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Anti-biofilm potential of aqueous *Eucalyptus* leaf extract against nosocomial pathogens: *Staphylococcus* and *Pseudomonas aeruginosa*

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Abstract
Biofilm infections pose a major challenge for the physicians and researchers worldwide due to their inherent recalcitrance towards antibiotic treatment and numerous survival and immune evading strategies.

The present study focuses on exploring alternative safe solutions to cater to the biofilm bacteria. It evaluated the anti- biofilm potential of aqueous leaf extract obtained from Eucalyptus against two clinically significant bacteria (*S. aureus* and *P. aeruginosa*) forming potent biofilms. Also, these bacteria have been shown to be highly resistant to the action of antibiotics making the situation worse.

In vitro biofilms were established for both the strains on microtiter plate surface and on Foley catheter surface. The treatment was done with Eucalyptus leaf extract (1:1) and the biofilm progression was monitored. Results indicates significant decline in the adhered viable load in both the treated bacterial species. Eucalyptus aqueous extract from leaves represented a potential alternative with significant anti-biofilm ability against *S. aureus* and *P. aeruginosa*. This calls for further exploring the potential of Eucalyptus extract in combination with antibiotic as combination systems for possible synergism against resistant clinical isolates.

Keywords: Biofilm, eucalyptus, antibacterial, coagulase negative staphylococci

Introduction

The emergence and spread of resistant bacterial strains is becoming a major global issue with the possibility of return of the pre-antibiotic era. One of the most frequently encountered nosocomial pathogens where antibiotic resistance is rising include *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *S. aureus* has become an important source of both community- and hospital-acquired infections worldwide. The treatment of staphylococcal infections is difficult due to its intrinsic ability to develop resistance to the deployed antibiotics and form potent biofilms. Moreover, the emergence of resistant strains i.e methicillin-resistant *S. aureus* (MRSA), glycopeptide intermediate *S. aureus* (GISA) and vancomycin intermediate *S. aureus* (VISA) have made management of such infections a major challenge [1, 2].

Similarly, *P. aeruginosa* is capable of acting as a persistent opportunistic pathogen in immune-compromised individuals such as elderly, burn and wound patients, AIDS patients, diabetics etc. causinga range of infections such as otitis media, urinary tract infections, catheter and foreign body infections, serious wound infections, chronic air way colonization(Cystic fibrosis) etc. The virulence factors secreted which include the exotoxins and endotoxins released by *P. aeruginosa* continue to cause chronic inflammation which makes infections difficult to treat leading to a life-threatening situation [3].

Both these bacteria are known to form biofilms, and the bacteria residing in biofilms have been shown to be highly resistant to the action of antibiotics [4]. The phenotypic resistance of bacteria residing in biofilm is due to the poor penetration of most of the antibiotics due to the biofilm matrix that acts as a barrier. Also, biofilm bacteria show slow growth and act as persister cells which are not killed or acted upon by majority of the drugs [5, 6, 7]. Cells associated with a biofilm are 100- to 1,000-fold more resistant than their free-swimming planktonic counterparts [8, 9]. Thus, under clinical conditions where biofilms play a major role in pathogenesis, including wounds in diabetic patients, catheter associated urinary tract infections and endocarditis, treatment failures are high despite the long duration of many treatments [10].

The worldwide escalation in antibiotic resistance rates calls for search of effective and safe alternatives to be investigated with priority. Although the development of resistance is unavoidable as it is a pivotal aspect of microbial evolution, but the urgent need for development of novel antibacterial products is equally and essentially prudent.

Herbal medicines have been long used as part of traditional therapy with proven minimal side effects [11, 12]. However, in recent years, interest has rekindled among the scientific community in investigating these as alternative medicine to address the challenge of drug resistance. Eucalyptus is one of the world's important and most widely planted genera [13]. Eucalyptus species is well known medicinal plant with biological and pharmacological properties. Out of this, *Eucalyptus globulus* is the main furnisher of essential oils which have proven antimicrobial, anti-inflammatory, anti-oxidant, anti-diabetic and even cancer fighting potential [14, 15]. The present study was focused on evaluating the anti-biofilm potential of aqueous extract obtained from *E. globulus* leaves against two most clinically significant pathogens (*S. aureus* and *P. aeruginosa*) capable of forming potent biofilms and associated with life threatening infections and issues of drug resistance. The study enables us to reinforce our understanding into the use of traditional herbal medicines as an effective and safe alternative to the commonly used antibiotics in preventing as well as treating biofilm infections.

