size). The filtrate obtained was extracted with acidified ethyl acetate (1 %, v/v, acetic acid) (supernatant/acidified ethyl acetate, 7 : 3, v/v) and finally concentrated and dried by rotary evaporator at 40 °C. The dried powder was reconstituted in acetonitrile and quantified by electrospray MS (ESMS) (Gao *et al.*, 2003). Peak intensities for C4-AHL (m/z=172) and *N*-dodecanoylhomoserine lactone (C12-AHL) (m/z=148 and 298), and their sodium adducts (m/z=194 and 230, respectively), were combined and converted to concentrations using a standard curve generated from the pure compounds (Adonizio *et al.*, 2008). Background readings from samples extracted with alkaline ethyl acetate were subtracted from those of the acid-extracted bacterial cultures before conversion, as the lactone ring is broken by alkaline hydrolysis, making AHLs too polar to be fully extracted into ethyl acetate.

***β*-Galactosidase assay.** The transcriptional activity of QS-gene promoters was assayed using PAO1-derived strains harbouring promoter–*lacZ* fusions: PlasI-$_{lacZ}$ (pPCS223), PlasR-$_{lacZ}$ (pPCS1001), PrhlI-$_{lacZ}$ (pLPR1), PrhlR-$_{lacZ}$ (pPCS1002), and, as a control, a promoterless *lacZ* fusion strain (pLP170). Cultures were grown in AB minimal medium and monitored under the same conditions as PAO1, and LSFE was added once the cultures reached OD_{600} 1.7. Assays for *β*-galactosidase activity were performed using ONPG, as described by Mathee *et al.* (1997).

Assessment of biofilm formation. Biofilms were cultivated in continuous-culture once-through flow chambers perfused with sterile AB minimal medium (Hentzer & Givskov, 2003). LSFE (10 mg ml^{-1}) was added to the AB minimal medium. Biofilm growth and development were examined by an LSM-510 META laser confocal scanning microscope (Carl Zeiss) equipped with an argon laser and a helium-neon laser for excitation of fluorophores. The tolerance of biofilms to tobramycin was assessed by introducing tobramycin (350 μg ml^{-1}) to the influent medium of 3 day-old PAO1 biofilms. After 24 h, biofilms were examined by scanning confocal laser microscopy (SCLM) (Rasmussen *et al.*, 2005). Bacterial viability in biofilm cultures was assessed using the LIVE/DEAD *Bac*Light bacterial viability staining kit (Invitrogen). The stain stock solutions of SYTO 9 and propidium iodide (PI) were diluted 2000-fold in AB minimal medium and injected into the flow channels. The staining was allowed to progress for 15 min with the medium flow arrested. Live SYTO 9-stained cells and dead PI-stained cells were visualized by SCLM using FITC and tetramethyl rhodamine isocyanate optical filters, respectively.

Another method was also employed to assess the anti-biofilm-formation properties of LSFE. In this assay, polystyrene microtitre dishes were assayed essentially as described elsewhere (O'Toole & Kolter, 1998), with a few modifications. Briefly, an overnight culture of PAO1 in AB minimal medium was diluted 1 : 100 in fresh medium and grown for another hour. After that, different concentrations of LSFE were pipetted into the wells of the microtitre dishes and incubated for 48 h at 30 °C. The medium was removed, and 100 μl of a 1 % (w/v) aqueous solution of crystal violet (CV) was added. Following staining at room temperature for 20 min, the dye was removed and the wells were washed thoroughly. Plates were rinsed to remove planktonic cells, and surface-attached cells were then quantified by solubilizing the dye in ethanol and measuring A_{650}.

Statistical analysis. All the experiments were performed independently in triplicate on pooled samples of biological replicates. The inhibition of violacein production was calculated from the formula $100-[(A_{585}$ of extract-treated sample/A_{585} of untreated sample) $\times 100]$ and expressed as mean ± SD values. The optical density of LSFE plus LB was measured at every concentration, and this was subtracted from the readings as background. Similarly, the percentage inhibition of biofilm formation, virulence factors and gene expression was calculated as $100-[(OD_{600}$ of extract-treated sample well/OD_{600} of untreated sample) $\times 100]$ and expressed as mean ± SD values. The effects of LSFE on each activity were analysed by analysis of variance (ANOVA) with a P value of 0.05, followed by Tukey's pairwise multiple comparison test on SPSS 16.0 (SPSS, Inc.).

