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RESEARCH ARTICLE

Inhibition of Antibiofilm Mediated Virulence Factors in *Pseudomonas aeruginosa* by *Andrographis Paniculata*

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ABSTRACT:
The focal intent of the study is about Quorum sensing (QS) which is well known for biofilm production, bacterial virulence and antibiotic resistance. Inhibition of quorum sensing in bacteria can reduce the risk of pathogenicity in all bacterial infection. In the present study the methanolic leaf extract of *Andrographis paniculata* has showed its anti quorum sensing activity against acyl homoserine lactone -dependent showed a concentration dependent (10-100 µg/ml) reduction in biofilm production in *Pseudomonas aeruginosa* and the inhibition level was upto 10-80%. Microtiter plate (MTP)assay and Light microscopic analysis has further confirmed the antibiofilm activity in *Pseudomonas aeruginosa* when treated with methanolic leaf extract of *Andrographis paniculata*. In addition the methanolic ectract of *Andrographis paniculata* itself showed no antibacterial activity in *Pseudomonas aeruginosa*.

KEYWORDS: Antibiofilm, Quorum sensing inhibition, Acyl homoserine lactone, *Pseudomonas aeruginosa*, Microtiter plate, *Andrographis paniculata*.

1. INTRODUCTION:

Pseudomonas aeruginosa is a gram negative, aerobic bacillus that can survive easily in minimal nutrient condition, which present abundantly in the environment. *Pseudomonas aeruginosa* can cause infections in plants, animals, as well as in humans. In humans, it acts as an opportunistic pathogen, commonly associated with immunocompromised patients, burn victims, ICU patients, and patients on longer antibiotic treatment. *P. aeruginosa* has been identified to have a paramount relevance in the development of cystic fibrosis (CF), another severe disease that results in a chronic deterioration of pulmonary function.

The decreased mucus clearance in the lungs of CF patients provides a suitable niche for the growth of many bacteria, and further the CF specific alteration in the receptor for the adhesins on pili of *P. aeruginosa*, facilitates *Pseudomonas* infection in CF lung[5]. It has been one of the most common nosocomial pathogens, prominently causing ventilator associated pneumonia, surgical site infections or wound infections, catheter associated urinary tract infections and bloodstream infections[10]. Almost all the clinical strains of *P. aeruginosa* have evolved as multidrug resistant, and have been commonly associated with low susceptibility to the fluoroquinolones, cephalosporins and carbapenems.

Then, colonization may proceed to acute infection or chronic infection, by the aid of its extracellular virulence factors. Chronic infection is characterized by a low production of virulence factors, biofilm formation and tissue damage, mainly caused by chronic inflammation. Acute infections involve extensive tissue damage, bloodstream invasion and dissemination, achieved by the

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production of several extracellular virulence factors such as elastases (LasA and LasB), exotoxin A, alkaline protease, exoenzyme S, rhamnolipids, phospholipase, pyocyanin, etc[3]. In the current scenario, very few treatment options are available for the management of Pseudomonas infections, and hence, there is an urgent need to develop new drugs with stronger efficacy and lower risks of resistance. Besides the development of new antibiotics or antibacterial drugs, medical research has been targeted towards the discovery of QQ compounds as anti-virulence drugs to fight the infections caused by *P. aeruginosa* [2].

Andrographis paniculata was considered to be a rich depository of bioactive compounds with antibacterial, antifungal, antiviral, antifouling, antibiofilm, immunosuppressant and cytotoxic activities. *Andrographis paniculata* are known to produce powerful anti-biofilm and anti-inflammatory compounds[3]. Similarly, the methanolic extract of *Andrographis paniculata* have shown a strong quorum sensing inhibitory (QSI) activity and acted as a good antagonist against the QS systems of *S. marcescens*[8]. Although, they are known for their bioactive potential, studies on its QSI properties remain scanty. Hence, the present investigation is aimed to study the effect of QSI activity of *Andrographis paniculata* extracts against the QS systems of *Pseudomonas aeruginosa* [11].

2. MATERIALS AND METHODS:

2.1Preparation of *Andrographis paniculata* leaf Extracts:

Andrographis paniculata leaves used in this present study were collected from well grown trees in Tamilnadu Agriculture University, Katuthotam, Thanjavur district, Tamilnadu[4]. The leaves were washed with tap water and then with distilled water. The washed leaves were shade dried and powered using blender. 5gm of powered sample were soaked in 50 ml of methanol for overnight. The methanolic phase was collected and dried at 55°C at hot air oven. The residues were collected and redissolved with deionized water. Finally stored at -20°C for further use.

2.2 Bacterial Strains and Their Culture Conditions:

Pseudomonas aeruginosa (*MTCC 8289*) used in this study was bought from the Microbial Type Culture Collection and Gene Bank, The bacterial strain was allowed to grow aerobically in Luria– Bertani slants. From the slants, sub culture were done and cultures were maintained in (LB) broth (Hi-Media, India) at an optimum temperature (30° C) and intensity was checked using UV spectrometer at OD 600nm before using for further analysis.

