Identification of natural compounds which inhibit biofilm formation in clinical isolates of *Klebsiella pneumoniae*

H Magesh¹, Arun Kumar², Ayesha Alam³, Priyam⁴, Uma Sekar⁵, Venil N Sumantran⁶ & Rama Vaidyanathan⁷*

¹Department of Biotechnology, Dr. M.G.R.Educational and Research Institute, E.V.R. Periyar Salai, Maduravoyal, Chennai 600 095, India
²Department of Microbiology, Sri Ramachandra University, Porur, Chennai 600 116, India
³Bhupat & Jyothi Mehta School of Biosciences, Indian Institute of Technology, Chennai 600 036, India

Received 14 November 2012; revised 18 June 2013

*Klebsiella pneumoniae*, an important opportunistic pathogen, exists as a biofilm in persistent infections and in-dwelling medical devices. With the objective of identifying natural compounds inhibiting biofilm formation in *K. pneumoniae*, 35 clinical isolates were screened, out of which 7 strong biofilm producers were identified. Six natural compounds were tested for their inhibitory effects on bacterial growth and biofilm formation by determining the minimum inhibitory concentration and minimum concentration for biofilm inhibition (MBIC) for each compound. The results show that reserpine followed by linoleic acid, were the most potent biofilm inhibitors. Reserpine, an efflux pump inhibitor was effective at biofilm inhibition at a concentration of 0.0156 mg/mL, 64-fold lower concentration than its MIC. Linoleic acid, an essential fatty acid was effective as a biofilm inhibitor at 0.0312 mg/mL, which is 32-fold lower than its MIC. Berberine, another plant derived antimicrobial, chitosan and eugenol had an MBIC value of 0.0635 mg/mL. Curcumin, a natural phenolic compound was effective at biofilm inhibition at a concentration of 0.25 mg/mL, which is 50 fold less than its MIC. Notably, the MIC and MBIC data on these 6 natural compounds was reproducible in all seven high biofilm forming isolates of *K. pneumoniae*. The present report is a comprehensive comparative analysis of the dose dependent inhibition of various natural compounds on biofilm formation in *K. pneumoniae*.

**Keywords:** Biofilm inhibition, Efflux pumps inhibitors, *Klebsiella pneumoniae*, Multi-drug resistance, Minimum inhibitory concentration

Persistent infections are a global challenge for human beings, claiming millions of lives every year and demanding huge medical and social resources. One common survival strategy employed by bacteria pathogens is to form a biofilm, an amorphous and dynamic structure that is not only resistant to antibiotics, but also resistant to host immune clearance¹. Biofilm formation is a two-stage biological process controlled by surface adhesins and cell-to-cell communication pathways. Aggregated bacterial cells protected and/or coated by extracellular matrix, are insensitive to both nutritional stimulation and hostile attacks. In the human body, biofilms may trigger persistent infections with chronic inflammation.

*Klebsiella pneumoniae* is an important opportunistic pathogen and is a common cause of urinary tract infections, respiratory tract infections, and septicemia, especially in immuno-compromised individuals². Factors that are implicated in the virulence of *K. pneumoniae* strains include the capsular serotype, lipopolysaccharide, iron-scavenging systems, fimbrial and non-fimbrial adhesions. The ability of bacteria to form a biofilm on host tissue surfaces is an important step in the development of infection.

As a correlation between biofilm formation and bacterial persistence has been established³, the possibility of using drugs targeting biofilm formation in combination with the current antibiotics is emerging as a potential therapeutic approach for this type of bacterial persistent infection. With an increase in reports of multi drug resistant infections, and the importance of biofilm formation resulting in decreasing susceptibility to antibiotics, there is an urgency to discover molecules targeting the inhibition of biofilm formation of bacteria. The objective of the present study was to identify natural compounds for their potential to inhibit biofilm formation. Natural products chitosan, eugenol, curcumin and linoleic acid

---

* Correspondent author
Telephone: 91 44 23782176
Fax: 91 44 2378 3165
E-mail: ramavaidy@gmail.com
which are known antibacterials\textsuperscript{4-7} and efflux pump inhibitors reserpine and berberine\textsuperscript{8} were analyzed for their potential to suppress biofilm formation in high biofilm forming strains of \textit{K. pneumoniae}. For each compound, we quantitatively compared the potencies for bacterial growth inhibition versus biofilm inhibition. Such data would enable selection of natural compounds which could synergize with established anti-microbials, and increase their therapeutic efficacy.