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RESULTS

LSFE inhibits production of AHL molecules and QS gene expression

LSFE inhibited autoinducer synthetase (*lasI* and *rhlI*) expression in PAO1, resulting in a decrease in AHL levels (Table 1). As compared with the untreated control, the decrease in C4-AHL levels was observed to be from 3.1 to 43.6 % in response to LSFE in a concentration-dependent manner (2–10 mg ml^{-1}). A significant decrease, ranging from 12.0 to 67.9 %, in C12-AHL levels corresponded to the addition of different concentrations. The most significant decrease was observed in *lasR* expression (5.2–74.5 % decrease), followed by *lasI*, *rhlR* and *rhlI* expression (2.5–69.2 %, 3.5–66.4 % and 4.2–53.9 % decreases, respectively) at 2–10 mg LSFE ml^{-1}. These results show that the reduction in AHL levels due to LSFE is as a consequence of the inhibition of *lasI* and *rhlI* gene expression in PAO1.

Anti-QS activity of LSFE

As LSFE inhibited AHL molecule production and QS-related gene expression, we further investigated the anti-QS activity of LSFE using *C. violaceum* 12472. In *C. violaceum* 12472, production of a purple pigment, violacein, is under QS control (Singh *et al.*, 2009a). Anti-QS compounds inhibit the production of violacein in *C. violaceum* 12472, making this strain excellent for screening (Singh *et al.*, 2009b). The ethanolic extract of *L. speciosa* fruit was compared with those of eight medicinal plants, i.e. *Bixa orellana*, *Boerhavia diffusa*, *Cinnamomum tamala*, *F. bengalensis*, *Polyalthia longifolia*, *Santalabum album* and *T. arjuna*, for their anti-QS potential. Lack of purple pigmentation from *C. violaceum* 12472 is indicative of QS inhibition (Fig. 1a). Out of nine medicinal plant extracts screened for anti-QS activity, LSFE proved to be the most effective. The anti-QS activity of LSFE was further determined to be concentration-dependent (5, 7.5 and 10 mg ml^{-1}) in an experiment in *C. violaceum* 12472 using the disk-diffusion bioassay (Fig. 1b). To confirm that LSFE inhibits QS signalling in *C. violaceum* 12472, we used the LB agar plate striking method. After 24 h incubation, LB plates showed full growth of the bacterium. Our results indicate that the reduced production of the violacein pigment in *C. violaceum* 12472 after treatment with LSFE was not due to the inhibition of growth of the bacterium.

Since violacein pigment production is highly regulated by QS in *C. violaceum* 12472, next we examined the inhibitory effect of LSFE on violacein production using a liquid culture of *C. violaceum* 12472 and spectrophotometric quantification. As shown in Fig. 2, violacein production was inhibited by LSFE, by 6.2 ± 0.72 to 77.3 ± 5.44 %, in a concentration-dependent manner (2–10 mg ml^{-1}).

Antibacterial versus anti-QS activity

Addition of LSFE at early stationary phase was chosen to limit any confounding effects on growth. When LSFE was added at the beginning of the cell cycle (time zero), there were changes (slight delay or acceleration) in the exponential phase (data not shown). This did not, however, affect the end-point cell density in most cases. To confirm an anti-QS mode of action, rather than changes in cell growth, a growth curve was taken to control for the latter (Fig. 3). Cultures of *C. violaceum* CV026 and PAO1 were grown to early stationary phase before addition of LSFE (Fig. 3). This ensured that LSFE had the same opportunity (length of time and cell density) to reach the time point at which QS-controlled virulence factors are produced. Stationary phase was reached in all samples (including the control) approximately 8 h after LSFE addition. The addition of LSFE did not significantly affect cell density or total protein concentration. Furthermore, the area surrounding a disk containing LSFE showed normal cell growth, when examined under higher magnification (data not shown). These results indicate that

Table 1. Effect of LSFE on AHL production and QS gene expression in *P. aeruginosa* PAO1

