



**EFFECT OF ESSENTIAL OILS ON BIOFILM FORMATION
BY *PROTEUS MIRABILIS***

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ABSTRACT

There is an increasing evidence of antibiotic resistance due to the formation of bacterial biofilm in various wounds and urinary tract infections (UTIs). Biofilm formation by *Proteus mirabilis* on urinary catheter has posed to be a great risk and further complicates the infection. The aim of the present study was to evaluate the effect of five essential oils, as, Garlic, Eucalyptus, Neem, Tulsi, and Clove, on the biofilm formation by *Proteus mirabilis* MTCC 425 strain. Out of all the five essential oils, Eucalyptus oil showed highest marked reduction in the biofilm formation by *P. mirabilis* MTCC 425. This was followed by a slight reduction in biofilm formation by Garlic, Tulsi, Neem and Clove oil. The Eucalyptus oil showed the maximum reduction in biofilm formation; hence it was used to check the inhibition effect on biofilm formation on catheter. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Eucalyptus oil against the *P. mirabilis* strain was also found out and the MIC was found out to be 300µl/ml and MBC to be 1200µl/ml

KEYWORDS: *Proteus mirabilis* MTCC 425, Catheter, Essential oils, Biofilm



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INTRODUCTION

In patients with abnormal urethras or due to the use of catheters many times, *Proteus mirabilis*, a Gram-negative, rod-shaped bacterium, causes catheter associated urinary tract infections.^{1, 2, 3} *P. mirabilis*, has a tendency to colonize the intestinal tract and then develops a reservoir for its spread into the periurethral region and then to the urinary tract. In the periurethral region, the catheter gets contaminated, which leads to the development of nosocomial infection.⁴ It also colonizes on the surface of kidney and bladder. More than *E.coli* and other urinary tract pathogens, *P. mirabilis* is found colonizing the urinary tract and kidney, as it can produce urease enzyme and some other virulence factor.⁵ On the surface of catheter, once the *P. mirabilis* has adhered and colonized, it forms a characteristic crystalline biofilm structure, which protects these bacteria from antibiotics and the immune response mounted by the host, thereby, aiding in perseverance of the infection. For the formation of the crystalline biofilm which blocks the catheter, very strong physical and chemical factors such as urease and capsule polysaccharide are produced.⁴ Once the mushroom-shaped crystalline biofilm is formed on the surface on the catheter and the epithelial surface of the urinary tract, the bacteria within the biofilm communicate with each other through quorum sensing, using signal molecules such as autoinducer-2 (AI-2), glutamine⁶, cyclic dipeptides and putrescine⁷, it is believed to help in the spread of the infection and resist antibiotic treatment.

The biofilm formation also leads to other complications such as bladder and kidney stones, which in turn leads to blocking the catheter⁴. In order to reduce the biofilm formation and thereby control the spread of the *P. mirabilis* growth further in the catheterized urinary tract, many compounds have been tested against the crystalline biofilm, such as nalidixic acid, tetra sodium EDTA, urease inhibitors, quorum sensing inhibitors⁸, Cinoxacin⁹, p-nitrophenylglycerol¹⁰, etc. Few antibiotics have shown better effectiveness against the biofilm, but due to the overuse of antibiotics, the potential development of resistance against these drugs limits its

application in this area⁸. To counteract this, few unique natural anti-biofilm compounds are now being tested against the biofilm formation on catheters by *P. mirabilis*. One such natural substance is the extract of a South American plant, *Ibicella lutea*, which affects the biofilm formation on glass and polystyrene^{8, 11}. Other natural compounds such as Resveratrol¹², *Millingtonia hortensis* L.¹³, Terpenes and terpenoids¹⁴, triclosan¹⁵, etc, have been shown to have both antibiofilm and antimicrobial effect on *P. mirabilis* strains.