Material and Methods

Bacterial Strains

S. aureus strains were isolated from skin and nares of healthy volunteers. For this, skin swabs and nasal swabs from n=12 healthy volunteers were taken in nutrient broth tubes. These swabs were streaked on nutrient agar plates and incubated at 37°C for 24 hours. Colonies so obtained on nutrient agar plates were further identified on the basis of a) Microscopic analysis: Gram reaction and morphology b) Colony morphology c) Catalase reaction d) Coagulase test e) Mannitol fermentation and growth on selective medium i.e Mannitol salt agar (MSA).Clinical strain of *P. aeruginosa* was procured as a gift sample from Department of Microbiology, Panjab University. It was further checked for its purity and identified again on the basis of Gram reaction, motility, oxidase test, green pigmentation in broth and on media plates. Strains isolated were later stored as slants at 4°C till further use. *S. aureus* strain S-2 was used for further experimentation and biofilm formation.

Extraction of Eucalyptus leaf extract

Sample collection and Pre-treatment

The Fresh leaves of the Plant (Eucalyptus leaves) were collected from Ropar, SAS Nagar, Mohali, Panjabi and placed in polyethylene bags and transported to the Microbiology lab of Food Science Department, MCM DAV College for Women, Chandigarh. Eucalyptus leaves were carefully washed using tap water and dried under sunlight to remove moisture. The dried leaves were milled/grinded manually using pestle motar. The powdered leaves were transferred to a glass sealed cans and placed in the refrigerator before the extraction process.

Extract preparation

The aqueous extract of dried plant leaves was made in distilled water. About 10 grams of leaf powder was taken and mixed in 100 ml of distilled water. The mixture was taken into 250 ml sterile conical flasks, plugged with sterile cotton and kept in Shaking Incubator at 150 rpm for 24 hours. The solution was then filtered through muslin cloth. This process was repeated three times after which a clear aqueous extract of the plant was taken. This was finally concentrated on water bath at 50°C to obtain the final aqueous extract which was labeled and stored in airtight containers at 4°C till further use.

Optimisation of *In vitro* Biofilm formation by *S. aureus* (Microtiter plate method)

S. aurues biofilm was grown in 96-well microtiter plate according to method described by Bedi *et al.* [16] Briefly, 100 µl of nutrient broth and 100 µl of bacterial culture (optical density i.e O.D at 600 nm = 0.3) equivalent to 10^8 CFU/ml of *S. aureus* (S-2) were added to the wells of microtiter plate and incubated at 37°C overnight. In each plate, control wells containing sterile media were included that acted as plate sterility control. After every 24 h, planktonic bacteria were removed and a set of two wells (corresponding to each day) were washed thoroughly 3 times with 0.85% NaCl. Adherent biofilm was scraped from two wells, suspended in 0.85% NaCl and vortexed for 3 min. Bacterial load of biofilm was enumerated by viable cell counting. For crystal violet staining, duplicate wells were stained with 0.1% of crystal violet stain for 10 min, gently washed with 200 µl fresh normal saline and then de-stained with 95% of ethanol. The content from wells was transferred to flat bottom plate and analyzed at 595 nm. In rest of the wells, spent medium was replaced with fresh sterile nutrient broth and microtiter plate again incubated at 37°C overnight. This procedure was repeated until 7th day of experiment.