With the objective of identifying natural compounds that have biofilm inhibition activity, high biofilm producing clinical isolates of multi drug resistant (MDR) \textit{K. pneumoniae} were first screened and subsequently the effects of the 6 test compounds were analyzed on biofilm formation. The analysis showed a dose dependent biofilm inhibitory activity for each of the 6 test compounds. The results from this analysis will be useful in designing combinatorial treatment of antibiotics and biofilm inhibitors.

\textbf{Materials and Methods}

\textit{Preparation of natural compounds}—The following natural compounds were dissolved at a concentration of 10 mg/mL in dimethylsulfoxide (DMSO, Loba-Chemie, Pvt. Ltd. Mumbai, India), reserpine (Sigma-Aldrich Co. LLC., USA), berberine (Sigma-Aldrich Co. LLC., USA), ciprofloxacin (Sigma-Aldrich Co. LLC., USA), eugenol (HiMedia Laboratories Pvt. Ltd., Mumbai, India), linoleic acid (Sisco Research Laboratories Mumbai Pvt. Ltd., Mumbai, India), chitosan (Axiogen Pvt. Ltd., India) and curcumin (M/S. Agrihub Pvt. Ltd., Tuticorin, India).

\textit{Bacterial strains and antimicrobial sensitivity test}—Clinical isolates (35) of multidrug resistant \textit{K. pneumoniae} were collected from a tertiary care hospital in Chennai during November 2009-February 2010. The isolates were numbered from 1 - 35. Based on the source of the isolate, a prefix U for urine, B for blood and S for sputum was given. The isolates were tested for their antimicrobial sensitivity to ciprofloxacin (5 \textmu g disk\textsuperscript{-1}, HiMedia Laboratories Pvt. Ltd., Mumbai, India), ceftaxime (30 \textmu g disk\textsuperscript{-1}, HiMedia Laboratories Pvt. Ltd., Mumbai, India), and amoxyclav (30 \textmu g disk\textsuperscript{-1}, HiMedia Laboratories Pvt. Ltd., Mumbai, India) using recommended guidelines. The isolates were scored for their antimicrobial resistance according to the CLSI guidelines\textsuperscript{9}.

\textit{Determination of minimum inhibitory concentration of natural compounds}—The MIC for each of the natural compounds was determined using the tissue culture plate method\textsuperscript{10,11}. The bacterial isolates were maintained in Brain heart infusion agar (HiMedia Laboratories Pvt. Ltd. Mumbai, India) plate and inoculated into 5 mL of Mueller Hinton broth (HiMedia Laboratories Pvt. Ltd. Mumbai, India) and incubated for 18 h at 37 °C in shaker. The overnight culture was adjusted to 0.5 McFarland standards [0.5 mL 1.17% (w/v) BaCl\textsubscript{2} \times 2H\textsubscript{2}O + 99.5 mL 1% (w/v) H\textsubscript{2}SO\textsubscript{4}]. Overnight culture (10 \muL, corresponding to 0.5x 10\textsuperscript{8}cfu) was added to 100 \muL of Mueller Hinton broth and incubated overnight.

To the 96-well micro titer plate 150 \muL of sterile Mueller Hinton broth (HiMedia Laboratories Pvt. Ltd. Mumbai, India) was added and two fold serial dilutions of the natural compounds were made starting with the first well by adding 50\muL of the test compound dissolved at a concentration of 4 mg/mL. To each of the wells 10\muL of the diluted culture (0.5 McFarland standard) was added. This resulted in the final concentration of the compound ranging from 2 mg/mL in the first well to 0.0078 mg/mL in the 9\textsuperscript{th}well. In curcumin, higher concentrations were used to determine its MIC. The tissue culture plate was then incubated at 37 °C in stationary condition for overnight. The growth of the bacterial culture was measured at a wavelength of 595 nm with Bio-Rad Model iMark Micro plate Absorbance Reader. Two types of negative controls and one positive control were used in each assay. The ‘Vehicle control’ contained the solvent used for dissolving the test compounds (10\muL DMSO) and 100 \muL of media in 10\textsuperscript{th} well. The ‘Media control’ lacked bacteria and plant compounds, and only contained media (100 \muL of MHB broth in the 11\textsuperscript{th}well). The ‘untreated control’ lacked plant compounds, but contained growing bacteria (positive control:10 \muL bacterial culture and 100 \muL of sterile MHB media in 12\textsuperscript{th} well).

