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Research Article

Study the effect of essential oils on microbial biofilm formation by *Klebsiella pneumonia*

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Abstract: Microbial infections associated with biofilms formation through quorum sensing have imposed a serious problem in their treatment using conventional antibiotics. This has prompted researchers to identify alternatives such as plant products as antimicrobial agents. Research on plant derived natural antimicrobials agents, has almost exclusively focused on their effects against planktonic micro-organisms, however, the biofilm forms are more resistant to antimicrobial agents and therefore more difficult to control, remain largely unexplored. In view of this, the present study evaluated the antibiofilm activity of five different plant essential oils i.e., Garlic (*Allium sativum*), Eucalyptus (*Eucalyptus grandis*), Neem (*Azadirachta indica*), Tulsi (*Ocimum sanctum*), Clove (*Syzygium aromaticum*) on the growth of the *Klebsiella pneumoniae* NCIM 2719 strain on the abiotic surface like urinary catheter. Out of the five essential oil tested, the Eucalyptus oil showed the maximum inhibition in biofilm formation on both tube and on the surface of the catheter.
Keywords: Urinary Tract Infection, *Klebsiella pneumonia*, biofilm, essential oils.

INTRODUCTION

Catheter associated urinary tract infection (CAUTI) has been one of the major nosocomial infection caused due to catheterization of patients for a long duration as studied in previous studies[1,2]. Many pathogens are responsible for CAUTI, but one of the most prevalent pathogen is *Klebsiella pneumoniae*, which forms a biofilm on the surface of the indwelling urinary catheter [3, 4]. The biofilm is formed with help of fimbriae that are present on the surface of the bacterial cells, through which they adhere on both the catheter as well as the host mucosal surface [4]. As a result of biofilm formation, the bacteria develop resistance against antimicrobial agents [5, 6] thus; chemotherapeutic agents are not effective enough to treat CAUTI. The biofilm formation also leads to many complications such as bacterimia, formation of bladder stones, etc. [7]. Some of the chemotherapeutic substances such as, Triclosan have shown to reduce the biofilm formation on catheters by *Klebsiella pneumonia* [8]. Although, it has been found that most of the antibiotics used against *K. pneumoniae* has been rendered ineffective due to the biofilm formation, which does not allow the antibiotic to enter inside the film and affect the individual bacterial cells [5].

Therefore, in order to limit the growth of the biofilm on the catheter surface or to reduce the number of bacterial pathogen present in the urinary tract, natural compounds are tested to replace the available chemotherapeutic agents. Many essential oils, consists of many plant secondary metabolites, have been used for a long time to kill different infectious pathogens. Most of the essential oils from Rosewood, Cedarwood, Lime, Orange, Rosemary, Sage, etc, have been shown to have bactericidal effect on *Klebsiella pneumonia* [9], but very few of them, such as, *Cuminum cyminum* seed essential oil [10], have been tested for biofilm inhibition formed by *Klebsiella pneumoniae* on the catheter.

In the present study, the effect of five selected essential oils i.e., Garlic (*Allium sativum*), Eucalyptus (*Eucalyptus grandis*), Neem (*Azadirachta indica*), Tulsi (*Ocimum sanctum*), Clove (*Syzygium aromaticum*), was tested on the biofilm formation by a *Klebsiella pneumoniae* NCIM 2719 on tubes as well as on catheters. The essential oil with the best inhibitory effect was selected and its effect was studied on the biofilm inhibition pattern on sterile urinary catheter.

MATERIALS AND METHODS

Klebsiella pneumoniae NCIM 2719 strain was obtained from National Chemical Laboratory, Pune, India. Isolates were grown and maintained in Luria-Bertani (LB) medium to give approximate 10^5 -10^6 CFU/ml. Five essential oils from plants as, Eucalyptus (*Eucalyptus globulus*), Garlic (*Allium sativum*), Clove (*Syzygium aromaticum*), Tulsi (*Ocimum Sanctum)* and Neem (*Azadirachta indica,)* were commercially available in local market. The Chemicals such as 1% Crystal Violet was obtained from Biolab Diagonostics (I) Pvt. Ltd., 95% Ethanol was prepared from Absolute alcohol made by S D Fine-Chem Ltd. and phosphate buffer saline (pH 7.3) was prepared in the laboratory itself. Sterile urethral catheter which was used to test the biofilm was manufactured by Romsons Scientific & Surgical Industries Pvt.ltd. Nonpyrogenic, 15ml High

clarity, screw cap Polypropylene Conical tubes, manufactured by BD Falcon were used.

Biofilm formation assay

Biofilm formation by *Klebsiella pneumoniae* strain in tubes was done based on the tube assay [11]. Two sets of three LB broth tubes were inoculated with 100µl/ml of culture inoculums. All the tubes were incubated for 96 hr along with their respective control tubes. After incubation the biofilm formation pattern of the organism in the tubes was observed.

Biofilm formation assay on catheter surface

The ability of *Klebsiella pneumoniae* NCIM 2719 strain to form biofilms on the urethral catheter was evaluated by biofilm assay [12]. Catheter was cut under sterile conditions into segments of 6 cm in length and incubated stationary in LB broth inoculated with bacterial culture. The tubes were incubated for 24 hrs. Next day, the catheter segments were transferred to fresh media. This step was repeated till 96hr. After incubation the biofilm formation pattern of the organism on the catheters was observed.

