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Original Article

Attenuation of quorum-sensing-dependent virulence factors and biofilm formation by medicinal plants against antibiotic resistant *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa use small signaling molecules such as acyl homoserine lactones (AHLs), which play an important role in release virulence factors and toxin for further establishment of host infection. Thus, involving with the QS system would provide alternative ways of preventing the pathogenicity. In the present study, totally six medicinal plants (*Terminalia bellerica*, *Celastrus paniculatus*, *Kingiodendron pinnatum*, *Schleichera oleosa*, *Melastoma malabathricum*, *Garcinia gummi-gutta*) were screened for anti-QS activity using biomonitor strain of *Chromobacterium violaceum* CV12472. The primary screening of antimicrobial activity of all the plant extracts have inhibited the growth of tested bacterial species. Of these at the sub-minimum inhibitory concentration the methanol extract of *T. bellerica* (0.0625–0.5 mg/ml) has significantly inhibited violacein production (20.07–66.22%) in *C. violaceum* (CV12472). Consequently, the extract of *T. bellerica* has reduced the production of pyocyanin, exopolysaccharide and biofilm formation in *P. aeruginosa* strains. Fluorescence and scanning electron microscopy analysis confirmed the reduction of biofilm formation in *P. aeruginosa* strains when treated with *T. bellerica*. GC–MS analysis showed the active compounds inhibited the production of virulence factors of *P. aeruginosa*. The results suggest the possible use of this *T. bellerica* as an anti-QS and anti-biofilm agent to control *Pseudomonas* infection. Interference of QS provides an important means for the inhibition of bacterial virulence and thus aids in treatment strategies.

1. Introduction

Pseudomonas aeruginosa is one of the most important opportunistic human pathogens. It is capable of infecting patients suffering from cystic fibrosis, chronic obstructive pulmonary diseases, immunocompromised patients and AIDS patients.[1] In addition, *P. aeruginosa* is able to cause severe burn wound infections, urinary tract infection, bloodstream infections, nosocomial infections[2] and meningitis.[3] In hospital environment, this organism can easily infect patients who are immunocompromised and it rarely infects healthy people and hospital coworkers.[4] *P. aeruginosa* releases a variety of virulence factors, which are mainly involved in the progression of disease through enforcing the adhesion, modifying the immune response, evading from phagocytosis and destroying the host tissue.[5] The ability of *P. aeruginosa* to communicate and coordinate with other cells in the population using small signaling molecules such as acyl homoserine lactones (AHLs) is called quorum sensing (QS).[6] QS is an intercellular communication system, which can efficiently control the gene expression in a cell-density dependent manner.[6] Once the threshold concentration is reached, the organisms release virulence factors for further establishment of host infection.[6] *P. aeruginosa* possesses two QS system namely *las* and *rhl* which utilize *N*-acyl homoserine lactones (AHLs) as signaling molecules.[7] These signaling molecules, synthesized by bacterial cells, diffuse out the cells and it binds to transcriptional regulators. There are two QS systems in *P. aeruginosa* (LasR/I and RhlI/R) which have been identified to regulate the expression of virulence factors. The LasI is essential for the production of the AHL

Abbreviations: AHLs, acyl homoserine lactones; QS, quorum sensing; EPS, exopolymeric substance; 3O-C12-HSL, *N*-(3-oxododecanoyl)-L-homoserine lactone; C4-HSL, *N*-butyryl-homoserine lactone.

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molecule, *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) and lasR is the transcriptional regulator.[8] The second QS system comprises RhlI and RhlR proteins. The RhlI synthase mediates the synthesis of the signal molecule, *N*-butyryl-homoserine lactone (C4-HSL) and RhlR is the transcriptional regulator.[9] The *las* system controls virulence factors such as LasB elastase, LasA protease, alkaline protease, exotoxin A and biofilm formation. Similarly, *rhl* system controls the production of pyocyanin pigment, rhamnolipids, LasB elastase and hydrogen cyanide.[7,10,11] These virulence factors are involved in cellular toxicity and acute infection.[12] Treatment strategies of *Pseudomonas* infections are greatly challenged by the emergence of drug resistant strains and also on account of biofilm formation by this bacterium.

Biofilms are a conglomerate of microbes enclosed in a self-secreted exopolymeric substance (EPS) that environs the bacterial population. It is mainly composed of polysaccharides, proteins, DNAs, lipids and other macromolecules.[13] The biofilm shields the bacterial population from clearance by the immune system and contributes to the pathogenesis of chronic infections such as cystic fibrosis and other pulmonary illnesses.[14] Biofilm formation and maintenance of its architecture are QS-dependent phenomena.[15] EPS is important for the development of biofilm matrix and provides an effective barrier that restricts the entry of antibiotics and antimicrobial peptides.[16] QS-controlled virulence factors and biofilm formation are vital for the development of acute and chronic infections, particularly in Gram-negative bacteria such as *P. aeruginosa*. These factors result in bacterial persistence and reduced sensitivity to antimicrobials.[16] Hence, there is an increasing need to search potent anti-QS and antibiofilm compounds from the natural sources. Medicinal plants and plants derived essential oils, that interfere the regulation of QS system and biofilm formation could be powerful allies for conventional antibiotics in the struggle against *P. aeruginosa* infections.[17]