The MIC was defined as the minimum concentration of the extract that did not allow any visible growth or turbidity of the organism in broth. MIC\textsubscript{90} refers to concentration of the test compound required to prevent the growth of 90% of organisms tested. For each compound the MIC was tested for the nine isolates in duplicates. The concentration at which all the isolates failed to grow is taken as MIC.

\textit{Screening of \textit{K. pneumoniae} for biofilm formation}—In this method, test strains were cultured on fresh brain heart infusion agar (BHI) plate and inoculated in sterile brain heart infusion broth and incubated overnight at 37 °C without shaking. The overnight culture was diluted to 0.5 McFarland standard in fresh BHI medium.
The modified Tissue Culture Plate (TCP) method was used for screening biofilm formation in *K. pneumoniae* isolates. In the TCP method, an overnight culture of each isolate was adjusted to a McFarland standard of 0.5. An aliquot of 10 µL of the culture was added to 100 µL of the fresh BHI broth and incubated overnight. After 24 h, the planktonic cells were aspirated, and wells were washed with phosphate buffer saline (PBS, pH 7.2) to remove free floating bacteria. Biofilms which adhered to the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet (0.1% w/v, aqueous solution, HiMedia Laboratories Pvt. Ltd., Mumbai, India). Excess stain was washed with deionized water and plates were dried. The absorbance of stained adherent bacteria (dried polysaccharides) were determined by Bio-Rad Model iMark Micro plate Absorbance Reader at 595 nm. To compensate for background absorbance, OD values from sterile medium well were averaged and subtracted from all test values. The experiment was repeated twice. Each isolate was analyzed in triplicate. Thirty five clinical isolates and a control strain MTCC432 was screened by the TCP assay.

Quantitation of biofilm data—Biofilm formation potential of all 35 test cultures could be quantitatively compared as the incubation was started with the same cell number for each of isolate. Further, free forming (planktonic) cells did not contribute to biofilm formation since they were removed at the start of the experiment. Therefore, varying amounts of biofilm formation by various isolates could be quantitated by comparing OD values of stained adherent cells. Isolates which gave an OD<0.120 were classified as non-adherent and weak biofilm producers; O.D. values of 0.120 to 0.240 were classified as moderately adherent and moderate biofilm producers; O.D. value of > 0.240 was classified as strongly adherent and high biofilm producers.

Microscopic determination of biofilm formation—The test strain was cultured in brain heart infusion broth. A sterile glass slide was kept in a sterile petriplate and overlaid with 20 mL of test strain inoculated in Brain Heart Infusion broth. After 24 and 48h of incubation, the slide was taken out aseptically and washed with phosphate buffer saline (pH 7.2) to remove free floating planktonic bacteria. The biofilm was fixed with 2% sodium acetate and stained with 0.1% crystal violet stain, washed and air dried. The slide was examined under Trinocular microscope at 100x oil immersion. Photomicrographs of adhered bacterial biofilms were recorded.

Biofilm inhibition assay—Only those isolates of *K. pneumoniae* which were classified as strong biofilm producers were used. Test compounds were dissolved in DMSO (10 mg/mL), and two fold dilutions were made to result in a final concentration ranging from 2-0.0078 mg/mL in the wells after the addition of the freshly diluted brain heart infusion broth culture containing 10^6 cfu of the strong biofilm forming isolate per well. After incubation for 24 h at 37 ºC, the tissue culture plate was washed, fixed and biofilms were stained and visualized as outlined above. The inhibitory effect of the plant compound on biofilm production was calculated by subtracting the media control. The MBIC is the concentration of the natural compound at which the biofilm formation was reduced to an Absorbance 595<0.12 OD. Each assay for MBIC determination was performed in triplicate.

