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EUCALYPTUS OIL PREVENTS STAPHYLOCOCCUS EPIDERMIDIS BIOFILM FORMATION

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ABSTRACT

Objective: To evaluate the antibacterial activity of the essential oil extracted from *Eucalyptus Globulus* cultivated in Lebanon against three Gram-positive bacteria (Staphylococcus aureus ATCC 25923, Enterococcus faecalis ATCC 29212 and Staphylococcus epidermidis CIP 444) and two Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa ATCC 27853). Also, the antibiofilm activity has been investigated against the biofilm produced by Staphylococcus epidermidis CIP 444. **Materials:** The essential oil extracted from Eucalyptus leaves were used in broth microdilution methods to test the minimal inhibitory concentration, and then the minimal bactericidal concentration was determined. The antibiofilm activity against Staphylococcus epidermidis was determined by quantification of total biofilm biomass with crystal violet. **Results:** The results have shown that Eucalyptus oil, have good antibacterial activities against the 5 bacterial strains used in this study. A negligible biofilm eradication but a promising biofilm prevention activity against Staphylococcus epidermidis was shown to be dose-dependent. **Conclusion:** Eucalyptus oil has a promising antibacterial activity and prevents Staphylococcus epidermidis biofilm formation, and therefore could be used in pharmaceutical and medical applications.

KEYWORDS: *Eucalyptus globulus;* Essential oil; Antibacterial activity; Antibiofilm prevention; antibiofilm eradication.

INTRODUCTION

In most environments, microorganisms evolve in a sessile mode of growth, designated as "biofilm", which is characterized by cells embedded in a self-produced extracellular matrix.[1] Bacterial biofilms are associated with a wide range of infections, from those related to exogenous devices, such as catheters or prosthetic joints, to chronic tissue infections such as those occurring in the lungs of cystic fibrosis patients.[2] Biofilms employ various defense mechanisms against attacks from antimicrobial agents.[3] Furthermore, the antibiotics available till date are ineffective for treating these biofilm related infections due to their higher values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), which may result in in-vivo toxicity.[4] Hence, it is critically important to design or screen anti-biofilm molecules that can effectively minimize and eradicate biofilm related infections. Therefore, the search for new natural resources having therapeutic effects remains currently of great interest. In recent years, extracted polysaccharides from seaweed have been extensively studied due to the numerous interesting biological activities including anticoagulant[5], antiviral[6], antioxidant[7], anticancer[8], anti-inflammatory[9] and antimicrobial[10] activities and antibiofilm effects.[11] In a previous study, we showed that alginate extracted from Laurus Nobilis growing in Lebanon exhibit a promising antibacterial and antibiofilm activities.[12]

Among the many genera of *Myrtaceae* family, *Eucalyptus* is one of the most cultivated plants in subtropical and Mediterranean regions, including Lebanon.[13] 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[14,15] as well as in the treatment of respiratory tract infections.[16-19], wound healing, diabetes and fungal infections.[20-22] In addition, *in vitro* and *in vivo* studies have indicated that polysaccharides and essential oil extracted from *Eucalyptus* exhibit various properties

depending on its geographical location and its species such as herbicidal, anti-inflammatory, antioxidant, anticancer, antibacterial, antiviral, and antifungal activities.[23-31]

However, to our knowledge, no study has been made to examine the efficiency of essential oil isolated from Lebanese *Eucalyptus* in eradicating or preventing biofilm formation. The aim of this study was thus to extract and evaluate the antibacterial and antibiofilm activities of essential oil extracted from *Eucalyptus* leaves cultivated in Lebanon.

MATERIAL AND METHODS

Plant material

Eucalyptus leaves were collected from the Lebanese University campus in Hadath, Beirut, in the year 2017. The samples were air dried at room temperature in the shade for a few weeks to a final moisture content of 10.0%. Before use, the dried samples were ground in a blender so that the particle size will be between 0.8-0.9 mm.

Essential oil extraction

The volatile oils of Eucalyptus leaves were obtained by of hydrodistillation process in the Clevenger apparatus. A total of 100 g Eucalyptus leaves were placed in a flask (2.5L) and hydrodistilled for 2.5 h. The oil samples were dried over anhydrous sodium sulphate and stored at 4 °C in the dark.