LSFE concn (mg ml^{-1})	AHL production*		Gene expression†			
	C4-AHL	C12-AHL	*lasI*	*lasR*	*rhlI*	*rhlR*
2.0	0.861 ± 0.16	0.95 ± 0.14	3527 ± 253	4893 ± 407	4099 ± 369	7729 ± 503
4.0	0.803 ± 0.18	0.81 ± 0.08	3169 ± 245	4314 ± 373	3831 ± 352	7016 ± 483
6.0	0.671 ± 0.15	0.69 ± 0.09‡	2722 ± 239	3427 ± 349‡	3475 ± 364‡	6232 ± 462‡
8.0	0.592 ± 0.14‡	0.48 ± 0.07‡	2283 ± 242‡	2451 ± 309§	2631 ± 282‡	4925 ± 382‡
10.0	0.501 ± 0.10§	0.35 ± 0.03§	1115 ± 193§	1316 ± 216§	1973 ± 251§	2693 ± 283§
Control	0.889 ± 0.12	1.09 ± 0.13	3617 ± 269	5162 ± 409	4278 ± 399	8011 ± 517

*AHL production is expressed in μM.

†Gene expression was measured via the β-galactosidase activity of *lacZ* gene fusion products and is expressed as Miller units.

‡Indicates significance at $P<0.05$.

§Indicates significance at $P<0.10$.

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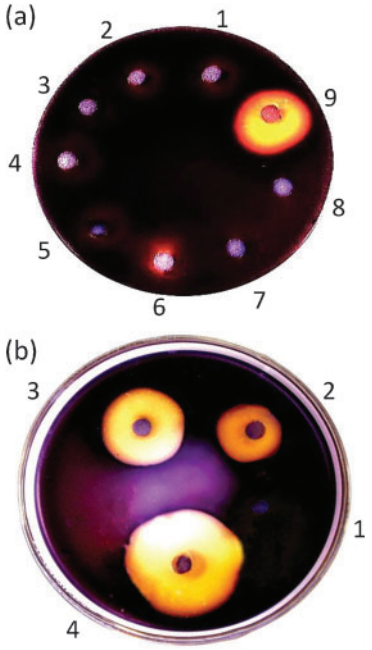

Fig. 1. Inhibitory effects of medicinal plants on violacein production in the biomonitor strain *C. violaceum* 12472. (a) The disk-diffusion method was used for the detection of anti-QS activity. *C. violaceum* 12472 was grown on LB agar and supplemented with kanamycin (20 μg ml^{-1}). Five millilitres of molten LB agar (0.3 %, w/v) was inoculated with 50 ml of a culture of this bacterium. The agar/culture solution was immediately poured over the surface of pre-warmed LB agar plates. The extracts to be tested were: (1) control (80 % ethanol); (2–8) *Bixa orellana* (bark), *Boerhavia diffusa* (roots), *Cinnamomum tamala* (leaves), *F. bengalensis* (fruits), *Polyalthia longifolia* (bark), *Santalabum album* (bark) and *T. arjuna* (fruits), respectively; and (9) *L. speciosa* (fruit) (5 mg ml^{-1}). The extracts were pipetted onto sterile paper disks, which were placed on the solidified agar. The plates were incubated overnight at 30 °C and examined for violacein production. Anti-QS activity was detected by a colourless, opaque halo zone with viable bacterial cells around the disk. (b) The concentration-dependent inhibitory effect of LSFE on violacein production in *C. violaceum* 12472: (1) control (80 % ethanol); (2–4) *L. speciosa* (5, 7.5 and 10 mg ml^{-1}, respectively).