P. aeruginosa (*in vitro* catheter assay)

Sterile Foley catheter pieces of 1 cm were cut and *P. aeruginosa* biofilm was allowed to develop under static conditions for 7 days. [17] The catheter pieces were transferred to fresh medium ever 24 h. Each day, the catheter pieces in duplicate were removed, rinsed three times with and cells were removed from the surface by scraping with a sterile scalpel blade. The cells were sonicated using a low-level sonication cycle and vortexed for 30 sec. Dispersed samples were centrifuged and the biofilm cells were suspended in one ml phosphate buffered saline (PBS), pH 7.4. Serial dilutions were prepared and plated on nutrient agar plates to determine the cell viability. This procedure was repeated until 7th day of experiment. For visualization of adhered biofilm cells [18] on catheter and within its lumen, two additional catheters were removed and rinsed with distilled water. After drying at room temperature for 15min, each catheter was cut longitudinally into two half to expose the catheter lumen.700µl of 1% crystal violet was added to catheters for 20 min. The stained biofilms were solubilized with 33% acetic acid, and absorbance was determined at 630 nm.

Antimicrobial susceptibility (well diffusion assay)

Preparation of Inoculum

Overnight cultures of test strains of *S. aureus* (S-2) and *P. aeruginosa* were prepared by inoculating isolates into nutrient broth and incubated at 37°C for 24 hr. Next day, cell density was adjusted with PBS, pH 7. 4 so as to obtain an O.D of 0.3 at 600 nm (corresponding to 10^8 CFU/ml).

Antimicrobial Sensitivity Testing

Agar well diffusion method was used to determine the zone of inhibition. O.D adjusted cultures of the two strains were spread plated using a sterile cotton swab over the entire surface of their respective Mueller Hinton agar plates. Petri plates were allowed to dry. About 3-4 wells in each plate of 6 mm diameter and 5 mm depth were punched in agar surface with the help of a sterilized borer for placing the samples. Different volumes of the undiluted leaf extract were dispensed into respective wells and gentamicin (10 µg) (Himedia) was used as a positive control. Dimethyl sulfoxide (DMSO) was used as negative control. The plates were then left at room temperature for 30 minutes and then incubated for 24 hours at 37°C. After incubation, the zones of inhibition were measured and the results reported in millimeters (mm). All the tests were run in triplicate and the average result was taken.

Effect of aqueous Eucalyptus extract on biofilm formation by *S. aureus* (S-2)

S. aureus biofilm was grown in 96-well microtiter plate as described above. To study the ability of the extract to prevent biofilm formation, the wells were treated by adding aqueous leaf extract (1:1) ratio with media. All the test wells were seeded with O.D adjusted *S. aureus* culture. In each plate, positive control wells containing media and test organism were included along with negative control wells containing media only. At each time point (on different days post-inoculation), wells were processed to determine the viable load and absorbance by crystal violet staining method as described above.

Effect of aqueous Eucalyptus extract on biofilm formation by *P. aeruginosa*

P. aeruginosa biofilm was grown on Foley's Catheter as described above. To study the ability to prevent biofilm formation, aqueous leaf extract (1:1) ratio was added to the flask containing cut pieces of catheter suspended in media broth. The test flask was seeded with O.D adjusted *P. aeruginosa* culture At each time point (on different days post-inoculation), catheter pieces shall be removed and processed to determine the viable load and absorbance by crystal violet staining method as described above. Similarly, control flask seeded with test organism and catheter was also put up along with test flask.

Statistical Analysis

All experiments were performed in duplicate and repeated at least three times on different days to validate the reproducibility of experiments. The effect of different treatments on biofilm eradication was evaluated by the Student's t-test and $P < 0.05$ was considered significant. Data were analysed using Excel software.

Results

A total of two strains of *S. aureus* and two strains of CoNS were isolated (Table-1). All the strains were gram positive cocci in bunches, catalase positive. *S. aureus* strains gave golden yellow colonies on nutrient agar. They were coagulase positive and showed mannitol fermentation. The colonies seen on selective media i.e Mannitol salt agar (MSA) exhibited change in colour to yellow confirming positive mannitol fermentation. The other two strains exhibited no change in color with non-mannitol fermentation on MSA plates. *S. aureus* (S-2) was selected for further experimentation and biofilm formation.

Note: Data on strain isolation and identification with pictoral representation of tests performed for both *S. aureus* and *P. aeruginosa* can be provided as supplementary data if required.