ANTIBACTERIAL TESTING

Bacterial strains, media and reagents

Three Gram-positive bacteria [Staphylococcus epidermidisCIP 444 (S. epidermidis), Staphylococcus aureus ATCC 25923 (S.aureus) and Enterococcus faecalis ATCC 29212 (E. faecalis)]and two Gram-negative strains [Escherichia coli (E. coli) ATCC 35218 and Pseudomonas aeruginosa ATCC 27853 (P.aeruginosa)] were used in this study. CIP 444 is a clinical strain which was isolated from a patient with infected implanted device, hospitalized in the Mignot Hospital of Versailles, France[32] This strain was identified and characterized for many features in the previous studies and deposited to be enclosed within the microorganisms of the collection of Institute Pasteur in 2007.[32-35] The other strains are ATCC. The strains were stored at -80 °C in glycerol stocks and used as required. Brain heart infusion, brain heart agar, and Mueller-Hinton broth were purchased from Himedia (Mumbai, India), prepared and then autoclaved as indicated by the manufacturer.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays

MICs and MBCs were determined by a microtiter broth dilution method as recommended by the Clinical and Laboratory Standards Institute.[36] The MIC was defined as the lowest extract concentration that yielded no visible growth.

Serial two-fold dilutions of essential oil in Mueller-Hinton broth were prepared in a 96-well plate (100 µL per well). Wells with no plant extract added were used as a positive growth control. A diluted bacterial suspension was added to each well to give a final concentration of $5x10^5$ CFU/mL, confirmed by viable counts.

1Wells without bacteria added were used as a negative growth control. The plates were incubated for 24 h at 37 °C. The contents of the wells showing no visible growth were placed on brain heart agar and the number of colonies was counted after overnight incubation at 37 °C. The MBC was defined as the lowest concentration of the product reducing the initial inoculum by≥99.9%. The MIC and MBC were determined for all strains. For each strain, at least three independent determinations were done and the modal value was taken.

ANTIBIOFILM TESTING

Biofilm formation

Assay of biofilm formation in polystyrene was performed essentially according to a standard procedure with some modifications.[32]. Actually, S. epidermidis CIP 444 was grown in trypticase soy broth medium overnight at 37 °C. Then the overnight cultured S. epidermidis CIP 444 suspension with defined volume at concentration of $4.16x10^5$ CFU/mL (confirmed by viable count) was added to a trypticase soy broth medium supplemented with 0.25% glucose. A total of 120 µL of this bacterial suspension was inoculated into each well of a sterile 96-well flat-bottom polystyrene tissue culture-treated microtiter plate (Corning®Costar® 3598,Corning, NY 14831, USA) except for column 12 which was used as a negative control and filled only with the sterile medium, and then the plates were incubated for 24 h at 37 °C. The next step included discarding the biomass and washing the microtiter plates with saline (0.9% NaCl) to remove any non-adherent bacteria, then drying the plates at room temperature for several minutes. After that, the remaining biofilm attached to the wall and the bottom of the wells was fixed by heating at 90 °C for 50 min, thus the plates were ready for treatment with the plant extracts.

Biofilm eradication activity

After the fixation of the formed biofilm as previously described, each well of the microtiter plate was filled with 120 µL of sterile physiologic water to be used as a diluent for the serial dilution of our plant extracts. A serial ½ dilution was then made with equal volume of the extract in the saline water in the wells except for column 11 which was used a positive untreated control, then the microtiter plates are incubated at 37 °C for 18 h. Tests were performed in quadruple. The wells were then washed 2 times by saline water, filled with 100 µL 0.1% crystal violet and left at room temperature for 10 min. The stain was then discarded and the wells were washed by saline water for 3 times. They were finally filled with 100 µL of physiologic water and the OD490 nm were measured.

Biofilm prevention activity

The ability of the extract to prevent biofilm formation was also investigated.One hundred microliters of TSB medium supplemented with 0.25% glucose and 100 µL of products were added to the first well of 96-well microplates and serial 1/2 dilution was done till 10^{th} well. A diluted bacterial suspension was added as inoculum to each well to give a final concentration of 5×10^5 CFU/mL. Wells lacking any product were used as positive control for biofilm formation. Wells without bacteria were used as negative control. The remaining steps were done as previously described in the biofilm formation part and OD were measured.

RESULTS

Oils extracted from *Eucalyptus*

The Volatile Oil amount of *Eucalyptus* leaves (V.O. amount mL/100g) = 0, 75.

Antibacterial activity of essential oil

The MIC and MBC values of essential oil extracted from *Eucalyptus* against Gram-positive and Gram-negative bacteria are summarized in Table 1. The MIC was defined as the lowest concentration of essential oil that inhibited the visible growth of a microorganism after overnight incubation. MBC was defined as the lowest concentration able to reduce the initial bacterial inoculum by > 99.9%.