Essential oils have been found since ancient times to have antimicrobial activity. Though few of the essential oil's effect as an antibacterial agent has been tested, such as Eucalyptus Oil and Garlic oil has been tested as an antibacterial agents on *P. mirabilis*^{16, 17, 18, 19}, but, their antibiofilm activity against *P. mirabilis* strains have not been assessed much. There are few reports on use of plant essential oils which are being tested for their antibiofilm activity on different pathogenic bacteria. Linalool-rich essential oil from *Croton cajucara* has been found to act against *C. albicans*²⁰, the essential oil of *Satureja thymbra* has also been shown to have a sufficient bactericidal effect on bacterial biofilms formed on stainless steel²¹, thyme and basil essential oil act against *Shigella* spp.²², trans-cinnamaldehyde effectively prevented uropathogenic *E. coli* biofilm on plates and catheters²³, and essential oil components such as α -pinene, 1,8-cineole, limonene, geranyl acetate and linalool, have been found effective against *L. monocytogenes* infection²⁴. The aims of the present investigation was to assess the antibiofilm formation activities of the five different essential oils including Garlic, Eucalyptus, Neem, Tulsi and Clove against the *P. mirabilis* MTCC 425, in a polypropylene tubes and on catheters and also to carry out the evaluation of the minimum inhibitory and microbicidal concentration of the most effective oil resulting to antibacterial activity.

MATERIALS AND METHODS

P. mirabilis culture was obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, and Chandigarh, India. The MTCC No. is 425. The bacterial culture was further subcultured and stored on Tryptone Soy Agar (TSA). Isolated colonies of *P. mirabilis* MTCC425 were inoculated in TSB medium for 24 hrs and then cultures were diluted with fresh TSB to give approximate 10^5 - 10^7 CFU/ml. Tryptone Soy Broth (TSB) media was used for conducting further experiments which was obtained from HiMedia Laboratories Pvt. Ltd. Five different essential oils viz. Garlic, Eucalyptus, Neem, Tulsi and Clove, were purchased from commercially available brands in the local market. The chemicals such as 1% Crystal violet was obtained from Biolab Diagnostics (I) Pvt. Ltd., 95% Ethanol was prepared from Absolute alcohol made by S D Fine-Chem Ltd. and Phosphate buffered saline (PBS) was prepared in the laboratory. Sterile urethral catheter was used to test the biofilm formation, which was manufactured by M/S Romsons Scientific & Surgical Industries Pvt.Ltd, Agra. BD Falcon, Nonpyrogenic, 15ml/High clarity, screw cap Polypropylene Conical tubes, manufactured by BD Biosciences, USA were used.

i. Biofilm formation assay^{25, 26, 27}

Biofilm formation by *P. mirabilis* MTCC 425 strain was done based on the tube assay based on the earlier methodologies with only with the modifications in the medium and volumes used. A set of two tubes of Tryptone Soy Broth (TSB) were prepared. One tube from each set was inoculated with inoculums of culture. The tubes were incubated for 96 hrs along with their respective control tubes. After incubation, the biofilm formation pattern of the organism was observed. This test was done in triplicates. After incubation the tubes were aspirated, washed with PBS and stained with 2 ml of Crystal Violet (1.0%) for 5 min and then each tube was washed again with PBS. The Biofilm was visualized and classified as strong, moderate, weak or negative. The amount of biofilm produced in each tube was then quantified by adding 3 ml of 95% ethanol which dissolved the crystal violet stain. This dissolved

stain which contained the bacteria present in the biofilm was then measured by UV2301Spectrophotometer (ISCE021) at 570nm.

ii. Biofilm formation on catheter assay²⁵

The ability of *P. mirabilis* MTCC 425 strain to form biofilms on catheter was evaluated on the urethral catheter, and was estimated by biofilm assay with the following modifications. A set of two tubes of sterile 6ml TSB were prepared. Catheter was cut under sterile conditions into segments of 6 cm in length and placed in all the tubes. One tube was inoculated with bacterial culture and the other uninoculated tube was considered as Control tube. They were incubated 24 hrs. Then, catheter segments were transferred to fresh media. This step was repeated for five days. After incubation for 96 hrs, the biofilm formation pattern on the catheter by the organism was observed. This test was done in triplicates.

After incubation the tubes were aspirated, washed with PBS and stained with 2 ml of Crystal Violet (1.0%) for 5 min and then each tube was washed again with PBS. The Biofilm was visualized and classified as strong, moderate, weak or negative. The amount of biofilm produced in each tube was then quantified by adding 3 ml of 95% ethanol which dissolved the crystal violet stain. This dissolved stain which contained the bacteria present in the biofilm was then measured by UV2301Spectrophotometer (ISCE021) at 570nm.