Statistical analysis—Statistical analyses were performed with MS-Excel 2010. Data are shown as mean ± SD unless otherwise stated (Figs 1 and 3). For each bacteria, the biofilm formation assay was performed in triplicates and the mean OD was taken for the analysis. The data from a total of 35 bacteria were considered for the test. The biofilm formation of the bacteria was significantly different at OD>0.240 and the level of significance were tested by Sign test as the data were not normally distributed (Shapiro–Wilk statistics). The null hypothesis H0: <0.240 against the alternative H1: => 0.25 was tested. Statistical significance was set at P<0.05.

Results

Antimicrobial sensitivity test—The bacterial isolates collected were highly resistant to antibiotics. Out of 35 isolates, 31 were resistant to cefotaxime, a third generation cephalosporin antibiotic, whereas 24 isolates were resistant to ciprofloxacin. All the isolates were resistant to amoxycillin, a combination of amoxicillin and clavulanic acid. In this study, we focused on screening the biofilm forming potential of all the 35 MDR isolates.

Classification of *K. pneumoniae* based on biofilm formation—The 35 MDR isolates of *K. pneumoniae* were analysed for biofilm formation using the TCP method and the results are shown in Fig. 1. Isolates were classified by their biofilm forming potential. Seven isolates were strong biofilm producers, 13 were moderate producers, and 15 were poor biofilm producers. *K. pneumoniae* MTCC 423 which was not part of this set of hospital isolates was included as an
internal assay control and found it to be a moderate biofilm producer. Statistical analysis showed that the seven strong isolates produced significantly greater amounts of biofilms relative to the moderate and poor biofilm producers. Based on intensity of biofilm staining, these seven isolates of *K. pneumoniae* were classified as strong biofilm producers. The magnitude of biofilm formation in these 7 strains was significantly greater than that produced by the poor (biofilm producers \( P=0.0019 \)) (Fig. 1). Among these seven strong biofilm producers, three (B15, B16 and B24) were isolated from blood samples; two from sputum samples (S13 and S20) and two were from urinary samples (U5 and U25). The highest biofilm producers were observed in the two urinary isolates U5 and U25, which gave an OD of 0.47 and 0.5 respectively. Biofilm formation in the strong biofilm producing strains was 4.5 fold greater than that of the poor biofilm producers. The Fig. 1 also provides a quantitative method for identifying and demarcating biofilm producing versus non-biofilm producing strains. Thus, biofilm producing strains when assayed by the modified TCP method gave an OD value of >0.120, whereas non-biofilm producing strains gave an OD value of <0.120 by this assay.

**Photomicroscopic analysis of the biofilm producers**—To visualize biofilm formation, one representative isolate from the categories of strong, moderate, and non-biofilm producers was analyzed by the microscopic slide assay (Fig. 2). The biofilm formation was clearly visible for the strong biofilm producer U25, followed by the moderate biofilm producer U6 at 24 h (Fig. 2A) and 48 h (Fig. 2B). The strong biofilm producer (isolate U5) also showed strong adherence to the slide (data not shown). The non-biofilm producers, the isolate U23 did not show biofilm formation even after 48 h. One of the strongest biofilm producers, isolate U5, was chosen for further studies for screening biofilm inhibitors.

**Determination of minimum inhibitory concentration of natural compounds**—To confirm the antibacterial activity of the 6 natural compounds (curcumin, eugenol, linoleic acid, chitosan, reserpine and berberine), their minimal inhibitory concentration was determined in 9 MDR isolates of *K. pneumoniae* including the 7 high biofilm forming isolates. Table 1 shows the MIC of each test compound for 9 MDR isolates. Eugenol was found to be the most potent antimicrobial compound, followed by linoleic acid, chitosan, reserpine, berberine and curcumin.

Fig. 1—Biofilm formation of 35 MDR *K. pneumoniae* determined by using the Tissue Culture Plate assay after incubation for 24 h at 37 °C. Grid lines drawn at 0.120 OD and 0.240 OD demarcate the strong, moderate and non-biofilm formers. Those isolates with OD >0.240 are considered strong, biofilm formers; 0.120 to 0.240 OD medium biofilm formers, and <0.120 OD non biofilm former. The magnitude of biofilm formation by the strong biofilm formers was significantly greater than that produced by the non-biofilm producers \( (P=0.0019) \).
Determination of minimum concentration of natural compounds required for biofilm inhibition

The modified TCP assay could quantitatively differentiate between strong, moderate, and weak biofilm producers with statistical significance (Fig. 1). These statistically significant results validated the biofilm assay method. Therefore, this assay was used to identify compounds which could significantly inhibit biofilm formed by strong biofilm producers. The lowest concentration of a test compound required to inhibit biofilm formation by a strong biofilm producer to the level of a non-biofilm producer (OD<0.12), was determined. This concentration was referred to as minimal biofilm inhibitory concentration (MBIC) of the test compound.

