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Inhibition of N-Acyl Homoserine Lactone Mediated Quorum Sensing in *Pseudomonas aeruginosa* by *Phyllanthus emblica* and *Quercus infectoria*

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Abstract:
Quorum sensing (QS) is a process that enables bacteria to communicate using secretd signaling molecules called autoinducer. *Pseudomonas aeruginosa* an opportunistic pathogen in immune compromised patients is under the regulation of two different acyl homoserine lactones (AHLs) as the quorum signal molecules: N-3-oxododecanoyl homoserine lactone ($3OC_{12}$) and N- butanoyl homoserine lactone (C_4). Technology is required to block the very basic mechanism of bacterial communication that appear to control toxin production, adherence, motility, proteolysis and other virulence factors leading to pathogenecity. Natural products especially plants used in traditional medicines in different organisms are a promising source for deriving molecules that can potentially inhibit QS. Our objective was to screen for the QS inhibitory properties of 2 high quality herbs such as *Phyllanthus emblica* and *Quercus infectoria.* Effect of these extracts on *P. aeruginosa* virulence factors such as pyocyanin, total protease, elastase and biofilm formation were quantified by standard protocols. The result indicate that between and among the extracts of these two plants the aqueous extract of *P. emblica* showed greater reduced activity of pyocyanin synthesis, proteolytic enzymes elastolytic enzymes and biofilm formation.
Keywords: *Phyllanthus emblica, Quercus infectoria, Pseudomonas aeruginosa,* Quorum sensing and Acylated homoserine lactones.

1. Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen, which is not normally a part of the normal human microbiota, but it may cause acute infections in hospitalized patients and immune- comprised individuals for example those burn victims. It also causes more chronic types of infection, for hospital-acquired pneumonia in patients receiving artificial ventilation and keratitis in users of extended-wear soft contact lens (Christian and Barbara, 1998).

The capacity of *P. aeruginosa* to produce such a diverse often overwhelming infection is due to an arsenal of virulence factors include exoproteases, siderophores, exotoxins and lipases. A mass of cells is required to produce sufficient quantities of these factors to influence the surrounding environment (Matthew ana Gail, 2003).

The concentration of these molecules are in direct proportion to the load or density of the bacteria; in other words there is a direct relationship between the concentrations of chemical signaling molecules and that of the bacteria present in a given environment. This is termed as Quorum Sensing (QS).

It allows bacteria to communicate with each other by synchronizing/ regulating the expression of several genes that are necessary for bacteria to behave in unison as a group. These bacterial activities are not useful when expressed by just one bacterium. QS languages exist which enables the bacteria to communicate within and between the species. But they can become beneficial to bacteria when expressed in group-based manner. For example: QS is responsible for bioluminescence in marine bacteria such as *Vibrio harveyi* and *V. fischeri*, biofilm formation by both *P. aeruginosa* and *Burkholderia capacia* in the lungs of cystic fibrosis patients (Daniel *et*

al., 2002) and formation of virulence determinants by *Staphylococcus aureus* (Anjali *et al*., 2008).

The QS systems of *P.aeruginosa* attract the particular attention because this species is a human pathogen. This is a well-studied model bacterium whose QS system is a core mechanism to develop the biofilm. It produces two different acyl-homoserine lactones (AHLs) as the QS signal molecules: N-3-oxododecanoyl homoserine ($3OC_{12}$) and N-butanoyl lactone (C_4) (Fuqua, 2006).

These bacteria also have two systems of the lux I – lux r type: the las system (Las R- Las I, $3OC_{12}$ HSL acting as autoinducer) controls synthesis of various virulence factors (proteases, Las A and Las B, exotoxin A) and rhl system (Rhl R- Rhl I C4 HSL acting as autoinducer) controls rhamno lipids synthesis and biofilm production.

The increasing emergens of bacterial strains resistant to antibacterial drugs is a major challenge. *P.aeruginosa* is one of the most problematic human pathogens, showing intrinsic resistence to many structurally unrelated antibiotics. Resistance mechanisms include lower outer membrane permeability or multi drug efflux pumps or production of antibiotic modifying enzymes. Previous exposure to antibiotics often leads to multi drug resistant strains and the protective effects of alginate (Givskov *et al*., 1996).Therefore technology is required to block very basic mechanisms of bacterial communication that appear to control toxin production, adherence, and motility to pathogenicity (Morten and Givskov, 2003).