From time immemorial, plants have been used as traditional medicines and are being considered as safe. *Terminalia bellerica* belongs to the family of 'Combretaceae' commonly known as bastard myrobalan. *T. bellerica* is a traditional folk medicine which has been used to treat various ailments.[18] There are various reports indicative of a wide range of pharmacological activities of *T. bellerica* such as antimicrobial, antidiarrhoeal, antidiabetics, antioxidant, antianalgesic, anti-inflammatory and antifibrotic.[18,19] The main objective of this study was to evaluate the anti-QS and antibiofilm activity of *T. bellerica* against the *P. aeruginosa*. To our best knowledge, this is the first study towards analyzing the anti-QS and antibiofilm potential of *T. bellerica* against *P. aeruginosa*.

2. Materials and methods

2.1. Collection of plants and methanolic extraction of medicinal plants leaves

Terminalia bellerica leaves were collected from Kuvempu Nagar, Mysore, Karnataka (between 12° 17′ 2.6376″ N Latitude 76° 37′ 43.7016″ E Longitude). *Celastrus paniculatus* leaves were collected from Sakleshapura hill, Sakleshapura, Hassan district, Karnataka (between 12° 17′ 2.6376″ N Latitude 76° 37′ 43.7016″ E Longitude). *Kingiodendron pinnatum*, *Schleichera oleosa*, *Garcinia gummi-gutta* and *Melastoma malabathricum* leaves were collected from Pilikula Nisargadhama, Mangalore, Karnataka (between 12° 17′ 2.6376″ N Latitude 76° 37′ 43.7016″ E Longitude) Plants were identified by taxonomically and the plants herbarium were maintained in the Department of Studies in Microbiology, University of Mysore, Mysore (Table 1). The plant leaves were collected in sterile polyethylene bags and brought to the laboratory. The leaves were washed thrice with sterile distilled water and the leaves were dried under shade. The dried leaves were grinded in a coarse powder by the mechanical grinder. Ten gram of coarse each powder was added with 100 ml of methanol and the extraction was kept for 48 h with agitation at 150 rpm. After 48 h the extract was filtered with a Whatman no. 1 filter. The filtered extract was dried using rotary flash evaporator in a hot condition (50 °C). The collected crude extracts were further dried with vacuum concentrate and stored at 4 °C for further analysis.

2.2. Bacterial strains and culture conditions

The clinical isolate of *P. aeruginosa* CI-01 (GenBank accession no. KU870518) was obtained from a meningitis patient admitted to Mysore Medical College and Hospital, Mysore, Karnataka. *C. violaceum* (CV12472) was routinely cultured aerobically in Luria-Bertani broth (LB) and the cultures were incubated at 30 °C for 24 h. *P. aeruginosa* PAO1 and *P. aeruginosa* CI-01 were cultured in Nutrient Broth (NB). The cultures were incubated at 37 °C for 24 h in a rotary shaker (100 rpm).

2.3. 16S rRNA gene sequencing

The genomic DNA was isolated using the phenol chloroform extraction method.[20] The pathogen was identified by 16S rRNA gene sequence analysis using the universal primers, 27F and 1492R as described previously.[21]

2.4. Antibiotic sensitivity test

A clinical isolate of *P. aeruginosa* CI-01 and *P. aeruginosa* PAO1 were sub-cultured in LB broth and cultures were incubated at 37 °C in a shaker incubator. The optical density (OD) was adjusted to 0.5 McFarland (1×10^8 CFU/ml) standard. The clinical isolate *P. aeruginosa* strains were swabbed on the Muller Hinton Agar (MHA) (Himedia, India) plates. The antibiotics discs (Himedia, India) were gently placed on the center of the agar plates. The plates were incubated at 37 °C for 24 h. After incubation, the zone of inhibition was measured using standard scale (Himedia, India) and the results were analyzed by the standard deviation. The test was performed in triplicates and the results were interpreted according

Table 1
Medicinal properties of selected plants tested for anti-QS activity against *C. violaceum* (CV12472).

Accession no	Species	Family	Parts	Medicinal value
MGMB-002	*Terminalia bellerica*	Combretaceae	Bark, fruits, seed and whole plant	Antimicrobial, antidiarrhoeal, antidiabetics, antioxidant, antianalgesic, anti-inflammatory and antifibrotic
MGMB-003	*Celastrus paniculatus*	Celastraceae	Bark, leaves, seed oil and root	Antibacterial, antiarthritic, anorexia, arthritis, asthma and cough
MGMB-004	*Kingiodendron pinnatum*	Fabaceae	Leaves	Antimicrobial and antioxidant
MGMB-005	*Schleichera oleosa*	Sapindaceae	Bark an oil	Antibacterial, analgesic, malaria, ache, lung cancer and burns
MGMB-006	*Melastoma malabathricum*	Melastomataceae	Leaves, roots, flowers and shoots	Ache, digestive disorder and astringent
MGMB-007	*Garcinia gummi-gutta*	Clusiaceae	Fruits and leaves	Antibacterial, type 2 diabetes mellitus, antiulcer and diuretic activity

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