Table 1: Strains isolated and their characteristic features

	Source	Gram Reaction	Colony Morphology	Catalase & Coagulase Reaction	Mannitol Fermentation
S. aureus(S-1)	Spoiled food sample	Gram Positive Cocci in bunches;	Golden Yellow colonies on Nutrient agar	(+);(+)	(+)
S. aureus –(S-2)	Nasal	Gram Positive Cocci in bunches;	Golden Yellow colonies on Nutrient agar	(+);(+)	(+)
CoNS (S-3)	Skin	Gram Positive Cocci in bunches;	White colonies on Nutrient agar	(+);(-)	(-)
CoNS(S-4)	Nasal	Gram Positive Cocci in bunches;	White colonies on Nutrient agar	(+);(-)	(-)

P. aeruginosa strain was Gram negative, rod, showing blue to blue-green pure isolated colonies on nutrient agar, it showed positive results for oxidase test and motility test.

Antimicrobial Susceptibility Test

The data showed that *S. aureus* and CoNS spp. were sensitive to 25 µl showing negligible zones at 5 and 10 µl while *Pseudomonas* spp. required 50 µl of eucalyptus extract (Table 2) to show a zone diameter of 8 mm. With an increasing dose of extract added to wells, the resulting diameter of the zone of inhibition also increased for all the test organisms. The results of the study revealed that eucalyptus extract has antibacterial activity against Gram-positive as well as Gram-negative bacteria resistant to commonly used antimicrobial agents.

Table 2: The mean zone of inhibition exhibited by various doses of aqueous leaf extract of Eucalyptus against various bacterial test strains

Test Strains	Different doses of undiluted aqueous Eucalypus leaf extract							Gentamycin (10µg)
	5 µl	10 µl	25 µl	50 µl	100 µl	150 µl	200 µl	
S. aureus (S-1)	-	-	+	++	+++	+++	+++	+++
S. aureus (S-2)	-	-	+	++	+++	+++	+++	+++
CoNS (S-3)	-	-	+	++	+++	+++	+++	+++
CoNS(S-4)	-	-	+	+++	+++	+++	+++	+++
P. aeruginosa	-	-	-	+	++	+++	+++	++
Zone of inhibition. –: < 5 mm, +: 5-9 mm, ++: 10-19 mm, +++: > 20 mm								

50 µl of gentamycin (10µg) was used as control and showed a zone diameter of 15 mm against *P. aeruginosa* while *S.*

aureus and CoNS spp. showed zone diameters greater than 20 mm with gentamycin.

***In vitro* Biofilm formation and Treatment with Eucalyptus aqueous extract *S. aureus* (S-2):** *S. aureus* formed biofilm on surface of 96 well microtiter plate showing day dependent increase in adhered bacterial cells(Log CFU/ml).Peak biofilm was formed by day 3 (8 log CFU/ml) with no further increase seen (Fig. 1). Higher load was maintained till day 7.