2.3 Antibacterial assay:

Antibacterial activity of the *Andrographis paniculata* leaf extracts with QSI potential was performed in Muller–Hinton agar (MHA) (Hi Media, India) by the method followed by the Clinical and Laboratory Standards Institute (2006). The 100 µl of test bacterial suspensions which were expected to have cell density equivalent to 0.5 McFarland standards (approximately 1×108 CFU/ml) were uniformly spread over the surface of the MHA plate. Then, the sterile paper disks (Hi Media, India) with a diameter of 10 mm loaded with various concentrations (50–100 µg/ml) of *Andrographis paniculata* extracts were placed over the plates and incubated at 30 °C for 24 h and observed for growth inhibition zone[5].

2.4 Growth curve analysis:

One percentage of overnight culture of bacterial pathogens (0.4 OD at 600 nm) were inoculated in 250 ml Erlenmeyer flask containing 100 ml of LB broth supplemented with various concentrations (10-100µg/mg) of *Andrographis paniculata* extract. The flasks were incubated at 37°C under 180 rpm in a rotatory shaker. The cell density was measured in UV–visible spectrophotometer at every one hour interval[4].

2.5 Biofilm formation in 24-well Micro Titre Plate:

The effect of *Andrographis paniculata* extract on the biofilm formation on *Pseudomonas aeruginosa* was determined by quantifying the biofilm biomass through Micro Titer Plate assay[9]. Briefly, 1% of overnight cultures with OD adjusted to 0.4 at 600 nm [6]. The test pathogen were added into 1 ml of fresh LB medium and cultivated in the presence and absence of *Andrographis paniculata* extract (10-100 mg/ml) without agitation for 16 h at 30 °C. After 16 h incubation, the planktonic cells in MTPs were removed by rinsing the wells. The wells were rinsed twice with sterile distilled water. The surface-adhered cells in the MTP wells were stained with 250 µl of 0.2% crystal violet (CV) solution (Hi Media, India). The solutions were left in MTP wells for 10 mins. Then the excess CV solution was removed. So, CV in the stained cells was solubilized with 1 ml of 95% ethanol. The biofilm biomass was quantified by measuring the intensity of CV solution. The intensity was measured at OD 650 nm using UV–visible spectrophotometer [7].

2.6 Biofilm Prevention Assay:

Biofilm prevention assay was done by adding 10 µl of over night culture of *Pseudomonas aeruginosa* in above mentioned cell density to 1 ml of LB broth in 24 well MTP containing glass slides (1×1 cm) and supplemented with and without *Andrographis paniculata* methanolic leaf extracts (10-100 µg/ml). Culture set up was incubated without agitation at 30°C for 18 h[9]. After the

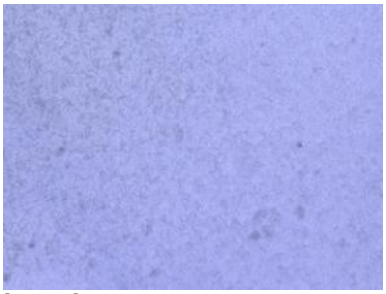
incubation, planktonic cells and media were discarded. The adherent cells which sticked in glass slides were gently rinsed twice with deionized water (Millipore–Milli-Q)[1].

2.7 Light microscopic analysis:

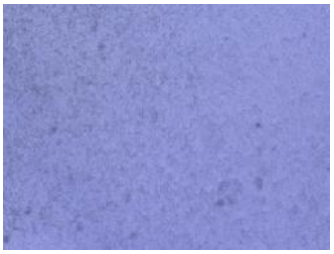
One percentage of overnight *Pseudomonas aeruginosa* cultures (0.4 OD at 600 nm) were added into 1 ml of fresh LB medium which containing cover glass of 1 cm^2 along with and without *Andrographis paniculata* methanolic leaf extracts (10-100 mg/ml). After 16 h of incubation, the cover glasses were rinsed thrice by using distilled water to remove the planktonic cells and biofilms. Then the cover glasses were stained with 0.2% CV solution. Stained cover glasses were placed on slides. The biofilm were pointed up and visible biofilms were visualized by light microscope at magnifications of 40X (Olympus CK ×41 Jenoptik Germany, ProRes C5)[9].