Using the strong biofilm producing isolate U5, clear and significant dose dependent inhibition of biofilm formation by the six different test compounds was demonstrated (Fig. 3). Reserpine was the most potent biofilm inhibitor, since 0.0156 mg/mL was sufficient to inhibit the biofilm formation completely (Fig. 3). Next, linoleic acid showed an MBIC at 0.0312 mg/mL. Chitosan, a known biofilm inhibitor, gave an MBIC value of 0.0625 mg/mL, as did berberine. Eugenol, an essential oil found in clove and Ocimum plants also gave an MBIC value of 0.0625 mg/mL. Finally, curcumin showed an MBIC at 0.25mg/mL.

Effect of the determined MBIC concentration against all the strong biofilm producers—Having
shown that biofilm formation by the isolate U5 is significantly inhibited by the 6 natural compounds (Fig. 3), it was tested if these compounds could also inhibit biofilm formation by all 7 strong biofilm producing isolates. The seven strong biofilm producing isolates of *K. pneumoniae* were most sensitive to biofilm inhibition by chitosan and reserpine. These 7 strains also showed similar levels of biofilm inhibition by linoleic acid, curcumin, berberine, and eugenol (at their MBIC concentrations). The raw data for inhibition by the six natural compounds in each of the seven high biofilm forming strains of *K. pneumoniae* is shown in Table 3. The results clearly prove that the MBIC concentration of the six natural compounds had reproducible biofilm inhibitory activity in each of the seven strong biofilm producing isolates of *K. pneumoniae*.

The comparative effects of six different test compounds on growth inhibition versus biofilm inhibition are summarized in Table 2. This comparison was done by measuring the ratio of MIC to MBIC value for each compound. Thus, a high ratio indicates that the concentration of the compound required to inhibit biofilm (MBIC) is significantly lower than the concentration required for inhibition of bacterial growth. The reserpine and berberine showed a 64 and 32 fold reduction in the concentration required for biofilm inhibition versus growth inhibition (MBIC versus MIC), respectively (Table2). Curcumin showed 50 fold reduction in its MBIC compared to its MIC. Linoleic acid and chitosan showed an 8-fold reduction in the MBIC versus MIC. The MBIC value for eugenol was 4-fold lower than its MIC value. Notably, the reduction in biofilm formation is unlikely to be due to the bactericidal effect of the compound, since the MIC values for each compound are significantly (4-64 fold) greater than the concentration of the compound which inhibited biofilm formation (MBIC). Therefore the data on MBIC truly reflect each natural compound’s ability to inhibit biofilm formation, and are not due to death of bacteria caused by the test compound.

![Fig. 3—Inhibition of biofilm in Isolate U5 with a strong biofilm forming capacity (OD-0.47) by different compounds. The effect of reserpine, berberine, linoleic acid, curcumin, eugenol and chitosan are shown. Controls (not treated with test compounds), was incubated with DMSO.](image)

**Table 2—Comparison of MIC vs MBIC of natural compounds.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (mg/mL)</th>
<th>MBIC (mg/mL)</th>
<th>Fold decrease in MBIC compared to MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reserpine</td>
<td>1</td>
<td>0.0156</td>
<td>64</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.25</td>
<td>0.0312</td>
<td>8</td>
</tr>
<tr>
<td>Berberine</td>
<td>2</td>
<td>0.0625</td>
<td>32</td>
</tr>
<tr>
<td>Chitosan</td>
<td>0.5</td>
<td>0.0625</td>
<td>8</td>
</tr>
<tr>
<td>Eugenol</td>
<td>0.25</td>
<td>0.0625</td>
<td>4</td>
</tr>
<tr>
<td>Curcumin</td>
<td>12.5</td>
<td>0.25</td>
<td>50</td>
</tr>
</tbody>
</table>