Biofilm inhibition assay

The anti biofilm activity of five selected essential oils (Eucalyptus, Garlic, Neem, Clove and Tulsi) was tested against *Klebsiella pneumoniae* by tube assay [11] in which 100 µl /ml of bacterial inoculums was inoculated in two tubes containing sterile LB broth. Sub-MIC concentration of 200µl/ml of each essential oils (Eucalyptus, Garlic, Neem, Clove and Tulsi) were added in each of the test tube with LB. Respective controls with only LB media as well as LB media with each essential oil was prepared and was incubated for 96hrs at 37^0C. The amount of biofilm produced in each tube was then quantified and measured. This test was performed in triplicate and the final reading was taken as an average of the three readings used further. The percentage reduction in biofilm formation was measured using the modified formula [13].

$$\% \text{ Reduction} = \frac{\text{Control OD} - \text{Test Sample OD}}{\text{Control OD}} \times 100$$

The graph was plotted with the obtained values and represented in the result section.

Biofilm inhibition assay on catheter surface

The most effective essential oil, which showed the maximum biofilm inhibition in the previous assay, was selected for and was subjected for biofilm inhibition assay on the catheter surface [12]. Inoculums with sterile LB broth with 5cm catheter tube were incubated with sub-MIC concentration of 200µl/ml of essential oil. Three tubes with controls were incubated at 37 °C for 96 hrs. The amount of biofilm produced in each tube was then quantified and measured by the method described earlier. This test was carried out in triplicates. The average readings were used to calculate the percentage reduction in biofilm formation.

RESULTS & DISCUSSION

Effect of Essential oils on Biofilm formation in tubes

The results obtained in the present study relieved that the tested five essential oils possess considerable reduction in biofilm formation. The optical density was measured for all the tubes. The final biofilm inhibition was calculated by subtracting the readings found of the inhibition of biofilm by the oils from the readings got from the control in which there were no oils added. The percentage reduction of biofilm was calculated and represented in the graph as seen below.

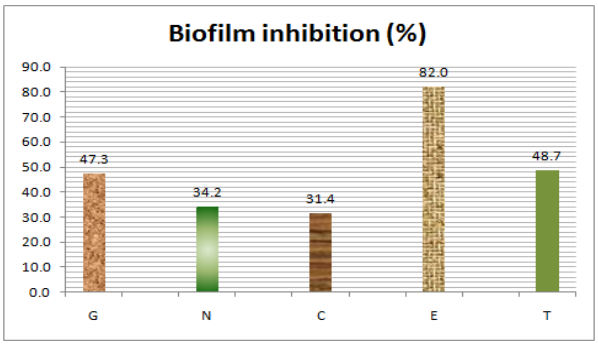

Fig 1: **Relative inhibition of Klebsiella pneumoniae biofilm by selected essential oils**
G: Garlic, N: Neem, C: Clove, E: Eucalyptus, T: Tulsi

Eucalyptus oil showed maximum inhibition of biofilm almost (82%), followed by Tulsi oil (48.7%), Garlic oil (47.3%), and Neem oil (34.2%) and finally clove oil showed least inhibition (31.4%). Eucalyptus oil with the maximum biofilm inhibition activity was selected to be the best oil among the rest of the oils in the present study and was further subjected to assess their biofilm inhibition activity on the surface of the urinary catheter.

Plants are important source of potentially useful biomolecules for the development of new chemotherapeutic agents. The first step towards this goal is to study their effect in, in vitro assay [14]. Many reports are available on the antimicrobial effect of essential oil as including Eucalyptus on the antibacterial effect on various pathogens including *K. pneumoniae*, as shown in the previous several studies [15]. However, not many reports are available on the studies of these essential oils for their antibiofilm activity. *K. pneumoniae* produced copious amounts of acidic polysaccharide capsules, which allowed it to adhere to epithelial cells and form biofilms on abiotic surfaces [16]. Recent studies have suggested that biofilm formation may be an important virulence factor for *K. pneumoniae* [17] In our study, the results showed that *Klebsiella* strain exposed to the sub-MIC concentration of the eucalyptus essential oil exhibited a reduction of

almost 82% in the OD_{595} reading compared to the control. The results of the present investigations correlate with the earlier results of the *Klebsiella* strains exposed to the sub-MIC concentration of cumin seed essential oil exhibited a reduction of two-fold or more in the OD_{595} reading compared to the control [10].

Effect of eucalyptus oil on biofilm formation on catheter

Based on the results obtained after performing the inhibitory effect of all the essential oils in the tube assay, eucalyptus essential oil was found to be most effective, was selected to assess the inhibition of biofilm formation on catheter. The optical density was measured for all the tubes. The final OD was obtained by taking the average of the three consecutive readings from each test repeat. The percentage reduction of biofilm was calculated by percentage difference formula. On catheter, the reduction in biofilm formation by Eucalyptus oil was found to be 13.1%.

The above result and discussion confers that the Eucalyptus oil shows the most reduction in the biofilm formed by *K. pneumoniae* strain used as compared to the other essential oils used, as Garlic, Neem, Tulsi and Clove oil. It showed significant reduction in both the tube assay and on catheters.

CONCLUSION

This study enables us to conclude that among the five selected essential oils, the Eucalyptus oil is highly effective with maximum inhibitory potential on biofilm formation by *K. pneumoniae* NCIM 2719. This study also demonstrated the inhibitory effect of most effective essential oil on the surface of the catheter with the sub-MIC concentration. Thus, the present study ascertains the value of plant derived molecules as a potential resource to combat with the challenges that are imposed by the current scenario of bacterial resistance. Additional *in vitro* studies would be needed to justify and further evaluate the potential of this oil as an effective biofilm inhibitor in treatment of such infections. Further phytochemical analysis responsible for these specific inhibitory activities is essential. Thus, the effect of these oils and its derived molecules could be of considerable interest for future development of new drugs.

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