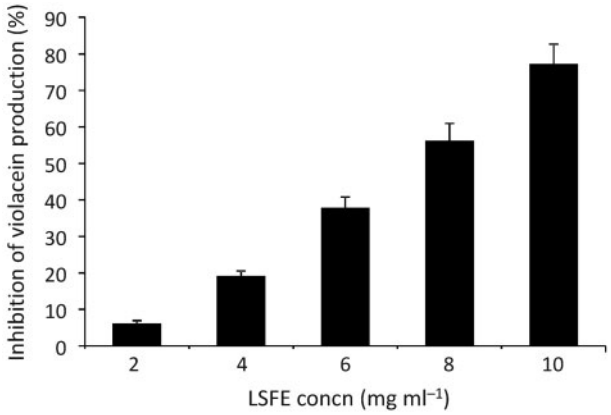

Fig. 2. Concentration-dependent inhibitory effect of LSFE on violacein production in *C. violaceum* 12472. Bacterial cells were grown in the presence or absence of LSFE, and incubated at room temperature for 10 min. Then, water-saturated n-butanol was added, and the mixture was vortexed and centrifuged at 13 000 r.p.m. (15 000 ***g***) for 5 min. The upper butanol phase containing the violacein was collected. The extracted violacein was quantified by A_{585}. Inhibition values were directly plotted as the means of replicate absorbance values against extract concentration; error bars, SD.

LSFE had no effect on the growth of *C. violaceum* CV026 and PAO1, and only inhibited violacein production, which was likely due to inhibition of the QS system.

LSFE inhibits QS-related virulence factor expression

LSFE at higher than 4 mg ml^{-1} had a significant effect on QS gene expression ($P<0.05$) in a concentration-dependent manner (Fig. 4a–c). To test the efficacy of LSFE in inhibiting PAO1 QS-controlled phenotypes, its effect on the production of some QS-controlled extracellular virulence factors, namely LasA protease, LasB elastase and pyoverdin, was investigated. The production of these virulence factors was partially or completely suppressed when PAO1 was grown in the presence of 2–10 mg LSFE ml^{-1}. LasA staphylolytic protease is a 20 kDa zinc metalloendopeptidase belonging to the β-lytic endopeptidase family of proteases (Kessler, 1995). There was a significant decrease in LasA protease activity (5.02–71.53 % compared with the control) when PAO1 was grown in the presence of 2–10 mg LSFE ml^{-1} (Fig. 4a). LasB elastase is a zinc metalloprotease capable of destroying or inactivating a wide range of biological tissues and immunological agents (Bever & Iglewski, 1988). LSFE showed a significant inhibitory effect on LasB elastase activity (2.53–58.48 %) at concentrations of 2–10 mg ml^{-1} (Fig. 4b). Pyoverdin promotes pathogenicity by stimulating bacterial growth and competing with mammalian transferrin for iron, the successful sequestration of which essentially starves the host tissues (Meyer *et al.*, 1996). LSFE treatment led to a significant reduction of pyoverdin production ranging from 3.80 to 51.42 % in a concentration-dependent manner (2–10 mg ml^{-1}) (Fig. 4c).

Effect of LSFE on biofilm tolerance to tobramycin

It has been shown that QS positively regulates biofilm formation in PAO1 and that biofilm cells are highly tolerant to antibiotic treatment (Rasmussen *et al.*, 2005). In one study, a QS mutant of PAO1 was more susceptible to SDS treatment than the wild-type counterpart (Davies *et al.*, 1998). Further, Hentzer *et al.* (2002) showed that biofilms treated with a QS inhibitor became susceptible to both SDS and tobramycin. Since LSFE demonstrated inhibition in the QS regulatory system, its effect on PAO1

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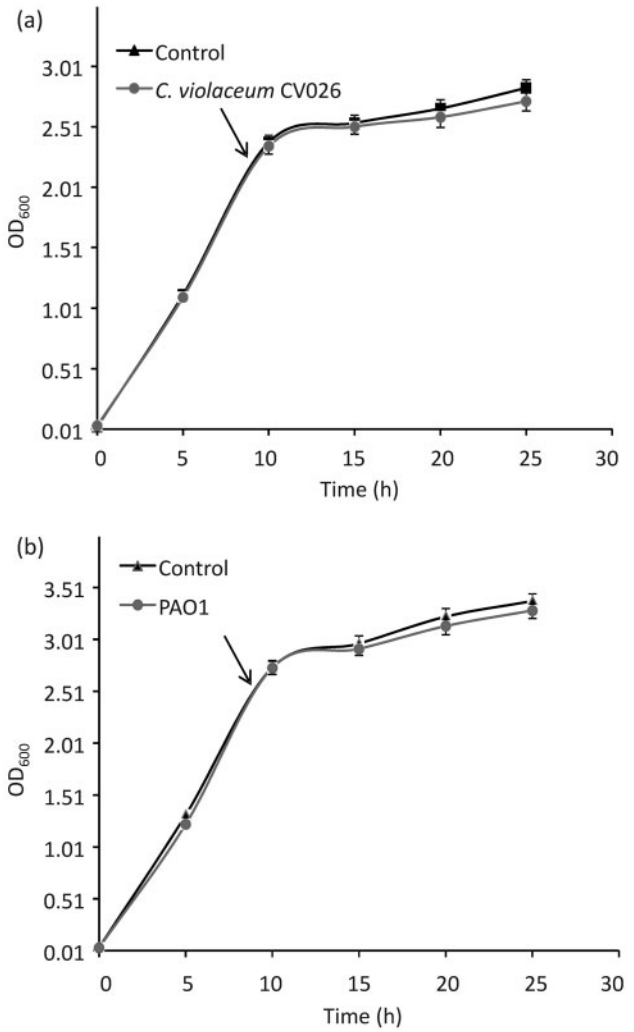