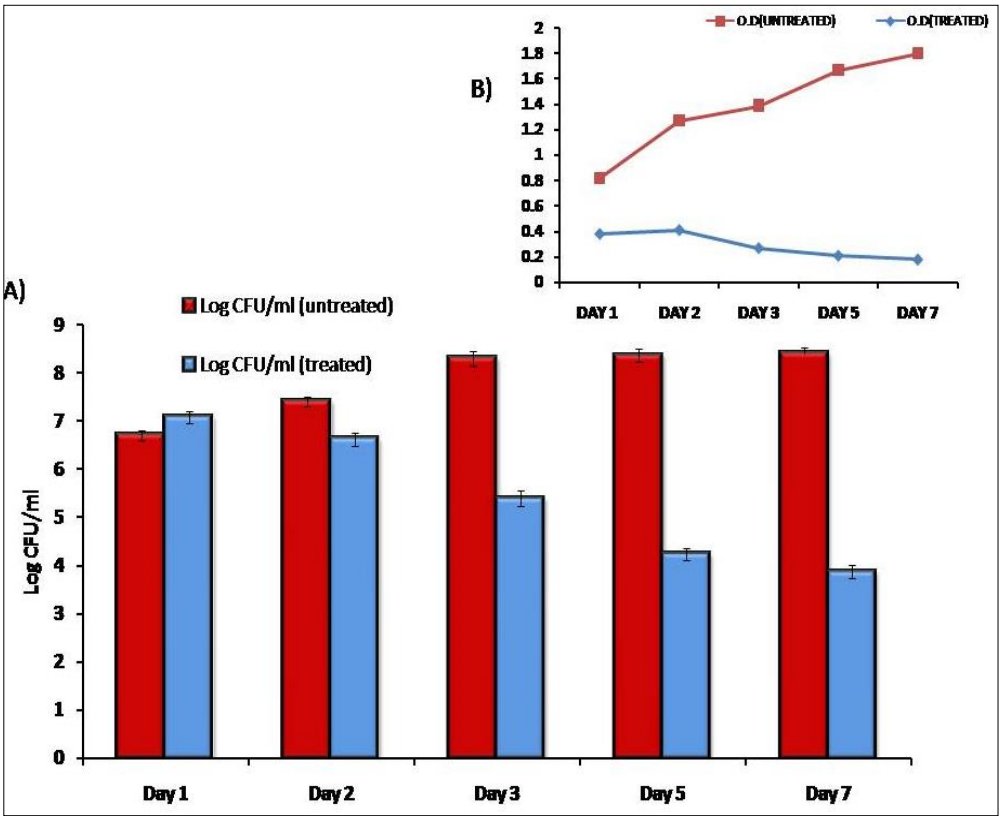

Fig 1: Comparison in terms of A) Log CFU/ml of *S. aureus* adhered on 96 well microtiter plate on different days between Eucalyptus extract treated and untreated wells and B) O.D (600 nm) of *S. aureus* adhered on 96 well microtiter plate on different days between Eucalyptus extract treated and untreated wells.

However, with eucalyptus extract treatment, significant decrease in adhered cells was seen by day 2 itself (p<0.05). By day 3, bacterial counts of 5.4 log CFU/ml was obtained with highly significant decrease of >3 log CFU/ml (p<0.05). Minimal adhered population was obtained in treated wells by day 7 (3.87 log CFU/ml) (Fig.1).Similarly, there was significant decrease in the absorbance as well as seen in treated samples. Absorbance did not increase beyond a value of 0.4 (on day 1) with sharp decline seen by day 3 and thereafter. However, in control wells, absorbance of adhered population showed a day dependent increase with peak value of 1.6 (Fig.1).

As seen in Fig.2, the crystal violet staining of biofilm cells in untreated wells clearly showed increase in adhered cell biomass on different days where a layer of cell formation was quite visible whereas, in wells treated with Eucalyptus extract, by day 7 there was minimal adherence of *S. aureus* seen and eventually it cleared thereafter.

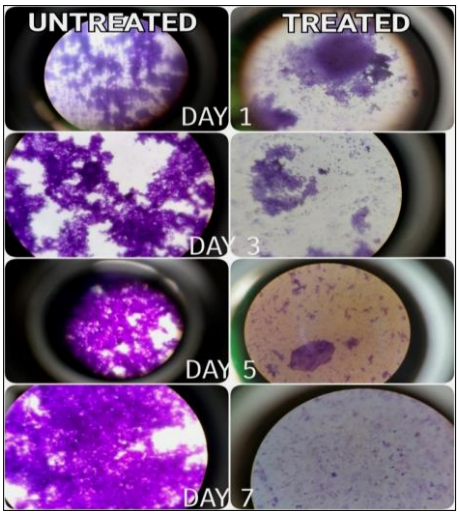

Fig 2: Pictoral representation of microscopic view of various stages of formation of biofilm on successive days as seen through crystal violet staining.

P. aeruginosa

In vitro biofilm formation of *P. aeruginosa* was established on Foley's catheter. Progression of biofilm formation was evaluated on basis of absorbance (O.D) and adhered bacterial load (CFU/ml) on different days post inoculation.

