Phytochemical screening, \textit{in vitro} anti quorum sensing activity and antioxidant activity of extracts of \textit{Plumeria alba}, \textit{Pisonia alba} and \textit{Cynodon dactylon}

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ABSTRACT

Biofilm formation is a major strategy of drug resistant bacteria to thrive and cause infection. This ever increasing drug resistance demands for new drugs and treatment. Our study investigated the potential of phytochemicals in inhibiting biofilm formation through screening of anti quorum sensing activity. Ethyl acetate extracts of leaves of \textit{Plumeria alba} and \textit{Cynodon dactylon} and methanol extract of leaves \textit{Pisonia alba} were assayed to determine their ability to inhibit the \textit{in vitro} biofilm formation. \textit{C. dactylon} extract showed an inhibition of 94.64±0.44% at 20 µg/mL concentrations against the bio film producing clinical isolate \textit{Pseudomonas aeruginosa}, whereas \textit{Plumeria alba} and \textit{Pisonia alba} extracts showed 90.30±0.27% and 88.25±0.82% inhibition activity respectively. \textit{C. dactylon} extract also exhibited considerable antioxidant activity of 65.42±0.787 through DPPH assay. Thus it indicates that these plants could be prospective agents in treating clinical pathologies through inhibition of cell communication.

INTRODUCTION

One of the biggest challenges in the clinical sector is the increasing drug resistance. A wide approach of survival for infectious bacteria is to form biofilm. They form a protective cover by biofilm and resist killing by biological and chemical bactericidal. This resistance can be attributed to a number of properties including blocking the permeation of antibacterial agents into the matrix of biofilm, multidrug efflux pump expression and enzymatic action of drug modifying enzymes. (Grant and Hung, 2013) (Stewart and Costerton, 2001) (Mah and O'Toole, 2001). Biofilm also helps to protect the pathogenic microorganisms against host immunity (Bakkiyaraj \textit{et al.}, 2010) (Baldassarri \textit{et al.}, 2006). The property of biofilm formation can be attributed to quorum sensing. Quorum sensing signals by individual bacterium known as auto inducers aid communication between the various bacteria. This helps them to operate together rather than exerting individual action (Kaufmann \textit{et al.}, 2008). This implies that quorum sensing inhibitors (QSIs) can be potent to alleviate the bacterial biofilm formation thus making them susceptible to antibiotics (Brackman \textit{et al.}, 2011). Recent reports demonstrate that certain biofilm associated bacteria return to planktonic stage by the secretion of D-amino acids and cis-2-decenoic acid, activating the disassembly of the biofilm. This strengthens the implication that small molecule QSIs can inhibit biofilm formation (Kolodkin-Gal \textit{et al.}, 2010) (Davies and Marques, 2009). Using natural products as biofilm inhibitors is safe and less toxic than using synthetic inhibitors. They are more specific than the synthesized chemical inhibitors (Koo and Jeon 2009).

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Plants are rich and inexhaustible source of active ingredients. Since time immemorial, medicinal plants have been known to contain bioactive compounds and were used in some form or other for treatment of various ailments (Rahmatullah et al., 2010). These compounds are normally accumulated as secondary metabolites in all plant cells and play a pivotal role in the modern medicine or as the lead compounds for new drug discovery (Vijayalakshmi and Ravindran, 2012). Plant derived constituents are looked upon as a new therapeutic agents which might also be acts as a nontoxic inhibitors of quorum sensing, thus controlling infections without encouraging the appearance of resistant bacterial strains. The antimicrobial efficiency of plants are as a result of the secondary metabolites produced, say phenols, tannins, flavonoids. (Karuppiah and Mustaffa, 2013) (Koo et al., 2003). Hence, there is a strong quest for bio-screening plant extracts for anti-biofilm and quorum sensing inhibitory activity, followed by isolating the compounds responsible for this activity (Zaki et al., 2013).