3. RESULTS AND DISCUSSION:

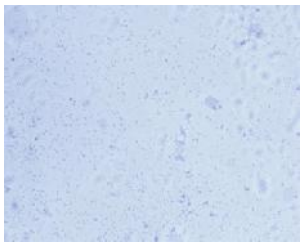
Biofilms are a highly dense matrix-encapsulated population which was attached to the surfaces[9]. The biofilm formation in *Pseudomonas aeruginosa* is a major virulence factor which is controlled by methanolic leaf extract of *Andrographis paniculata*. Biofilm has the ability to resist host immune response. It also resists conventional antibiotics. So, the control measures are required to prevent the biofilm formation in bacterial cells[9]. In the present study, biofilm images revealed that the *Andrographis paniculata* extracts effectively disturb the biofilm formation as shown in light microscopic analysis. In this study *Pseudomonas aeruginosa* was used as target pathogenic model to know anti-biofilm activity of *Andrographis paniculata*. The influence of methanol extract of *Andrographis paniculata* was assessed for its ability to inhibit biofilm formation in *Pseudomonas aeruginosa*. The minimum inhibition of biofilm was 10µg/ml and maximum inhibition of biofilm was 100µg/ml. The order to analyze the antibiofilm efficiency of *Andrographis paniculata* extract in inhibiting biofilm formation, *Pseudomonas aeruginosa* cells were allowed to grow in MTP having glass slide in presence and absence of *Andrographis paniculata* extract and the results were identified and visualized under a light microscope (Fig.1 & 2).



A. Untreated control

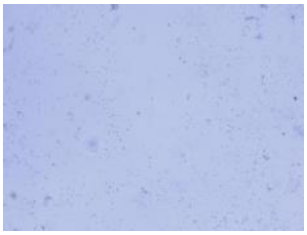


B. Treated with 10µg of extract

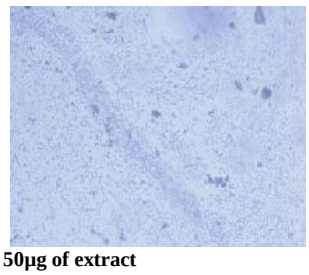


C. Treated with 15µg of extract

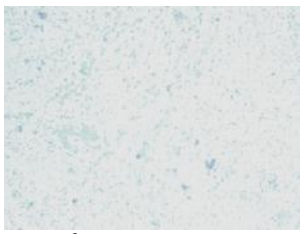
D. Treated with 20µg of extract



E. Treated with 25µg of extract



F. Treated with 50µg of extract



G. Treated with 100µg of extract

Figure 1: Light microscopic view of *Pseudomonas aeruginosa* treated with *Andrographis paniculata.*

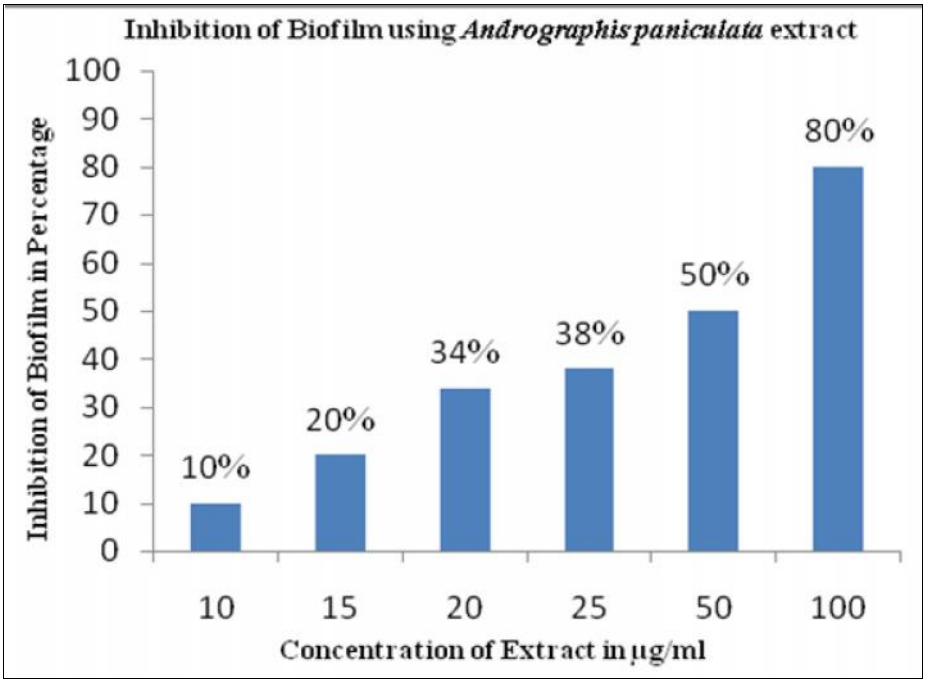


Figure 2: Graphical representation of biofilm inhibition in *Pseudomonas aeruginosa* using *Andrographis paniculata*

4. CONCLUSION:

In the present study, since the *Andrographis paniculata* extract were made by using methanol, the possibility of anti-biofilm activity was been ruled out. Therefore, it is envisaged that the active principle exhibiting QSI activity might be an analogue of AHL molecule. However, the extract needs further purification and characterization to find out the active principle with the antibiofilm inhibitor activity. In conclusion, the reduction in QS and the end effect on virulence factors production without affecting the bacterial growth provided some insight into the potential of *Andrographis paniculata* as QS inhibitors. Therefore, it is concluded that the QSI potential of these *Andrographis paniculata* could be used as anti-biofilm drug to combat with *Pseudomonas aeruginosa* and also with other bacterial infections.

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