**Table 1—Minimum inhibitory concentration (MIC) of natural compounds in 7 strong and 2 moderate biofilm producing *K. pneumoniae* isolates**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Isolate</th>
<th>MIC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Curcumin</td>
</tr>
<tr>
<td>1</td>
<td>U5</td>
<td>12.5</td>
</tr>
<tr>
<td>2</td>
<td>S13</td>
<td>nd</td>
</tr>
<tr>
<td>3</td>
<td>B14</td>
<td>12.5</td>
</tr>
<tr>
<td>4</td>
<td>B15</td>
<td>nd</td>
</tr>
<tr>
<td>5</td>
<td>B16</td>
<td>nd</td>
</tr>
<tr>
<td>6</td>
<td>S20</td>
<td>nd</td>
</tr>
<tr>
<td>7</td>
<td>B24</td>
<td>nd</td>
</tr>
<tr>
<td>8</td>
<td>U25</td>
<td>12.5</td>
</tr>
<tr>
<td>9</td>
<td>P34</td>
<td>nd</td>
</tr>
</tbody>
</table>

MIC mg/mL

Nd=not determined
Discussion

The objective of the present study was to identify natural compounds which can inhibit biofilm formation in clinical isolates of *K. pneumoniae*. Towards this, 35 clinical isolates were screened, out of which 7 strong biofilm producers were identified (Fig. 1). The effects of eugenol, linoleic acid, chitosan, curcumin and efflux pump inhibitors berberine and reserpine were tested for their inhibitory effects on bacterial growth (Table 1). Their effect on the biofilm formation was analyzed in a representative high biofilm forming strain U5 (Fig. 3) to determine the minimal concentration required for inhibiting biofilm formation. Further this concentration was used against each of the high biofilm formers to find out if the biofilm formation was inhibited in each of the high biofilm formers (Table 3). The present result (Table 2) show that while antimicrobial activity of natural compounds requires a higher concentration to inhibit biofilm formation potential can be inhibited by much lower concentrations (MBIC). There are two reasons which could explain why the observed MBIC values are significantly lower than the MIC values for the 6 natural compounds. First, the biofilm inhibition assay tests the effect of compounds on the formation of biofilm, and not on penetration of compounds into pre-formed biofilms. This could explain why the biofilms in the present study are highly sensitive to small concentrations of the natural compounds. Second, the compounds tested, may have inhibited receptors and molecules involved in the quorum sensing pathway which is required for biofilm formation. It is well known that quorum sensing pathways are inhibited by very low concentrations of natural compounds.

Among the compounds tested, the compound with the least MIC against *K. pneumoniae* strains, was eugenol (0.125 mg/mL), followed by linoleic acid at 0.25 mg/mL; chitosan and reserpine at 0.5 mg/mL, berberine at 2 mg/mL and finally curcumin at 12.5 mg/mL. When the minimal concentrations for biofilm inhibition (MBIC) for each of the test compounds were analyzed in the isolates which produced the strongest biofilms, it was found that reserpine was most potent and effective at 25.7µM or 0.0156 mg/mL. This data shows that biofilm formation is highly sensitive to inhibition by low concentrations of reserpine. Reserpine is an alkaloid derived from *Rauwolfia vomitoria* or *Rauwolfia serpentine* root bark. Reserpine is used to treat high blood pressure and as a tranquilizer in patients with mental disorders. It is approved by the US FDA as a hypertensive drug. Reserpine has also been reported to be an inhibitor of both mammalian and gram-positive bacterial efflux.

Inhibitors of efflux pumps have been reported to inhibit biofilm formation. The efflux pump inhibitors (EPI): 1-(1-naphthylmethyl)-piperazine (NMP) thioridazine, and Phe-Arg β-naphthylamide (PAβN), were found to inhibit biofilm formation. In this context, the present data on the potent biofilm inhibitory activity of reserpine is consistent with the literature. Since reserpine is neurotoxic at higher levels, it may be possible to develop formulations of antibiotics with very low concentrations of reserpine to target tough biofilm forming bacterial infections.