The results as depicted in Fig. 3, indicate that there was a day dependent increase in both adhered biomass and absorbance of adhered population. Peak load was seen on day 3 with a bacterial count of 7.61 log CFU/ml which was maintained till day 5. This correlated well with the increase in O.D on different days post inoculation. Peak absorbance was also seen by day 5 reaching to a value of 0.45 at 600 nm. Therefore, by day 3 young biofilm was formed on the surface of catheter pieces and peak was maintained till day 7.

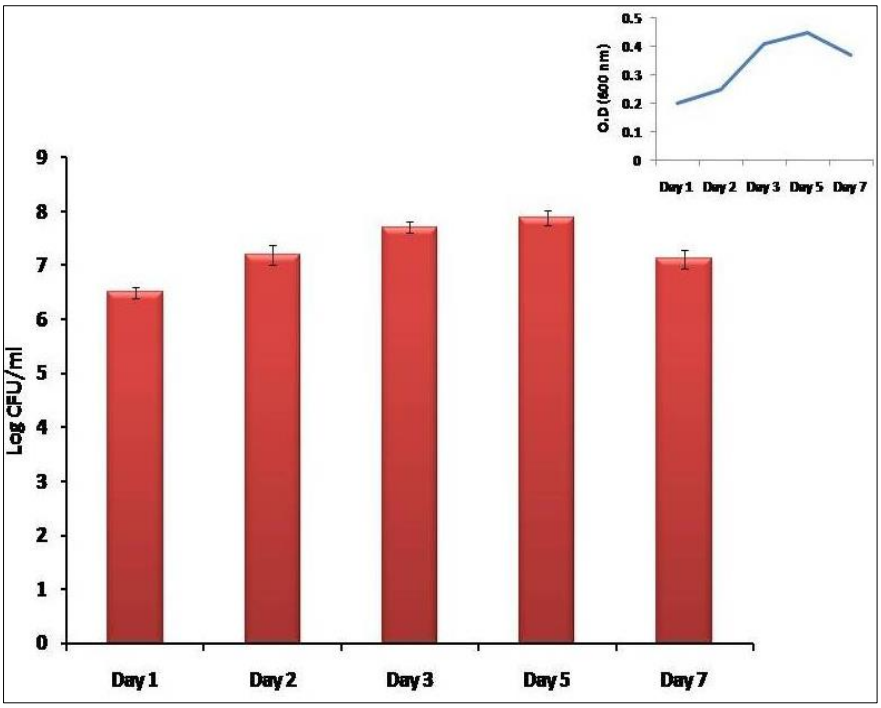

Fig 3: *In vitro* biofilm formation of *P. aeruginosa* on Foley's catheter determined in terms of CFU/ml adhered and optical density.

Treatment with eucalyptus extract on *P. aeruginosa* biofilm

Results (Fig. 4) showed a day dependent decrease in the adhered population (CFU/ml) as compared to untreated control. By day 3, there was a significant decrease of more than 2 log CFU/ml and by day 5 and 7, negligible load was seen adhered on the treated catheters with minimal count of 2.15 log CFU/ml. This indicates that eucalyptus extract has significant potency in decreasing the adhered population of this bacteria inhibiting the formation and maturation of biofilm on catheter surfaces. Results of absorbance (i.e O.D) on different days post treatment also supported this finding.

