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Reduction and removal of *Pseudomonas aeruginosa* biofilm by natural agents

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ABSTRACT

Biofilms protect the pathogens from inhibitory effect of antibiotics and immune cells. *Pseudomonas aeruginosa* is an important pathogen, and one of the hallmarks of *Pseudomonas aeruginosa* infection is its capability to adhere to, and propagate on medical devices, such as catheters, contact lenses, and wound dressings by forming strong biofilms. Therefore, identification of potent agents, capable of disinfecting *Pseudomonas aeruginosa* biofilms holds a significant value in designing effective biofilm control strategies, and therapeutic interventions. In an attempt to search for effective biofilm controlling agents, four different plant extracts were tested, using quantitative spectrometric method, for their ability to reduce and remove *Pseudomonas aeruginosa* biofilms. Several of the plant extracts were identified as strong biofilm controlling agents against *Pseudomonas aeruginosa*, where the extract of *Daphne mucronata (*Kuttital or Pipal) and *Azadirachta indica* (Neem) were most efficient in reducing and removing *Pseudomonas aeruginosa* biofilms. Our study identifies that several plant extracts can be effectively used to control of *Pseudomonas aeruginosa* biofilms. Indicating the importance of natural agents as potential antibiofilm and antimicrobial agents.

Keywords: Biofilm, Pseudomonas aeruginosa, Plant extracts

1. INTRODUCTION

Many microbial species have evolved to survive in stressful environments by self-assembling in highly organized, surface attached, and matrix encapsulated structures called biofilms [1]. The ability to attach to solid surfaces and the subsequent formation of an organized bacterial biofilm community are important steps in the pathogenesis of chronic bacterial infections and persistence in host tissues [2-4]. Biofilms protect the pathogens from inhibitory effect of antibiotics and immune cells, because it prevents their effective penetration [5-8].

Pseudomonas aeruginosa has emerged as an important pathogen during the past two decades. It is a causative agent in both nosocomial as well as community acquired infections, especially among patients with burn wounds, cystic fibrosis, acute leukemia, organ transplants, corneal infections, and intravenous drug infections [9-17]. One of the hallmarks of *Pseudomonas aeruginosa* infection is its capability to adhere to and propagate on medical devices, such as catheters, contact lenses, and wound dressings[18]. The adherence is aided by several microbial factors, in which biofilm formation holds a key position [12, 19, 20], and which is partly driven by flagella and type IV pili [21]. *Pseudomonas aeruginosa* grows a strong biofilm in the lungs of cystic fibrosis patients [22, 23]. Among the causative agents of pyelonephritis, *P. aeruginosa*has been proven to have the maximum biofilm-forming ability, and has been the cause of thechronic urinary tract infections [24].*Pseudomonas aeruginosa* is also the most commonly isolated organism in the patients of contact lens-related Microbial Keratitis[8, 25-29].

Therefore, identification of potent biofilm controlling agents in *Pseudomonas aeruginosa* holds a significant value in designing effective biofilm control strategies, and designing therapeutic interventions.

Several efficient qualitative and quantitative techniques have been described for rapid and efficient detection of biofilms [30, 31] and simultaneous screening of different natural agents for their anti-biofilm potential [32], where, many of these anti-biofilm agents reported are synthetic or of chemical origin [33, 34]. In our current study, an efficient quantitative spectroscopic technique was employed to analyze the potential of natural agents (plant extracts)in simultaneously reducing and removing pre-formed *Pseudomonas aeruginosa* biofilms.

2. MATERIALS AND METHODS

2.1. *Pseudomonas aeruginosa* culture and biofilm forming potential

In this study, we have used *Pseudomonas aeruginosa* isolate 6 (henceforth referred as IIDRL-PA-6), previously isolated in our laboratory from patients suffering from the contact lens related keratitis, and characterized to be a dominant biofilm former [29]. The IIDRL-PA-6 was maintained on Trypton Soy Agar (Sigma-Aldrich) plates at 37 ^{0}C.

2.2. Antimicrobial activity of Plant extracts

Antimicrobial activity of several plant extracts has been well documented against many pathogens including *Pseudomonas aeruginosa* [16, 35-49]. In this study, we evaluated the antimicrobial and antibiofilm activity of plants extracts indicated in Table 1, against IIDRL-PA-6 biofilm [50].

