SHORT COMMUNICATION

Leaf extract of *Azadirachta indica* (neem): a potential antibiofilm agent for *Pseudomonas aeruginosa*

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This study establishes that leaf extracts of *Azadirachta indica* or neem used in traditional medicine has potential as an anti-biofilm agent against clinical isolates of *Pseudomonas aeruginosa*.

Keywords

*Pseudomonas aeruginosa*; *Azadirachta indica* (neem); biofilms; scanning electron microscope.

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*Pseudomonas aeruginosa* is well known for its ability to form biofilm on indwelling medical devices. These biofilms are difficult to remove because of their high tolerance to conventional antibiotics. Therefore, there is a need to look for alternative agents such as medicinal plants, which can eradicate or inhibit biofilm effectively. This study evaluated the role of neem in inhibiting biofilm formation by *P. aeruginosa*. Factors contributing to adherence and biofilm formation were also studied. Results demonstrated that neem leaves extract was quite effective in disrupting formation and structure of biofilms. Moreover, the level of exopolysaccharide, alginate, hydrophobic interactions and uroepithelial cell attachment, which contributes to biofilm formation, was also affected significantly. Results confirm the effectiveness of neem extract in inhibiting biofilm formation. Such studies can lead to the discovery of safe antimicrobial drugs from natural sources without the risk of resistance.

*Pseudomonas aeruginosa* is the third most common pathogen associated with number of human infections such as pulmonary tract, urinary tract, burn wounds, and blood infections (Ehrlich *et al.*, 2002). These infections lead to significant morbidity and mortality in immunocompromised patients. *Pseudomonas aeruginosa* employs quorum sensing (QS) to regulate its virulence potential including wide array of virulence traits, and formation of biofilm, which result in chronic and recurrent infections (Bjarnsholt *et al.*, 2010). Microorganisms within a biofilm become 50–500 times more resistant to antimicrobial agents and hence are more difficult to eradicate (Gilbert *et al.*, 2002). This resistance is attributed to the presence of alginate and the existence of microbial cells in stationary phase. Moreover, the age of the biofilm greatly influences the outcome of a therapeutic regime (Ito *et al.*, 2009). The traditional mode of biofilm eradication relies upon a higher dose of antibiotics, but increased tolerance of biofilm to these antibiotics makes the eradication difficult (Kallel *et al.*, 2008).

Therefore, there is a need to search for alternative therapies for the treatment of various infections with negligible side effects (Ahmad *et al.*, 1998). Herbal or medicinal plants have proved a good choice. Medicinal plants have many substances such as peptides, unsaturated long-chain aldehydes, alkaloidal constituents, essential oils, phenols and water extracts, methanol and butanol soluble compounds, which show significant antibacterial properties against a number of human pathogens (El Astal *et al.*, 2005).

*Azadirachta indica* (neem) is world-renowned medicinal plant, having long history of usage in various ailments in Indian traditional medical system (Ayurveda, Unani, Tibetan), since time immemorial. Each part of neem, including leaves, bark extracts, oil, and products made from neem have medicinal properties. Neem products have been proved antihelminthic, antimicrobial, and act as a contraceptive and sedative agent (Ganguli, 2002). Although neem has been proved antimicrobial in nature (Aarati *et al.*, 2011), to the best of our knowledge, its role has not been evaluated in biofilm-associated infections. Therefore, this study was undertaken to evaluate the role of neem in biofilm formation.
Standard strain (PAO1) and two clinical isolates (PA3 and PA7) of *P. aeruginosa* were employed in this study. For QS signals estimation, *E. coli* MG4 was used as reporter strain.

Neem extract (NE) was prepared according to the method of Agbenin & Marley (2006). Fresh neem leaves were weighed, sterilized (1% sodium hypochlorite), and were washed thrice with sterile distilled water. Leaves were ground in mortar and mixed with 10 mL sterile distilled water. The mixture was allowed to stand for 4 h, and the homogenate was filtered (Whatman No.1). Filtrate was plated on nutrient agar to check its sterility. Sub-MIC showing no growth inhibition was used for all further experiments.

Catheter pieces were transferred to fresh medium after every 24 h. Each day, catheter pieces were removed in duplicate and rinsed thrice with phosphate-buffered saline (PBS 0.1 M, pH 7.2). Catheter surfaces were scraped with a sterile blade and were given a low-level sonication cycle. The dispersed sample was centrifuged, and the cells were suspended in 1 mL PBS. Cells were plated on nutrient agar to determine log CFU (Mittal et al., 2006).