Berberine, an antimicrobial compound had significant biofilm inhibitory activity at 0.0625 mg/mL, which was 32-fold lower than its MIC. Berberine is a plant alkaloid present in extracts of *Hydrastis Canadensis*, *Coptischenssis*, *Berberisa quifolium*, *Berberis vulgaris*, and *Berberis aristata*. Berberine extracts and decoctions have demonstrated significant antimicrobial activity against a variety of organisms including bacteria, viruses, fungi, ...
protozoans, helminths, and Chlamydia. Currently, the predominant clinical uses of berberine include bacterial diarrhea, intestinal parasite infections, and ocular trachoma infections. Berberine has been patented as a treatment against protozoan induced diarrhea. The present data suggest that it may be possible to use berberine as adjuvant therapy along with antibiotics to control biofilm forming bacterial infections.

The next most potent biofilm inhibitory compound was found to be linoleic acid, with an MBIC value of 0.0312 mg/mL (111.2 µM). Linoleic acid showed an 8-fold decrease in MBIC value compared to its MIC (Table 1). Long chain fatty acids such as linoleic acid have been shown to be inhibitory to the growth of gram positive bacteria. Linoleic acid (1-10mM) has been found to inhibit biofilm formation in *E.coli* K127. This inhibition has been attributed to the surfactant action of linoleic acid, which results in cell death due to increased permeability of the bacterial membrane. In this report, it has been shown for the first time, that linoleic acid also has a strong anti-biofilm activity in *K. pneumoniae*, a major human pathogen. Linoleic acid is a naturally occurring omega-6 essential fatty acid, and is recommended for patients with autoimmune diseases such as multiple sclerosis. Linoleic acid is also an essential constituent of skin cell membranes and is used in many beauty creams. The present data suggest that antimicrobial formulations for topical applications with a linoleic acid may have an advantage since they will also inhibit biofilm formation. In addition, it may be possible to develop coating of indwelling devices to prevent biofilm formation.

Chitosan, a hydrophilic biopolymer with antimicrobial activities was effective as biofilm inhibitor which is consistent with literature which has shown that chitosan-coated surfaces have anti-biofilm properties in vitro against some bacterial and fungal species. Eugenol has been found to inhibit biofilms in *Pseudomonas spp.*, *Candida albicans* and oral bacteria in dental plaque. Consistent with these reports, in the present analysis, eugenol was effective at biofilm inhibition at 0.0625 mg/mL or 381µM in *K. pneumoniae*. This data suggest that eugenol could be useful for controlling *K. pneumoniae* biofilms. Curcumin has been found to inhibit biofilm formation at a concentration of 8 µg/mL against *Helicobacter pylori*. This is the first study where the biofilm inhibition potential of curcumin against *K. pneumoniae* has been reported. Even though the MBIC concentration of curcumin is higher than that of the other compounds tested, there is potential in using curcumin in combination with antibiotics, since curcumin is known to be non-toxic at higher concentrations and it can be used in combination with antibiotics.

The present report is a comprehensive comparative analysis of the effects of various natural compounds on MIC and biofilm inhibition in strong biofilm forming isolates of *K. pneumoniae*. The results showed that reserpine, followed by linoleic acid are the most potent as biofilm inhibitors. While reserpine at higher concentration can be a neurotoxin, it can be used at low concentrations in combination with antibiotics. Linoleic acid as a natural fatty acid with biofilm inhibitory activity has much promise for use in topical application formulations and coating of indwelling implants.

The present report on the strong biofilm forming potential of *K. pneumoniae* strains is consistent with a study that found higher levels of biofilm formation by *K. pneumoniae* strains associated with pyogenic liver abscess. It has been shown that higher capsular polysaccharide production leads to higher biofilm formation. While it is clear that these natural compounds inhibit the biofilm formation, it would be interesting to find out the mode of action of these compounds. In addition, it is also important to find out if any of these compounds can disperse pre-formed biofilms. This report is significant since some of the compounds tested such as linoleic acid and curcumin found to inhibit biofilm formation are already used as a nutritional supplement or as an alternative medicine for different diseases. Adjuvant therapy with these compounds would be very useful in controlling stubborn biofilm forming infections of *K. pneumoniae*.

**Acknowledgment**

RV thanks CSIR, New Delhi for financial assistance. HM was supported by a SRF grant from CSIR.

**References**