iii. Measurement of Inhibition of Biofilm^{25,29}

The anti biofilm activity of five selected essential oils (Eucalyptus, Garlic, Neem, Clove and Tulsi) was checked against *P. mirabilis* MTCC 425 by tube assay. Twelve tubes containing 2 ml sterile TSB were prepared. The first tube was labeled as negative control, which contained only the TSB media, the second tube was labeled as positive control, which contained 2ml TSB media, and 100 μ l /ml of bacterial culture. Five set of two tubes for each essential oil (Eucalyptus, Garlic, Neem, Clove and Tulsi) was prepared in such a way that in each set, one tube contained only TSB media and sub-MIC level 200 μ l /ml of essential oil and the other tubes contained the

same and was inoculated with 100 µl /ml of bacterial culture. All these tubes were kept for incubation for 96 hrs. After incubation the tubes were aspirated, washed with PBS and stained with 2 ml of Crystal Violet (1.0%) for 5 min and then each tube was washed again with PBS. The Biofilm was visualized and classified as strong, moderate, weak or negative. The amount of biofilm produced in each tube was then quantified by adding 3 ml of 95% ethanol which dissolved the crystal violet stain. This

dissolved stain which contained the bacteria present in the biofilm was then measured by UV2301 Spectrophotometer (ISCE021) at 570nm. This test was done in triplicates and the final reading was taken as an average of all the three readings. The percentage reduction in biofilm formation for all the oils was measured by the following formula which is a modification of a formula used in a previous study:

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

A graph was plotted with the obtained values.

iv. Measurement of Inhibition of Biofilm on Catheter^{25, 29}

The anti biofilm activity of essential oil, which showed the maximum anti-biofilm activity in the previous test was checked against *P. mirabilis* MTCC 425 by tube assay. A set of four tubes of sterile TSB media was prepared with a sterile piece of catheter tube in it. One tube was labeled as negative control which contained only the media and the catheter. A second tube was labeled as positive control which was inoculated with 100 µl /ml of *P. mirabilis* MTCC 425 culture. The third tube contained only media and sub-MIC level, 200µl/ml of the best essential oil which showed the maximum anti-biofilm activity in the previous test and the catheter. The last tube was labeled as the test sample, which contained media, inoculums, the best essential oil and catheter. These tubes were incubated at 37 °C for 96 hrs. After incubation the

catheter segments were removed, washed and kept in a tube containing 6 ml of PBS for washing to remove media & planktonic cells. Catheter pieces were then incubated in 6ml of 1% crystal violet containing tube to stain the Biofilm formed and incubated for 5 minutes. Catheters were then washed with 6ml PBS twice to remove excess stain. Catheters were then washed in 95% ethanol containing tubes (6ml), for 25 mins at Room temperature to dissolve the crystal violet present on biofilm. It was quantified by using UV2301 Spectrophotometer (ISCE021) at 570nm. This test was done in triplicates and the final reading was taken as an average of all the three readings. The percentage reduction in biofilm formation for all the oils was measured by the following formula which is a modification of a formula used in a previous study:

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

A graph was plotted with the obtained values.

v. Minimum Inhibitory and Minimum Bacterial Concentrations¹⁶

The Minimum Inhibitory Concentration (MIC) assay was conducted Broth dilution method. Out of five essential oils, the oil showing maximum anti-biofilm activity was tested for

determining its MIC against bacterial culture. One set of seven tubes was prepared, containing 1ml of TSB. In six TSB tube different volumes of the oil showing maximum anti-biofilm activity, from 100µl to 1200 µl were added. In all TSB tubes 100µl/ml of clinical

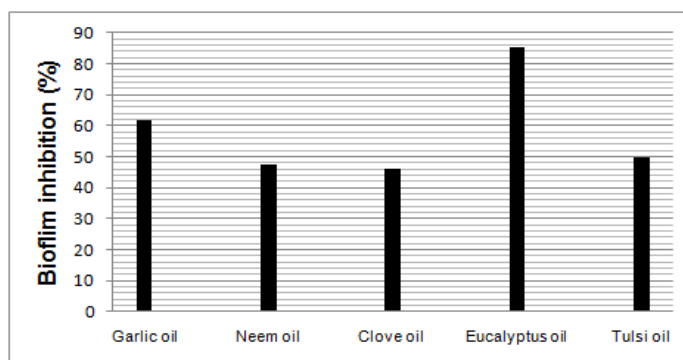
isolate was inoculated and was incubated at 37°C for 24hrs. 100µl of these assay broth were sub-cultured on the fresh media plates of Tryptone Soy Agar which were incubated at 37°C for 24 hrs. After 24 hrs of incubation the viable count was carried out based on which the MIC values were recorded. The MBC values were determined by removing 100 µl of bacterial suspension from the MIC tubes that did not show any growth and subcultured onto Tryptone Soy agar plates and incubated at 37°C for 24 hrs. After incubation, the concentration at which no visible growth was seen was recorded as the MBC.