Catheter pieces were fixed in 2.5% glutaraldehyde overnight at 4 °C followed by dehydration in gradient concentration of ethanol. Later, catheter pieces stubbed with gold ions were observed with SEM (JSM-6100, SM-Jeol, 20 KV) (Nickel et al., 1985).

Alginate was precipitated with an equal volume of culture supernatant and 2% (w/v) cetylpyridium chloride followed by centrifugation at 6000 *g* for 20 min. The pellet was suspended and precipitated with 5 mL of iso-propanol followed by centrifugation at 6000 *g* for 10 min. The pellet was suspended in 1 mL of normal saline. Alginate was determined using D-mannourate lactone as standard (Mathee et al., 1999). Total polysaccharide was determined by a standard method (Dubois et al., 1999).

The culture supernatant was mixed with an equal volume of p-xylene, and after phase separation, the optical density of aequous phase was taken and was expressed as percentage hydrophobicity (Rosenberg et al., 1980).

One milliliter each of bacterial suspension (10^8 CFU mL^-1^) and washed human uroepithelial cells (10^5 cells mL^-1^) were incubated at 37 °C for 1 h in shaker water bath. The mixture was centrifuged at 6000 *g* for 10 min. Smears prepared from pellet were stained with Giemsa. The average number of bacteria adhering to 30 UECs was counted thrice and was expressed per UEC (Sharma et al., 1987).

Experiments were repeated thrice to validate the reproducibility and were analyzed statistically using Graphpad prism to calculate *P* values.

*Pseudomonas aeruginosa* PAO1 and clinical isolates were grown in the presence of different concentrations of NE. 10% NE showing no growth inhibition as compared with control was selected as subinhibitory concentration (data not shown), and used for further experiments.

In the case of PAO1, accumulation of biofilm was maximum on day 5, which later showed a slight decline until day 7. Both the strains (PA3 and PA7) also showed a similar trend; however, PA3 showed less biofilm production as compared with PA7. Biofilm formation was significantly (*P* ≤ 0.01) reduced in all three strains with a peak on the 5th day in the presence of NE (Fig. 1). SEM of 5-day-old biofilm demonstrated that the surface of catheter was completely covered with biofilm cells along with large amount of exopolysaccharide in control. Rod-shaped bacteria were deeply embedded in thick extracellular glyocalyx, while in the case of NE-treated biofilms, a change in architecture of the biofilm was observed. Thin biofilm was formed with less exopolysaccharide (Fig. 1).

Five-day-old biofilm cells were used for the estimation of other factors. Levels of total polysaccharides, alginate, and cell surface hydrophobicity decreased significantly when *P. aeruginosa* was grown in the presence of NE as compared with their control (*P* ≤ 0.01). The average number of bacteria adhering to UECs was also reduced significantly in the presence of NE (*P* ≤ 0.01, Table 1).

Biofilm-originated infections are increasing with increased use of indwelling medical devices. Approximately 60% of all nosocomial infections are due to biofilms (Bjarnsholt et al., 2005a). Restricted penetration and presence of persister cells make the survival of biofilm much easier (Stoodley et al., 2002; Harrison et al., 2004). Therefore, it becomes essential to search for novel antimicrobial substances, which can inhibit biofilm formation and/or disrupt established biofilms. With this approach, the potential effect of neem on *P. aeruginosa* biofilms was explored.

Fresh neem leaves were used because they carry primary ingredients responsible for medicinal properties. Although the bacteriostatic activity of neem leaves extract has been demonstrated against *P. aeruginosa* in vitro (Maragathavalli et al., 2012), literature regarding its efficacy on biofilm is completely lacking. Therefore, to evaluate the role of NE on biofilm, biofilm was developed in the absence and in the presence of NE. Biofilm formation was more significant in strains PAO1 and PA7 as revealed from log CFU count than that observed in PA3. In the presence of NE, all three strains showed significantly reduced biofilm formation as indicated by decreased log CFU count (*P* ≤ 0.01). Earlier, Pai et al. (2004) reported the effectiveness of neem leaves extract against biofilms of *Streptococcus sanguis* in the oral cavity. NE reduced the plaque index and bacterial count significantly as compared with a control group. Polaquinii et al. (2006) also showed inhibition of *Candida albicans* biofilm by neem leaves extract. However, our study is the first to demonstrate the role of NE on biofilms of *P. aeruginosa*.