The blockage of the QS systems not only attenuates *P. aeruginosa* but also renders biofilm highly susceptible to treatment with conventional antibiotics. Filamentous fungi produce a battery of secondary metabolites, some of which are in clinically used as antimicrobial drugs. During the screening of 100 extracts from *Penicillum* sp., 33 where found to produce QS inhibitory (QSI) compounds (Cowman, 1999).

Control of infections by inhibiting microbial growth has been a primary approach of microbial chemotherapy. An emerging problem associated with continual indiscriminant use of this therapeutic strategy is the selection of resistant bacteria with higherlevel tolerance against these broad-spectrum antibiotics. Development of novel antibiotics that interfere with metabolism coupled with continued discriminant use of antibiotics will only lead to evolution of new resistance mechanism and pathways of bacteria.

Natural products especially plants used in traditional medicines in different cultures are a promising source for deriving molecules that can potentially inhibit QS. These plants can offer a large an attractive repertoire for the discovery of QS inhibitors (cowman, 1999). Recently various higher plants were also known to secrete AHL signal mimic substances that are capable of interfering with bacterial QS (Daniel *et al*., 2002).

Although the chemical nature of the active compounds from plants and their effect on bacterial QS could be one of the intrinsic plants defense mechanism against pathogenic invades. Using AHL analogues as the antagonists to interfere with QS dependent bacteria might use as a strategy in controlling in bacterial pathogens (Harling and Cui, 2005). There are of particular importance as these phytochemicals have been used for thousands of years for the treatment and management of diseases (Rasmuseen and Givskov, 2006). The secretion of enzymes that destroy the autoinducers and the production of autoinducer antagonists, are used by competitor bacteria and susceptible eukaryotic host to render QS bacteria mute and deaf respectively. Analogues synthetic strategies are now being explored for the development of novel antimicrobial therapies (Stephan and Bonnie 2001).

Our objective was to prove that *Phyllanthus emblica* and *Quercus infectoria* which plays a major role in Siddha, Ayurvedic and Unnani system of medicine and to understand their mechanism of action and

investigate their effect on the expression of QS regulated virulence factors in *P. aerusinosa.*

Materials and Methods

Plant Material and Extract Preparation:

The galls of *Quercus infectoria* and fruit of *Phyllanthus emblica* used in this study were obtained from the local market and were identified based on its physical characteristics.

Air dried and powered plant materials approximately from 100 to 300g were submitted to sequential maceration with 500ml of ethanol ant water at room temperature. After each step the extracts were filtered and the solvents were removed under vacuum at 30^0C until dry ethanol, aqueous extracts were obtained.

Strains and Media:

Pseudomonas aeruginosa MTCC 2453 and *Chromobacterium violaceum* 2656 were collected from Microbial Type Culture Collection Center. IMTECH, Chandiragh. The purchased culture was propagated in the nutrient broth and incubated at 37^OC for 24 hours of incubation. The grown culture was subcultured at regular intervals of 30 days and stored at 4^OC for future use. The Luria Bertani(LB) medium was used throughout the study.

Culture Condition:

For all assays except those for biofilm formation the culture condition were as follows. Overnight cultures of *Pseudomonas aeruginosa* were grown in LB medium at 37^0C with shaking. The cultures were then diluted 100- fold in fresh LB medium and allowed to grow to an optical density at 600nm (OD_{600}) 1.7(early stationary phase). At this point, the culture was divided into 10ml aliquots and additional 1ml of fresh medium containing plant extract or media control was added to a final concentration of 1mg/1ml extract. Cultures were recovered at late stationary phase (approximately 72h after addition). The cells were separated from the growth medium by centrifugation at 10,000xg for 10min (Turbo et al., 2003).

AHL Bioassay:

The extracted plant material was placed directly on to LB plates spread with *Chromobacterium violaceum* supplemented with AHL. Plates were incubated at 30^0C and QS

inhibition was detected by a ring of colorless. Purified halogenated furanone (100µg) was used as a positive control for QS inhibition and ethanol (20µl) as a negative control. The ethanol was allowed to evaporate from the control and sample discs before testing to eliminate toxicity.