To pursue for natural biofilm inhibition activity we have studied Plumeria alba, Pisonia alba and Cynodon dactylon plant extracts. These medicinal plants have been known to produce bioactive compounds which are used to treat various ailments. Pisonia alba belonging to the family Nyctaginaceae is an evergreen tree used as a diuretic (Sunil et al., 2009). Plumeria alba of the family Apocynaceae, a laticiferous small tree or shrub and the latex is used to alleviate ulcers and herpes. The seeds of this plant have haemostatic potential and bark been used as a plaster over tumours (Chaudhuri et al., 2015). Cynodon dactylon of the family Poaceae, commonly known as Bermuda grass has been traditionally used against diabetes and jaundice, kidney and urinary diseases and so on (Jarald et al., 2008).

It is a universally accepted fact that lately microorganisms have become resistant to several antimicrobials. With the advancement in technology, the bioactive potential of plant derived compounds can be utilized against antibiotic resistant microorganisms (Al-Hussaini and Mahasneh, 2009). Plant derived constituents are looked upon for new therapeutic and anti-pathogenic agents which might be nontoxic inhibitors of quorum sensing, thus controlling infections without encouraging the appearance of resistant bacterial strains (Hentzer and Givskov, 2003).

The phytochemicals’ properties can be analyzed and used against microbial growth patterns as plants having phytochemical properties possess strong antioxidant activity. So we performed the anti quorum sensing capability of various extracts of the three plants. Subsequently their antibiofilm potential was also assessed. Potent extracts which showed antioxidant property were analysed for their phytochemicals.

MATERIALS AND METHODS

The plants were collected freshly from the campus of VIT University, Vellore, Tamil Nadu, India in the month of July 2015 and identified by Dr. Benjamin Prasad Kumar, Department of Plant Biology and Biotechnology, Voorhees College, Vellore. A voucher specimen was maintained in our laboratory by an accession number BV/VIT/PPCD/12.07.2015-45 to 47.

Bacterial strains used

Chromobacterium violaceum (MTCC 2656), an indicator strain for screening quorum sensing (QS) inhibitors, was purchased from MTCC, IMTECH (Chandigarh, India). A total of 3 clinical isolates, Staphylococcus aureus, Salmonella typhi and Pseudomonas aeruginosa were employed in the study.

Chemicals and media

Methanol, Chloroform, Ethyl acetate, Petroleum ether, DPPH, other chemicals and dehydrated culture media utilized for the study were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). All chemicals involved were of analytical grade.

Extract Preparation and solvent extraction

Freshly collected leaves of Plumeria alba, Pisonia alba and Cynodon dactylon were surface sterilized with 0.5% of sodium hypochlorite for 15 minutes and cleaned thoroughly with repeated washing of distilled water. After shade drying the leaves were grounded to get powder and proceeded to extraction technique, which included 5 different solvents with increasing polarity viz., Petroleum ether, Chloroform, Ethyl acetate, Methanol and Distilled water. The dried extract was collected and stored in vials at 4 °C for further analysis.

Anti-quorum sensing (AQS) activity of the plant extracts

The AQS activity of the plant extracts were checked against an indicator organism, Chromobacterium violaceum (MTCC 2656). C.violaceum served as a QS system producing a visible violet coloration (Nithya et al., 2010). For the assay the prospective QS inhibitors were added in different concentration in wells containing 10 μL (1:100 dilution) of the bacterial culture. The plate was read at OD660 upon incubation at 37 °C for 24 hours. The bacterial cells with DMSO served as control.

Microtitre plate based biofilm formation assay

For detection and confirmation of biofilm formation capability of the clinical isolates Staphylococcus aureus, Salmonella typhi and Pseudomonas aeruginosa, we employed microtitre based technique. Freshly grown culture was diluted to 1:100 into fresh trypticase soy broth for biofilm assays. 100 μL of the dilution per well was added in a 96 well polystyrene microtitre dish.