Fig. 3. Influence of LSFE on growth of *C. violaceum* CV026 and *P. aeruginosa* PAO1. Extracts were added during the early stationary phase (approx. 8 h), as indicated by arrows. The flasks were incubated at 27 °C with 180 r.p.m. agitation for 24 h in a shaking incubator. The data represent mean ± SD values of experiments performed in triplicate.

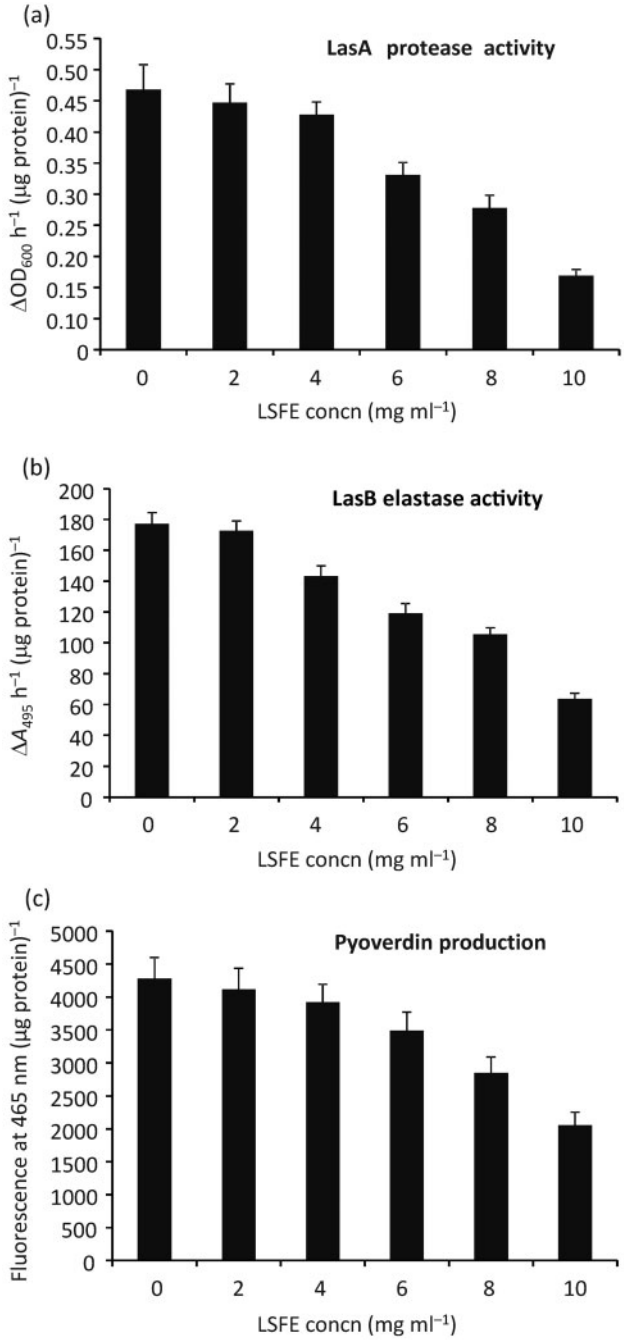

Fig. 4. Effect of LSFE on *P. aeruginosa* PAO1 virulence factors. (a) LasA protease activity (change in OD_{600} h^{-1} (µg protein)$^{-1}$, (b) LasB elastase activity (change in A_{495} h^{-1} (µg protein)$^{-1}$, (c) pyoverdin production expressed as fluorescence at 465 nm (µg protein)$^{-1}$ (excitation λ=405 nm).