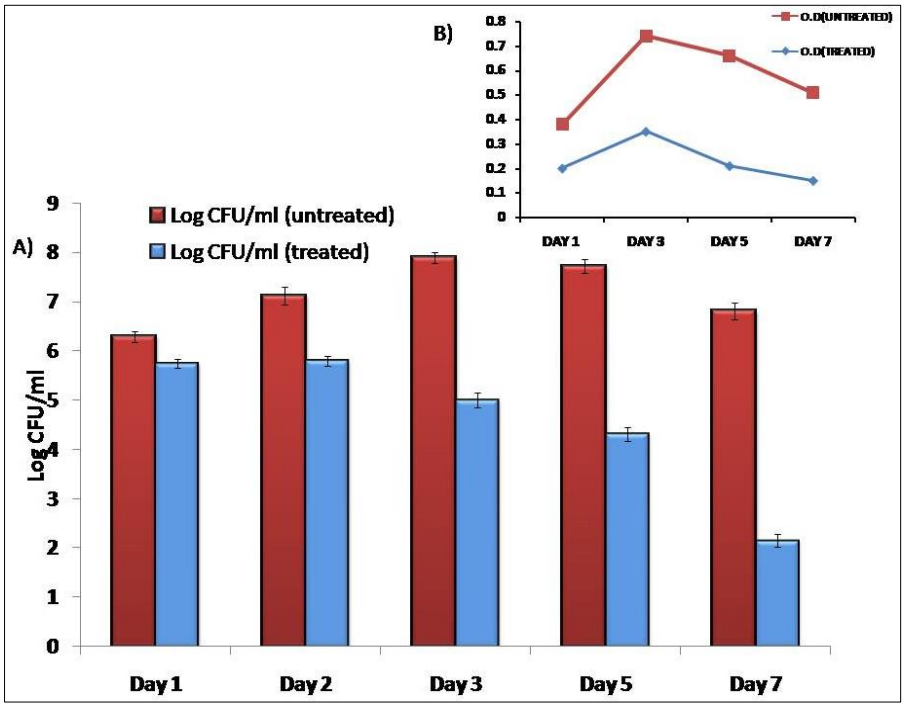

Fig 4: Comparison of A) adhered cell load (Log CFU/ml) of *P. aeruginosa* and B) absorbance on catheter surface treated with Eucalyptus extract.

Discussion

Biofilm infections pose a major medical problem especially in the hospital settings. Among the bacterial community, both *S. aureus* and *P. aeruginosa* are the leading bacterial species involved in causing range of serious nosocomial infections associated with high degree of recalcitrance towards antibiotic therapy due to their biofilm forming ability [19, 20, 21]. Biofilm producing bacteria secrete certain chemicals that protect them from disinfectants, antimicrobials and phagocytic host immune systems [22]. Moreover, biofilm bacteria are far more protected and resistant to antibiotic attack than their planktonic counterparts [23]. This makes treatment and eradication of biofilm infection very challenging with heavy burden on the patient prolonging their hospital stay and associated sufferings.

Eucalyptus extract presents a favorable alternative to be explored against biofilm bacteria. The medicinal properties of *Eucalyptus* are mainly due to Eucalyptol (also known as 1, 8-cineole), one of the ingredients of *Eucalyptus* oil (EO) contained within the leaves. Several studies have investigated the therapeutic effects of *Eucalyptus*, which has been used as an antiseptic and for relieving symptoms of cough, cold, sore throat [24, 25] as well as in the treatment of respiratory tract infections [26-29], wound healing, diabetes and fungal infections [30-32]. In addition, *in vitro* and *in vivo* studies have indicated that polysaccharides and essential oil extracted from *Eucalyptus* exhibit various antimicrobial properties. However, limited work has been focused on studying the anti-biofilm potential of Eucalyptus leaf extract against nosocomial pathogens. The present study focuses on the potential of aqueous extract of Eucalyptus leaf on the biofilm forming ability of two clinically significant bacteria i.e *S. aureus* and *P. aeruginosa* using *in vitro* assays.

Eucalyptus leaf extract was tested using well diffusion assay against the test species. The data showed that while *S. aureus* and CoNS spp. showed significant inhibitions at 25 µl extract, similar inhibition was observed for *Pseudomonas* spp. at 50 µl of eucalyptus extract. This indicated higher concentration of extract was required against *P. aeruginosa*. Results are similar to those reported by Trivedi and Hotchandani [33] with higher volumes required by the above gram negative test bacteria. Coagulase negative staphylococci (CoNS) has recently emerged as an opportunistic nosocomial pathogen especially in immune-compromised patients, due to its ease in incorporating different resistant mechanisms to antimicrobial genes, favored by the biofilm environment[34]. Eucalyptus extract showed potent inhibition against two strains of CoNS isolated from human nares in our laboratory. The results of the study revealed that eucalyptus extract has antibacterial activity against Gram-positive as well as Gram-negative bacteria resistant to commonly used antimicrobial agents. The difference in bacterial sensitivity toward eucalyptus extract can be attributed to the differences in the structural components of the matrix elaborated by each bacteria as well as the subpopulations of bacteria showing differential gene expression in unfavorable conditions and thus greater resistance [35].