RESULTS

1. Effect of Essential oils on Biofilm formation in tubes

The results show that there was a considerable reduction in biofilm formation in tubes by all the essential oils which were used. The Optical Density (OD) was measured for all the tubes kept and the final OD 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 average reading was considered and the percentage reduction of biofilm was calculated and represented in the graph as seen below.

Graph 1
Relative inhibition of *Proteus mirabilis* biofilm by selected essential oils



2. 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 oil was found to be most effective, and was used to assess the inhibition of biofilm formation on catheter. The optical density was measured for all the tubes kept and the final OD 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 average reading was considered and the percentage reduction of biofilm was calculated by percentage difference formula. The Eucalyptus oil was found to reduce the biofilm formation on catheter by 43.65%

3. MIC and MBC Assay

After observing the inhibitory effect of Eucalyptus oil (200 µl/ml), on biofilm formation, the Minimum Inhibitory

Concentration (MIC) as well as the Minimum Bactericidal Concentration (MBC) assay was performed using Eucalyptus oil with concentration ranging from 100 µl/ml to 1200 µl/ml. Equal amount of the assay broth (100 µl) was plated to get viable count and to ensure the antibiofilm and antimicrobial activity of the oil. The minimum inhibitory concentration (MIC) was observed at 300µl/ml, while the minimum bactericidal concentration (MBC) of eucalyptus oil was found to be 1200µl/ml.

DISCUSSION

Biofilm-forming bacterium constitutes a major medical problem because of the decreased vulnerability of bacteria, within the biofilm, to host defenses and antibiotic treatments. Many drugs, antibiotics and plant extracts have been under assessment for their ability to reduce

such biofilm mediated resistance of several pathogens. Present study evaluated the effect of essential oils against the biofilm formation on the urinary catheter formed by *P. mirabilis* MTCC 425 strain, which commonly infect the urinary tract in bed-ridden patients in India. To our comprehension, this is the foremost account of the effect of the selected essential oils (Garlic, Eucalyptus, Neem, Tulsi and Clove), on CAUTI. The various essential oils used in this study, have previously been tested as either antimicrobial or as antibiofilm agent for various microbial pathogens, however for the first time it is being tested on biofilm formation by *P. mirabilis* MTCC 425 strain. The antibacterial or sometimes antibiofilm property of all the mentioned essential oils has been tested on pathogens such as *Escherichia*, *Salmonella*, *Staphylococcus*, *Klebsiella*, *Proteus*, *Bacillus*, *Mycobacterium tuberculosis*¹⁹, *Candida albicans*²⁰, and *Listeria monocytogenes*²⁴. Furthermore, the effects of various essential oils have been checked against CAUTI mostly caused by *Staphylococcus epidermidis*, *Escherichia coli*²³ and *Candida*²⁸. Here, we have specially shown the effect of these five essential oils on biofilm formed on catheter by *P. mirabilis* MTCC 425 strain.

The percentage reduction of biofilm on tubes was found maximum for eucalyptus oil (85.30%), followed by garlic oil (61.68%), tulsi oil (49.63%), neem oil (47.65%) and finally clove oil (46.16%). Eucalyptus oil also showed marked reduction in biofilm formation on urinary catheter. The most effective essential oil out of the five essential oils used was found to be Eucalyptus oil. Hence, the present study confirms the effectiveness of the eucalyptus oil to treat *P. mirabilis* infection. The inhibitory effect of the selected essential oil was

specifically on the biofilm, as concentration of the essential oil used during antibiofilm assay (200 µl/ml) was lesser than that of the MIC (300 µl/ml) & MBC (1200 µl/ml) assay. For this reason, the concentration of oil used is affecting the biofilm forming ability of the pathogen rather than just reducing the numbers of bacterial cells.

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 *P. mirabilis* MTCC 425 strain. Thus, the study ascertains the value of plant derived remedies and active biomolecules as a potential resource to combat with the challenges imposed by bacterial drug resistance. Further phytochemical analysis is essential to identify the specific active components which might be responsible for the inhibitory activity. The effect of these oils and derived molecules can be further studied in vitro to observe the associated effects which could be of considerable interest for future development of new drugs.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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