biofilms was further investigated. Two sets of PAO1 biofilms were allowed to form for 72 h in flow chambers in which the medium was either without or with 10 mg LSFE ml^{-1} for 24 h. The effect of LSFE treatment was assessed by LIVE/DEAD staining. As in Fig. 5(c, d), it was found that most of the cells in the biofilm treated with LSFE died, when compared with an untreated biofilm (Fig. 5a). We further evaluated the inhibitory effects of LSFE on the biofilm-formation potential of PAO1 using an ELISA-based CV assay. PAO1 forms biofilms, a partially QS-controlled phenomenon (Davies *et al.*, 1998), in which cells are organized into layers and enmeshed in a matrix of mucoid polysaccharides (Adonizio *et al.*, 2008). A switch to the biofilm mode of growth confers increased antibacterial resistance and creates a considerably more severe infection in the lungs of patients with cystic fibrosis (Rasch *et al.*, 2004). LSFE inhibited PAO1 biofilm formation in a concentration-dependent manner (Fig. 5c). There was a significant decrease in biofilm formation when bacteria were

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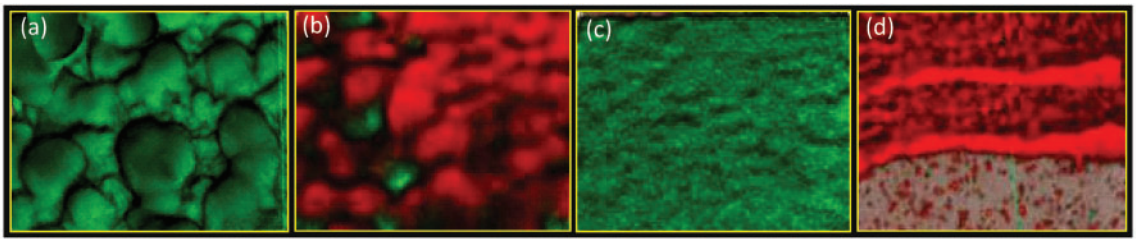

Fig. 5. Sensitivity of LSFE-treated *P. aeruginosa* PAO1 biofilms to tobramycin. SCLM photomicrographs of PAO1 biofilms grown in the absence or presence of 10 mg LSFE ml^{-1}. After 3 days, the biofilms were exposed to 350 μg tobramycin ml^{-1} for 24 h. Bacterial viability was assayed by staining using the LIVE/DEAD *Bac*Light Bacterial Viability kit: red areas are dead bacteria, and green areas are live bacteria. The biofilms were exposed to (a) no treatment (control), (b) 350 μg tobramycin ml^{-1}, (c) 10 mg LSFE ml^{-1}, (d) 10 mg LSFE ml^{-1} plus 350 μg tobramycin ml^{-1}.

grown in the presence of 2 mg ml^{-1} (7.0 % decrease), 4 mg ml^{-1} (16.3 % decrease), 6 mg ml^{-1} (30.2 % decrease), 8 mg ml^{-1} (54.6 % decrease) and 10 mg LSFE ml^{-1} (83.7 % decrease) when compared with the untreated control (Fig. 6).

DISCUSSION

Bacterial intercellular communication, or QS, controls the pathogenesis of many clinically important organisms. QS blockers are known to exist in marine algae and have the ability to attenuate bacterial pathogenicity. We hypothesized that medicinal plants traditionally used as medicines might also contain QS blockers. To test this hypothesis, nine medicinal plants were screened for anti-QS activity in *C. violaceum*. Of the plant extracts, LSFE showed significant QS inhibition. The virulence of *P. aeruginosa* strain PAO1 arises from its capacity to degrade host tissues with proteases and toxins, and to evade antibiotic attack by forming biofilms. Autoinducer production, biofilm formation and the virulence factors examined in this study are under QS control (Meyer *et al.*, 1996; Singh *et al.*, 2009a, b). Thus, LSFE was examined for its ability to interfere with QS-dependent production of PAO1 virulence factors (LasA protease, LasB elastase and pyoverdin) and gene expression (*las*/*rhl*). In addition, we examined the ability of LSFE to inhibit biofilm formation and AHL molecule synthesis.