For quantification, 2-3 replicates were maintained. The microtitre plate was incubated for 24 hrs at 37°C. Upon incubation the cells were dumped by flipping the plate. The plate was cleared of any planktonic cells and media. To the wells 125μL of 0.1% crystal violet dye was added. For staining the plates were incubated at room temperature for 15 to 20 minutes. For quantification of biofilm, the stain was solubilised by addition of
125 μL of 30% acetic acid to the wells. This was maintained static for 10 minutes. Thereafter the contents were added to a new microtitre plate and optical density was analysed at 570 nm with 30% acetic acid as blank (O’Toole, 2011).

**Effect of the plant extracts on growth of biofilm isolates**

The action of plant extracts on the growth of biofilm formers were analysed by a concentration based study. 20-100μg/mL of methanol extract of *Pisonia alba* and ethyl acetate extract of *Cynodon dactylon* and *Plumeria alba* were prepared as working solutions. 100 μL of overnight cultures *S. aureus*, *S. typhi* and *P. aeruginosa* adjusted to McFarland standard 0.5 was added in to wells followed by 100 μL of the extracts. After incubation at 37°C for 24 hours the plate was read spectrophotometrically at OD

DMSO and bacterial suspension served as blank and negative control respectively. All treatments and control were maintained in triplicates (Antunes et al., 2010).

**Inhibition efficacy of plant extracts on biofilm formation**

100μL of each working solution varying from the concentrations of 20-100μg/mL was added to the wells containing 100 μL of isolates in trypticase soy broth. The plate was incubated at 37°C for 24 hours. The plate was washed with sterile phosphate buffered saline to remove all unattached cells and 0.4% crystal violet was added to stain the plate.

The plate was de-stained for 30 minutes with 95% ethyl alcohol. At OD

Minimum biofilm inhibitory concentration (MBIC) of the extracts was decided based on the visible destruction of the biofilm and noticeable decline in the absorbance in comparison with the control (Dhamodaran et al., 2010) (Sanchez et al., 2016).

**Antioxidant assay**

**DPPH Radical scavenging assay**

Antioxidant activity of the solvent extracts was assessed by % DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging method. From the primary stock prepared by dissolving 0.01 g of each plant extract in 1mL of methanol. 200μL was added to make up to a final volume of 2 mL which is the secondary stock solution (Chaudhuri et al. 2015).

A series of working solutions viz., 20, 40, 60, 80 and 100μg/mL was made from the secondary stock to a final volume of 2 mL (Baldassarri et al., 2006). Ascorbic acid served as a standard. 1mL freshly prepared DPPH (was added to all test samples, incubated at 20°C for 40 minutes in dark and their absorbance was measured at 517nm. DPPH radical scavenging assay was calculated according to the formula below:

\[
\% \text{ inhibition} = \frac{OD_{\text{control}} - OD_{\text{test}}}{OD_{\text{control}}} \times 100
\]

where, OD\text{control} is the optical density of the DPPH radical + methanol, OD\text{test} is the optical density of DPPH radical + sample extract/standard (Nithya et al., 2010).

**Screening for Phytochemicals**

In order to analyze the various constituents of the extracts, they were screened for various phytochemicals namely saponins, carbohydrates, proteins, phenolics, sterols, flavonoids, alkaloids, terpenoids and glycosides according to standard protocols (Ho et al., 2015).

**Analysis of variance**

The data were examined statistically by Analysis of Variance (ANOVA) using GraphPad Prism 6.0 (GraphPad software Inc. San Diego, USA)

**RESULTS AND DISCUSSION**

**Successive solvent-solvent extraction**

Macerated extraction of phytochemicals by various extracts yielded 0.2-1% of total dry weight of the leaves of all the plants.

**Anti-quorum sensing activity of the plant extracts**

The indicator organism *Chromobacterium violaceum* was found to be inhibited by *Cynodon dactylon* (Ethyl acetate extract) by 80.34% followed by *Pisonia alba* (Methanol extract) by 49.56% and *Plumeria alba* (Ethyl acetate extract) by 47.90%. The rest of the extracts showed negligible amount of inhibition of the pigment, thus these three extracts were chosen for further studies.