2.3. Preparation of aqueous plant extracts

The 5% aqueous extracts of plants, mentioned in Table 1, were prepared and used in the study. Briefly, 2.5 g dried leaves/bark/powder/ berries were soaked into 50 ml autoclaved distilled water and boiled for three minutes for three times, with two minutes interval between each boiling time. The extract or supernatant was collected, centrifuged thrice for 5 minutes at 5000 rpm, until clear supernatant was obtained. The supernatant was filtered and sterilized using 0.2 um filter (Micropore filters), and freezed at -20 ^{0}C until further used. Maximum a week old extracts were used in the study otherwise fresh extracts were prepared.

2.4. Determination of antimicrobial potential of plant extracts by Disk-diffusion method

Antimicrobial activity of plant extracts was determined using Disk-Diffusion assay [51]. Briefly, 1 mL of IIDRL-PA-6 culture suspension was uniformly spread on two Nutrient Agar plates. Four sterile paper disks (6 mm in diameter; Becton, Dickinson & Co.) were placed on the surface of the agar plates, and were impregnated with 10 µL of 5% aqueous plant extracts. Plates were incubated for 24-48 hours at 37 °C. Antibacterial activity was determined by measuring a zone of inhibition around a disk, following a 24-48 hour incubation [52].

Table - 1: List and nature of plant extracts tested for their antimicrobial and antibiofilm activity against IIDRL_PA_6

Botanical Name	Extract	Concentration
Camellia sinensis	Aqueous	5 %
Daphne mucronata	Aqueous	5 %
Trigonellafoenum-graecum	Aqueous	5 %
Azadirachtaindica	Aqueous	5 %

Table - 2: Antimicrobial and antibiofilm activity of four different plants extracts

Plants extracts	Zone of inhibition (mm)	Biofilm Reduction (%)	Biofilm Removal (%)
Camellia sinensis	19±1	27.06	39.79
Daphne mucronata	12±1	40.08	46.02
Trigonellafoenum-graecum	19±1	27.2	41.39
Azadirachtaindica	9±2	30.1	51.08

The table shows antimicrobial activity, extracts measured in terms of zone of inhibition (mm), and antibiofilm (biofilm reduction and removal potential) potential of plant extracts.

2.5. Disinfection and Removal of *Pseudomonas aeruginosa* Biofilms

A quantitative spectrophotometric method, as described by *Pitts, et al,*[32], was used, with modification, to measure the biofilm disinfection and removal efficacy of the plant extracts described in Table 1. This method allows a rapid detection of concentration–dependent anti-biofilm activity of various agents [32]. The experiment was performed in two ways. In first experiment, the anti-biofilm activity of test agents was evaluated during incubation i.e. while the biofilm was being formed. Briefly, IIDRL-PA-6 culture was inoculated in 5-ml TSB and grown to stationary phase. The culture was diluted 1:100 in the tryptone soy broth (TSB) and 100 µl of diluted culture was pipette in total 10 wells, two wells for each test agent, one for blank (B) and one for control (C), of a fresh 96-well,non-tissue culture treated microtiter plate. One hundred micro litre of each test agent was inoculated in each well, and plate was covered and incubated at 37 ºC for 24 hours.

In the second experiment, anti-biofilm activity of the test agents was evaluated after incubation i.e. on pre-formed biofilm. Briefly, micro titre plates were inoculated as mentioned above and incubated at 37 ºC for 24 hours. After incubation, plates were washed with sterile water to remove planktonic cells and 200 µl of each plant extract was inoculated in each well, with exception of Blank and Control wells. Plates were incubated with the plant extract for a period of 1.5 hour.

After incubation, four small trays were set up in a series, and 1 to 2 inches of autoclaved tap water was added to the last three, while first tray served as waste. Planktonic bacteria were removed from the micro titer plates by vigorously shaking the plates over the waste tray. Wells were washed, by submerging the plates in the first water tray and then emptied over waste tray by vigorous shaking. Subsequently, for biofilm staining, 125 ul of 0.1% crystal violet solution was added to each well and incubated for 10 min at room temperature. Following incubation, the stain was emptied over the waste tray and plates were washed consecutively in each of the next two water trays with vigorous shaking to remove all liquid. Subsequently, the plates were inverted and vigorously tapped on paper towels to remove all the contents and left to air dry. Finally, the dye was solubilized by adding 200 µl of 95% ethanol to each well of the plate, and incubating the plate for 10-15 minutes at room temperature. In the next step, contents of each well were mixed by repeated pipetting, and then 125 µl of the crystal violet-ethanol solution was transferred from each well to a separate well of a new optically clear flat-bottom 96-well plate. Optical densities (OD) of each of these 125-µl samples were measured at a wavelength 630 nm.