In the biofilm mode of growth, *P. aeruginosa* produces glyocalyx, an extracellular polysaccharide, and organic polymer such as alginate, which play an important role in the structural development of biofilm (Sutherland, 2001; Toutain et al., 2004). Exopolysaccharide is responsible for binding the cells together in a highly hydrated polymer network. Although alteration in cell surface hydrophobicity of *P. aeruginosa* by different antibiotics has been shown previously (Kustos et al., 2003), the effect of NE on cell surface hydrophobicity has not been evaluated yet. Therefore, NE was evaluated for its role on *P. aeruginosa* hydrophobicity. Cell surface hydrophobicity was assessed based on binding of hydrocarbons to cells by interaction with p-xylene and
was expressed as percentage hydrophobicity (Obuekwe et al., 2008). Significant decrease in percentage hydrophobicity of all three strains was observed in the presence of NE, indicating its potential in inhibiting the initial step of biofilm formation. Attachment, a necessary step in colonization of host mucosal surface in invasive infections, enables the pathogen to avoid elimination by body fluids. *Pseudomonas aeruginosa* exhibits maximum adherence to UECs (Daifuku & Stamm, 1986). Results showed that the adhesion of *P. aeruginosa* to UECs was reduced significantly in the presence of NE, indicating that NE can inhibit the adhering ability of *P. aeruginosa*.

Table 1 Effect of neem extract on quorum-sensing signals, exopolysaccharide, alginate, cell surface hydrophobicity and uroepithelial cell adhesion by biofilm cells of *Pseudomonas aeruginosa* standard strain PAO1, and selected clinical isolates PA7 and PA3

<table>
<thead>
<tr>
<th><em>Pseudomonas aeruginosa</em> strains</th>
<th>Exopolysaccharides (µg mL⁻¹)</th>
<th>Alginate (µg mL⁻¹)</th>
<th>Cell surface hydrophobicity (percentage)</th>
<th>Uroepithelial cell adhesion (average number of bacterial cells per UEC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NE treated</td>
<td>Control</td>
<td>NE treated</td>
</tr>
<tr>
<td>PAO1</td>
<td>0.72 ± 0.02</td>
<td>0.58 ± 0.029</td>
<td>0.825 ± 0.02</td>
<td>0.425 ± 0.1</td>
</tr>
<tr>
<td>PA7</td>
<td>0.58 ± 0.018</td>
<td>0.39 ± 0.022</td>
<td>0.745 ± 0.05</td>
<td>0.515 ± 0.09</td>
</tr>
<tr>
<td>PA3</td>
<td>0.42 ± 0.02</td>
<td>0.29 ± 0.019</td>
<td>0.530 ± 0.07</td>
<td>0.410 ± 0.08</td>
</tr>
</tbody>
</table>

Results are expressed as mean value ± standard deviation.

Fig. 1 (a) The inhibition of biofilm formation in the presence of neem extract as estimated in terms of log CFU of biofilm generated on catheter surface from day 1 to day 7. Results are expressed as mean value ± standard deviation. (b) Photomicrograph of 5-day-old biofilm of *Pseudomonas aeruginosa* formed on catheter surface showing well-formed biofilm with bacterial cells embedded in copious amounts of exopolysaccharides (X 6500). (c) Biofilm formed in the presence of neem extract, where clear morphology of cells with very little exopolysaccharide was observed (X 6500).
Exposure to selected concentrations of NE also changed the morphology of rod-shaped cells along with significant reduction in exopolysaccharide amount. It indicated that NE was also effective in altering the structure of biofilm, which may result in the penetration of antibiotics easily and hence may be effective even at low doses. Bedi et al. (2009) demonstrated that biofilm cells of Klebsiella pneumoniae became more susceptible to amoxicillin when used in combination with bacteriophage treatment. Phage treatment disrupted the structure of biofilm through which antibiotic diffused to the interior of biofilm. Further, alginate also showed significant reduction in biofilms formed in the presence of NE in all three strains. Alginate produced by P. aeruginosa is capable of binding antibiotic molecules, thereby significantly reducing their penetration. As NE was able to reduce alginate significantly, the diffusion of antibiotics may later become easier.

The results suggest that neem leaves possess antibiofilm property and reinforce the possibility of employing NE in the eradication of biofilm infections. Neem either alone or in combination with antibiotics can be explored as a potent biofilm-eradicating agent. As global scenario is changing toward the use of nontoxic plant products having medicinal value, hence extensive research is required on neem for its better economic and therapeutic utilization.

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References