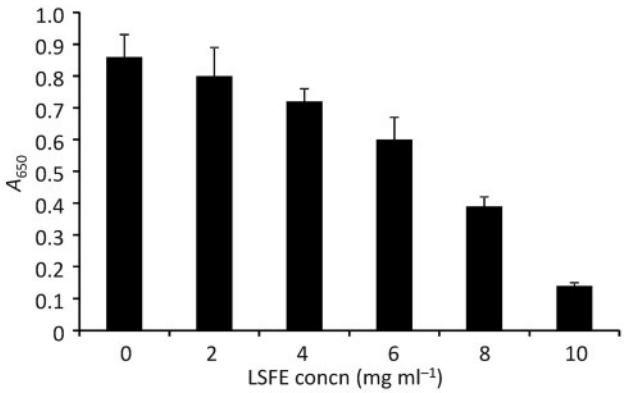

Fig. 6. Concentration-dependent inhibitory effect of LSFE on biofilm formation in *P. aeruginosa* PAO1. An overnight culture of *P. aeruginosa* PAO1 in AB minimal medium was diluted 1 : 100 in fresh medium and grown for another hour. After that, different concentrations of LSFE (2–10 mg ml^{-1}) were added and incubated for 48 h at 37 °C. The medium was removed, and 100 μl of a 1 % (w/v) aqueous solution of CV was added and incubated at room temperature for 20 min, after which the dye was removed and the wells were washed thoroughly. Plates were rinsed to remove planktonic cells, and surface-attached cells were quantified by solubilizing the dye in ethanol and measuring A_{650}.

LSFE inhibits cell–cell signalling

The production of violacein in *C. violaceum* 12472 is regulated by QS. *C. violaceum* uses LuxR/LasR receptors to interpret and communicate via QS signals (Hentzer *et al.*, 2002). LuxR functions as a transcription factor and induces the expression of several genes (Lyon & Muir, 2003). In the present study, LSFE demonstrated interference with violacein production in *C. violaceum* 12472 in a concentration-dependent manner (Fig. 1a, b). Moreover, the extract did not seem to affect the growth of *C. violaceum* CV026 and PAO1 (Fig. 2). Collectively, the data seem to suggest inhibition of autoinducer-mediated cell–cell signalling. Since LSFE might contain phytomolecules that are structurally very similar to natural furanones, it is likely that their mode of action is similar. The inhibitory potential of the extract was comparable with the reported activity of furanones from *D. pulchra* (Ren *et al.*, 2002). Since furanones are highly reactive molecules, it has been suggested that they can only serve as model compounds in research (Huber *et al.*, 2003).

LSFE affects biofilm tolerance to tobramycin

PAO1 is known to form biofilms on various surfaces and under different conditions (Hentzer & Givskov, 2003). Biofilm formation in PAO1 is suggested to be positively regulated by QS, specifically by AHL-mediated cell–cell signalling (Hentzer *et al.*, 2002; Rasmussen *et al.*, 2005). Since

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certain extracts from medicinal plants demonstrated inhibition of AHL-mediated cell–cell signalling, we further hypothesized that the inhibitory action of LSFE may also influence biofilm formation in PAO1. The observations from the biofilm assay supported this hypothesis. Indeed, the extract tested caused a decline in biofilm formation that was related to AHL-mediated cell–cell signalling. In contrast, classical antibiotics used for the treatment of *P. aeruginosa* infections, such as tobramycin and piperacillin, are required in 100- to 1000-fold higher concentrations to eradicate biofilm bacteria compared with their planktonic counterparts (Hentzer & Givskov, 2003). Furthermore, LSFE was found to inhibit biofilm formation by as much as 9.71–82.44 % at 2–10 mg ml^{-1}. In comparison, *D. pulchra*-derived furanone has been reported to inhibit biofilm formation by 55 % at 60 μg ml^{-1} (Ren *et al.*, 2002). Together, these results seem to suggest that LSFE suppresses biofilm formation in a growth-inhibitory fashion and plausibly in a QS-dependent manner. However, biofilm development and establishment are complex multi-factorial processes, which are governed by a combination of environmental factors and the genotype (Kjelleberg & Molin, 2002). Therefore, the possibility of LSFE acting via some other factors cannot be ruled out.