Measurement of anti-biofilm efficacy called Percentage Reduction/Removal was calculated from blank, control and test OD, using equation:

Percentage Reduction/Removal = $[(C\text{-}B) - (T\text{-}B) / (C\text{-}B)]*100\%$

Where B = absorbance of blank (no biofilm, no treatment), C = absorbance of control (biofilm, no treatment) and T = absorbance of test (biofilm and treatment)

3. RESULTS

3.1. Antimicrobial potential of plant extracts

In this study, antimicrobial potential of four plant extracts were evaluated. The extracts of *Camellia sinensis* and *Trigonellafoenum-graecum*, with 19±1 mm zone of inhibition exhibited most potent antibacterial activity (Table 2), followed by *Daphne mucronata*, which produced zone of inhibition of 12±1(Table 2).

3.2. Antibiofilm potential of plant extracts

In this study, antibiofilm (biofilm reduction and removal) potential of four plant extracts was also evaluated. Extracts of *Daphne mucronata*and *Azadirachta indica* displayed most potent biofilm reduction (40% and 30%, respectively) and removal (46% and 51%, respectively) potential, as compared to the untreated control (Table 2), while extracts of *Camellia sinensis* and *Trigonellafoenum-graecum* only exhibited strong biofilm removal potential, 39.8% and 41%, respectively (Table 2).

4. Discussion

In this study, we have evaluated the antimicrobial and antibiofilm activity of four different plant extracts. We found that the extracts of *Daphne mucronata* and *Azadirachta indica* exhibited substantial antimicrobial and antibiofilm(reduction and removal) potential.

*Daphne mucronata*is a source of wide range of bioactive secondary metabolites, particularly coumarins, flavonoids, lignans, triterpenoids, coumarinolignans, daphnecin etc. [53]. It also contains a novel daphnane-type diterpene ester, gnidilatimonoein,which inhibited adhesion of tumor cells to fibronectin-coated culture wells, and inanimal models[54, 55]. Our study identifies *Daphne mucronata* as a potential source of anti-biofilm agent. The mechanism of biofilm inhibition needs to be elucidated in further studies.

We have found that the leaf extracts of *Azadirachta indica*(neem)effective in disrupting formationand structure of biofilms formed by *Pseudomonas aeruginosa* (IIDRL-PA-6)[56, 57].It has been shown that *Azadirachta indica*affects and disrupts important components, which are involved in biofilm formation, such as the level of exopolysaccharide, alginate, hydrophobic interactions and uroepithelial cell attachment[56].The antimicrobial and antibiofilm activities of the plant may be associated with the presence of various bioactive compounds such as tetranortriterpenes, including nimbin, nimbinin, nimbidinin, nimbolide, and nimbidic acid [58].

Moreover, our study showed the anti-bacterial potential of *Camellia sinensis*and *Trigonellafoenum-graecum* against *Pseudomonas aeruginosa* (IIDRL-PA-6). *Camellia sinensis* has been known for its antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis, Shigellaflexneri, Shigelladysenteriae, and Vibrio*spp., including *Vibrio cholerae*). The antibacterial activity of *Camellia sinensis*has been attributed to catechins, theaflavins, isoflavins and flavonols [59]. The antibiofilm activity of *Camellia sinensis*may be because of acid polysaccharide CS-F2, and Tea catechinepigalloctechingallate, which has also been implicated to have anti-adhesive effects against *Helicobacter pylori*, *Propionibacterium acnes*, and *Staphylococcus aureus* [60, 61].

Our study appears to be the first study to report antibiofilm potential of *Trigonellafoenum-graecum* against *Pseudomonas aeruginosa.* The antibiofilm property of the plant material is due to the presence of many active phyto chemicals including vitamins, flavonoids, terpenoids, carotenoids, cumarins, curcumins, lignin, saponin[62].

5. CONCLUSION

In conclusion, our study provides a strong evidence of the anti-biofilm potential of the local ethnopharmacologic plants against *Pseudomonas aeruginosa.* These extracts can be further tested to identify active antibiofilm agents in the extracts.

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