In vitro biofilm of selected *S. aureus* strain was established on abiotic surface by employing the Microtiter dish assay that allows the formation of a biofilm on the wall and/or bottom of a microtiter well. The ease, low cost and flexibility of the microtiter plate assay has made it a critical tool for the study of biofilms [36]. Eucalyptus treated well (1:1) showed significant decline in the adhered population of biofilm cell load with negligible load seen by day 7 as compared to control wells. This indicates potent anti-biofilm activity in the aqueous leaf extract against this Gram positive bacteria. The leaf extract was added soon after addition of bacteria i.e in the initial stages of biofilm formation. Although adherence with initial load of 6 log CFU/ml did occur on day 1 but by day 3 and beyond, the biofilm cells were significantly reduced to 3 log CFU/ ml. The reduction in bacterial numbers is possibly due to successful inhibition of microbial respiration and increased plasma membrane permeability, resulting in death of bacterial cells after massive ion leakage [37, 38].It may also happen due to hydrophilic nature of bacterial cell wall. In the present study, aqueous extract so tested have shown strong antibacterial potential against *S. aureus* biofilm cells. Results emphasize that by further increasing the extract concentration from 50% and above, increased inhibition may be expected. Also, eucalyptus extract can be used in combination with another anti-biofilm agent or antibiotic to obtain synergistic killing. The same fact has also been stressed by other workers [39, 40].

Pseudomonas aeruginosa is an opportunistic human pathogen that is especially adept at forming surface-associated biofilms such as catheter associated urinary tract infections (UTIs) [41, 42]. *P. aeruginosa* is responsible for 12% of all nosocomial UTIs [43]. To study the biofilm forming ability, we simulated *in vitro* biofilm formation by test strain on Foleys urinary catheters. Peak biofilm was formed by day 3 on the catheter surface. However, Eucalyptus extract showed significant day dependent decrease in the biofilm adhered bacterial numbers with minimal load of 2 log CFU/ml by day 7.Sambhyal *et al*. [44] who studied the anti-biofilm effect of EO against *P. aeruginosa* showed that biofilm cells exhibited 65.43% sensitivity to eucalyptus oil. Similarly, Lu and co-workers concluded that eucalyptus is a potential alternative to chemical treatment to control the spoilage of refrigerated pork meat by *P. aeruginosa*. They indicated the killing mechanism of Eucalyptus extract or oil includes increasing the membrane permeability and leakage causing morphological deformities in the biofilm cell structure [45]. However, Quartin and co-workers showed that nano emulsions containing Eucalyptus oil did not present antimicrobial activity against *P. aeruginosa* as the Nano emulsion contained only 5% of active oil. This emphasizes of using higher concentration of the Eucalyptus component for enhanced killing effects [46].

Another indication from the above results highlight towards using the Eucalyptus extract in a combination system with another anti-biofilm antibiotic for higher inhibition and lesser chances of developing resistance as seen in other combination treatments. Pereira *et al*. [47] showed synergistic effects between extracts of *Eucalyptus globulus* leaves and antibiotics against several isolates from respiratory tract infections (*Pseudomonas aeruginosa*).

The results depicted that Eucalyptus aqueous extract from leaves represented a potential alternative with significant anti-biofilm ability against *S. aureus* and *P. aeruginosa*. It significantly decreased the adherent cell population on both the surfaces (mictotiter plate and catheter surface). Eucalyptus extract can thus be used alone or in combination with an antibiotic to be used as a coating agent on various catheter surfaces as a measure to prevent the initial adherence and biofilm formation in hospital settings. Future *in vivo* studies are essential to augment the fact that Eucalyptus extract has anti-biofilm potential against *S. aureus* and *P. aeruginosa*by employing suitable animal infection models.

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