LSFE inhibits production of virulence factors

P. aeruginosa proteases LasA and LasB are believed to play a major role in pathogenesis via host tissue degradation (Kessler *et al.*, 1998). LSFE resulted in a significant decrease in the LasA and LasB activities of PAO1. This was in agreement with an earlier study (Adonizio *et al.*, 2008), in which the authors observed that medicinal plants from Florida showed inhibitory effects on LasA and LasB activity. It is likely that the extract downregulated LasA and LasB activity. In comparison, recent studies on garlic extract (at 2 %, w/v) show a 50 % decrease in LasB activity (Rasmussen *et al.*, 2005), whereas purified halogenated furanone from *D. pulchra* (10 μM) induces an approximately 90 % decrease (Hentzer *et al.*, 2002). LSFE caused a significant reduction of pyoverdin production. Mixed results with respect to pyoverdin production have been observed with furanones. A naturally occurring furanone from *D. pulchra* [(5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone] actually increases production of pyoverdin (Ren *et al.*, 2002), whereas furanone C-30, a synthetic derivative of a compound from *D. pulchra*, confers a 90 % reduction in pyoverdin levels (Hentzer *et al.*, 2002). Furthermore, Adonizio *et al.* (2008) observed that *Conocarpus erectus* induced a substantial decrease in pyoverdin activity (91 % decrease) when compared with the control. There was also no significant change in cell growth corresponding to that in pyoverdin production, leaving an anti-QS effect as the most plausible explanation.

LSFE attenuates virulence gene expression

QS inhibition occurs via a number of different mechanisms, the most well known being signal mimicry, which has been observed with garlic extract (Rasmussen *et al.*, 2005) and furanones (Manefield *et al.*, 2002). The QS system can be interfered with in a number of other ways, including acylase or lactonase activity (Dong *et al.*, 2001), signal binding, inhibition of genetic regulation systems, or interruption of downstream virulence and biofilm genes (Whitehead *et al.*, 2001). The current view of the *P. aeruginosa* QS hierarchy suggests that *las* controls *rhl*, with virulence proteins further downstream (Schuster & Greenberg, 2006). The virulence proteins LasA protease and LasB elastase are generally governed by the *lasI/R* system (Schuster & Greenberg, 2006); however, pyoverdin is believed to be under *rhlI/R* control (Brint & Ohman, 1995). Biofilm production is under partial QS control (Davies *et al.*, 1998). The redundant and autoregulatory nature of the QS system is quite convoluted (Venturi, 2006). This, when compounded with the complex phytochemistry of LSFE, inhibits precisely linking AHL production, QS gene expression and virulence factor production.

In summary, our work here shows that LSFE inhibits the expression of both autoinducer synthetases (*lasI* and *rhlI*) and their respective receptors (LasR and RhlR), which results in the inhibition of biofilm formation and the reduced production of virulence factors affected by the QS-signalling mechanism. LSFE significantly lowered the pathogenicity of *P. aeruginosa* PAO1 through interference with signalling molecule (C4-AHL and C12-AHL) production. This would strongly suggest an anti-QS rather than an antibacterial effect. Research is currently under way to purify active biomolecules from LSFE.

Extracts from vegetables, fruits, herbs, and terrestrial and medicinal plants have long been a source of medicines, and as such, there have been many ethnobotanically directed searches for agents to treat infections. However, most studies focus solely on bactericidal effects. Since LSFE in this study shows no cidal activity, QS inhibition remains a potential mode of action.

To conclude, the effect of LSFE on *P. aeruginosa* PAO1 is quite complex, and perhaps extends beyond the domain of the QS hypothesis. However, the reduction of QS gene expression and signalling molecule production, and the end-effect on virulence factor production, provide some insight into how it could be used in future to combat *P. aeruginosa* and other bacterial infections.

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