**Microtitre plate based biofilm formation assay**

Clinical isolates were screened for biofilm formation by static biofilm formation test. Isolates *Staphylococcus aureus*, *Salmonella typhi* and *Pseudomonas aeruginosa* showed biofilm formation, however *P.aeruginosa* was found as the best biofilm forming isolate. According to Nyenje, 2013, strength of biofilm formation, however

**Effect of the plant extracts on growth of biofilm isolates**

The three plant extracts were screened for their effect on antimicrobial activity against the active biofilm producer. The result revealed less than 55% inhibition. The highest activity was exhibited by *Pisonia alba* at 100μg/mL (Table-I).
Table 1: Growth inhibition exhibited by plant extracts against *P. aeruginosa*

<table>
<thead>
<tr>
<th>Plant Extracts</th>
<th>Growth Inhibition at concentrations as below</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentrations (µg/mL)</td>
</tr>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Plumeria alba</td>
<td>15.29</td>
</tr>
<tr>
<td>Pisonia alba</td>
<td>45.10</td>
</tr>
<tr>
<td>Cynodon dactylon</td>
<td>42.08</td>
</tr>
</tbody>
</table>

**Inhibition efficacy of plant extracts on biofilm formation**

Bacteriocidal activity indicates negative results which imply that the bacteria are found resistant to the extracts. However, the extracts exerted significant activity against biofilm formation. The bacterial biofilm formation was considerably disturbed as is indicated in Fig 1, by the percentage inhibition. The variation was visibly clear in the microtitre as reduction in crystal violet stained cells adhering to the surface of the titre base. *P. aeruginosa* was inhibited at the highest by *C. dactylon* with a percentage of 94.64±0.44. The inhibition percentage exhibited by *Plumeria alba* and *Pisonia alba* was 90.30±0.272 and 88.25±0.827 respectively. The minimum biofilm inhibitory concentration was determined using the microtitre plate technique which was found to be 20 µg/mL for *C. dactylon*, 40 µg/mL for *Pisonia alba* and 60 µg/mL for *Plumeria alba*.

**Phytochemical screening**

Phytochemical screening of *Plumeria alba* exhibited the presence of saponins, glycosides, cardiac glycosides, flavonoids and terpenoids while *Pisonia alba* showed the presence of saponins, glycosides, alkaloids and flavonoids. *Cynodon dactylon* expressed glycosides, flavonoids and terpenoids. Based on the phytochemical content ethyl acetate extract of *Plumeria alba* and *C. dactylon* and methanol extract of *Pisonia alba* were retained for further studies. Figure 3 depicts the phytochemical composition of the plant extracts. Our findings show that *Cynodon dactylon* exhibit significant anti-biofilm at a concentration of 20 µg/mL. This can be attributed to the phytochemical constituent of *C. dactylon*. From the composition of phytochemicals it can be proposed that the flavonoids content contributes to biofilm inhibition. Flavonoids from various sources are potent anti-biofilm agents which inhibit biofilm by bacteria such as *Streptococcus mutans*, *Aeromonas hydrophila* and *Escherichia coli* (Koo et al., 2000) (Vikram et al., 2010) (Sanchez et al., 2016). Lee et al. (2010) reported the action of phloretin, a flavonoid at concentrations of 25 and 50 g/mL against biofilm of *E. coli* O157:H7 by 89 and 93%. However this is the prime report on anti-biofilm activity of *C. dactylon* against *P. aeruginosa*. In addition the extract shows considerable amount of antioxidant activity.

**CONCLUSION**

Briefly, *C. dactylon* can be a prospective antibiofilm agent which can act against *P. aeruginosa* induced nosocomial infections and the antioxidant activity adds to the beneficial effects. The phytochemicals are a means of biofilm inhibition while antibiotics can wipe out the bacterial population.

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REFERENCES


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