Total Proteolytic activity:

The protein concentration of the cell supernantants were determined by Lowry method. The total proteolytic activity of the culture supernatants was estimated according to a method described earlier. Briefly to 100ul of culture supernants 900µl of 0.5% azocasein prepared in 50mM tris buffer containing 2mMCaCl2. Samples were incubated at 37^0C for 30 min. after this 15%TCA (100ml) was added to stop the reaction and centrifuged (8000 rpm, 40C, 10min). The absorbance of the supernatants from both controls and treatments was measured at 440nm (Oliver *et al*., 1951).

Elastolytic Activity:

The elastolytic activity of the LB medium culture supernatants was determined by using elastin Congo red. A 100ul aliquot was added to 900ul of ECR buffer (100mM Tris, 1mM CaCl2, pH 7.5) containing 20mg ECR. This mixture was then incubated with shaking for 3h at 37^0C. Insoluble ECR was removed by centrifugation and the absorption of the supernatants was measured at 495nm. Cell-free LB medium alone and LB medium with plant extracts were used as negative controls. Activity was expressed as the change in the OD_{495} per µg protein (Kalai mathee *et al*., 2008).

Photometric Pyocyanin Assay:

1.5ml of broth culture was centrifuged at 14000rpm for 5min, 1ml of supernatants was mixed with 1.5ml of chloroform and left to separate. The blue organic layer was mixed with 1ml of 0.2MHcl, whereby the blue mixture turns

red and red form of pyocyanin moves into the aqueous layer. This was re-centrifuged prior to measuring the absorbance at 520nm (Duncan sharp *et al*., 2009).

Polyvinyl Chloride Biofilm Formation Assay:

The effect of plant extracts on the attachment phase of biofilm formation was measured by using the polyvinyl chloride biofilm formation assay. Briefly overnight cultures of *Pseudomonas aeruginosa* were re-suspended in LB medium in the presence and the absence of plant extracts. After 10h incubation at 30^0C, the biofilms in the microtitre plates were visualized by staining with crystal violet solution. The plates were rinsed to remove planktonic cells and the surface attached cells were then quantified by solublizing the dye in ethanol and measuring the absorbance at OD_{650} (Kalai mathee *et al.*, 2008).

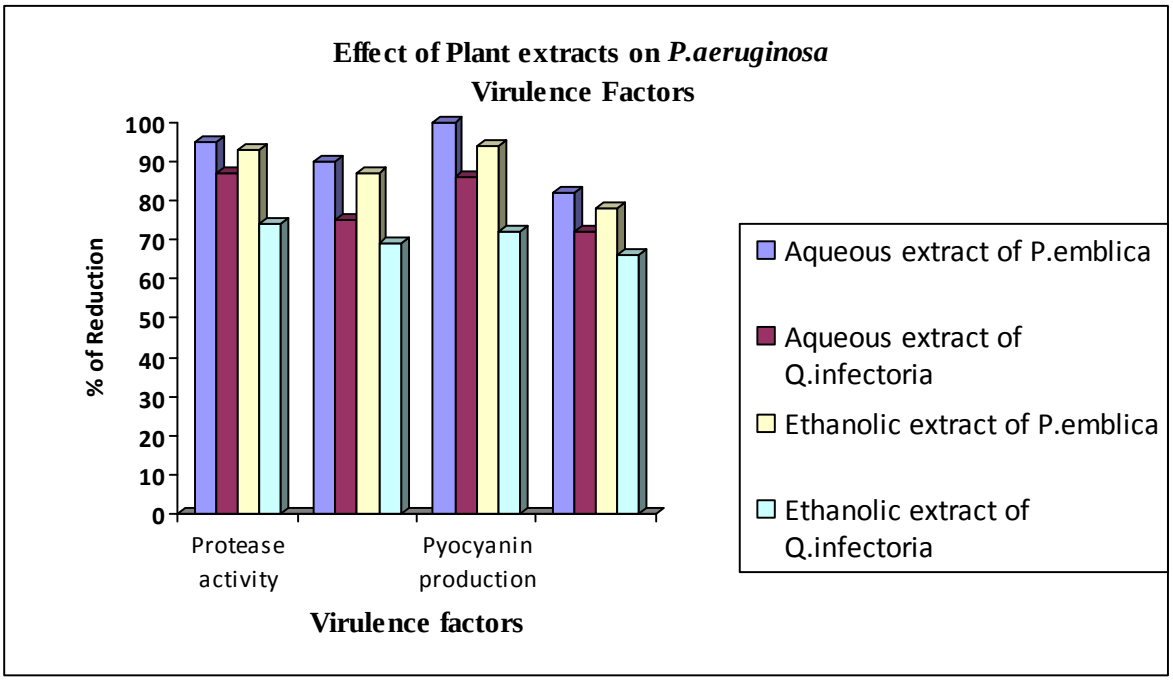
Result and Discussion

AHL Bioassay:

Loss of purple pigment in *Chromobacterium violaceum* is indicative of QS inhibition by the plant products introduced. This anti-QS activity of the two species as aqueous and ethanol extracts were screened using the *Chromobacterium violaceum* bioassay.Control discs containing halogenated furanone, gentamycin and ethanol was included. As expected a zone of growth inhibition was observed with gentamycin, a zone of QS inhibition (halo) was seen with a furanone and no inhibition was with ethanol.Strong activity was observed in both the aqueous and ethanol extract of *P. emblica* and aqueous extract of *Q. infectoria*. Low activity was detected with ethanol extract of *Q. infectoria* using this strain.

Total Proteolytic Activity:

We measured the ability of the extracts to decrease the total proteolytic activity in *P. aeruginosa*. The highest activity was observed by aqueous extract of *P. emblica* which decreases the proteolytic activity in cultures by 95%. The second highest activity was observed by ethanolic extrtact of *P. emblica. Q. infectoria* was effective in decreasing the total proteolytic activity when compared to the control. Four extracts from two medicinal plants *P. emblica*, and *Q. infectoria* was examined for their anti QS activities against *P. aeruginosa*. The virulence of *P. aeruginosa* is owed to its capacity to degrade host tissue with tissue with proteases and toxins (Kalai *et al*., 2008).



Las B Elastase Activity:

Las B elastase is a zinc mettaloprotease capable of destroying or inactivating a wide range of biological tissues and immunological agents. There was a significant decrease in Las B activity compared to that of the control when strain *P. aeruginosa* was grown in the presence of aqueous extract of *Q. infectoria* (75%) and ethanolic extract (69%). The growth of organism in the presence of aqueous and ethanolic extract of *P. emblica* showed an increased activity of 90% and 87% respectively.

Pyocyanin Assay:

Pyocyanin (1-hyroxy-5-methylphenazine) is an antibiotic pigment secreted by*P. aeruginosa* which allows the bacteria to preferentially grown in an environment and is regulated by genes controlled by QS. Pyocyanin is a secondary metabolite and has antimicrobial activity against several species of bacteria, fungi and protozoa. Due to its ability to interfere with the redox metabolism in the target organism. We investigated the effect of our plant extracts on the ability of the bacteria to synthesize and secrete this virulent pigment. Our results indicate that all the extracts significantly decreased the formation of the pigment in the bacteria.

Aqueous extract of *P. emblica* had the maximum activity and reduced the formation of pyocyanin by over 100%. This was followed by the ethanolic extract of *P. emblica* and aqueous extract of *Q. infectoria*. The lowest activity among the samples tested was observed in ethanolic extract of *Q. infectoria* in the bacteria by 72% compared to control.

Biofilm Formation:

P. aeruginosa has the ability to form biofilm a partially QS controlled phenomenon in which cells are organized into layers and enmeshed in a matrix of mucoid polysaccharides. 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. There was a significant decrease in biofilm formation compared to that of the control when strain *P. aeruginosa* was grown in the presence of aqueous extract of *P. emblica* (82%), ethanolic extract of *P. emblica* 78%, aqueous extract of *Q. infectoria* 72% and ethanolic extract of *Q. infectoria* caused 66% decrease in biofilm formation; however this result was marginally significant. Biofilm formation *Q. Virginiana* exhibited an effect on biofilm production. Disruption of the Qs system with furanones has also been shown to inhibit biofilm growth. Previous work with garlic and *D. pulchra* furanones showed a qualitative change in biofilm morphology and a reduction in thickness (Morten *et a*l., 2002).

Microbiological research is now focused on the development of molecules that are structurally related to autoinducers. Such molecules have potential use as antimicrobial drugs aimed at bacteria that are quorum sensing to control virulence. Similarly, the biosynthetic enzymes involved in autoinducer production and the autoinducer detection apparatuses are viewed as potential targets for novel antimicrobial drug design.

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