Results showed that essential oil extracted from *Eucalyptus* exhibited bacteriostatic and bactericidal activity against Gram-positive strains (*S. aureus*, *E. faecalis*, and *S. epidermidis*) and Gram-negative ones (*E. coli* and *P. aeruginosa*). Toward Gram-negative bacteria, the essential oil exhibit the same inhibitory activity. Regarding both inhibitory and killing effect of essential oil, S. epidermidis was globally the most sensitive among the Gram- positive bacterial strains with MIC value equivalent to 0.38 mg/mL and MBC value equivalent to 0.75 mg/mL (table 1). The ratio MBC/MIC was equal to 2, highlighting the bacteriocidal activity of the essential oil extracted from *Eucalyptus*. In conclusion, the essential oil extracted from *Eucalyptus* showed an antibacterial activity against the tested strains, and it was found more efficient against *S. epidermidis*, a Gram-positive bacterium.

Table 1: Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the essential oil extracted from *Eucalyptus* leaves against selected microorganisms.

Bacterial strain	MIC (mg/mL)	MBC (mg/mL)
S. aureus	1.5	>1.5
E. faecalis	0.75	1.5
S. epidermidis	0.38	0.75
E. coli	0.75	1.5
P. aeruginosa	0.75	1.5

Biofilm eradication activity

Our results showed that essential oil extracted from Eucalyptus globulus had visually low biofilm eradication capacity. The highest eradication capacity is about 15% for the lowest oil concentration used of about 0.1%. The eradicating capacities decreased with the increase of the oil concentration. It is about 14%, 7%, 10% and 3% at 0.2, 0.4, 0.8 and 1.56 % of oil concentration respectively. At higher oil concentrations (3.12, 6.25, 12.5, 25 and 50%) the eradicating capacities reaches negative values from -4 to -26% showing no eradication capacity compared to positive control (figure 1).

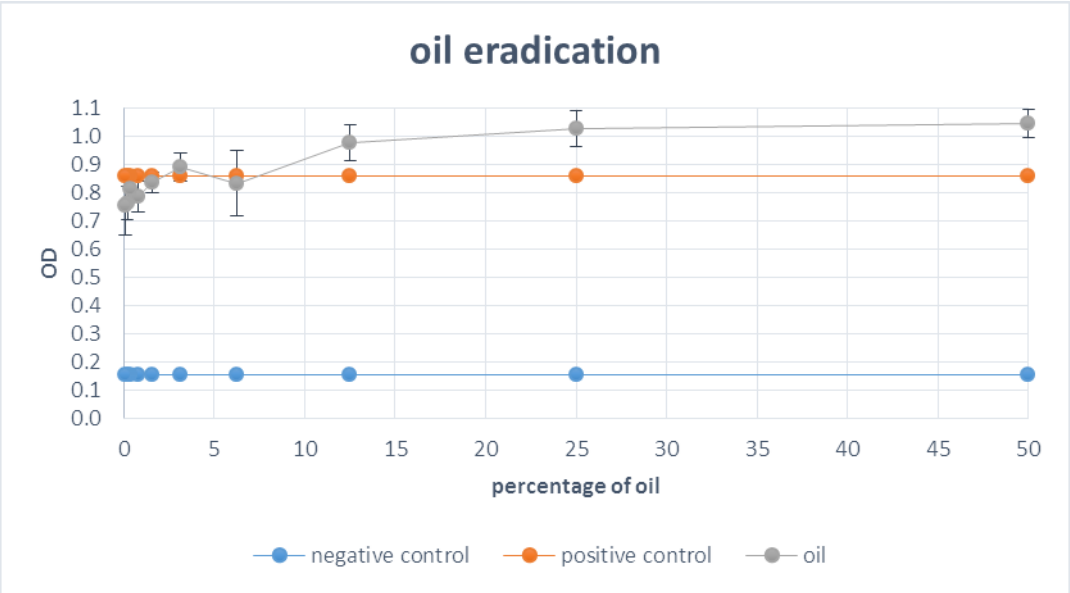

Figure 1: Eradicating capacity of essential oil extracted from *Eucalyptus Globulus* against S.*epidermidis* biofilm at different concentrations.

Biofilm prevention activity

Initial attachment of bacteria to a solid surface is the first step in biofilm formation. Therefore, extract prevention of biofilm formation by S. epidermidis was also investigated. Our results showed that essential oil extracted from *Eucalyptus Globulus* has visually an important ability to prevent S. epidermidis biofilm formation with the highest prevention capacity of 94% found at the highest oil concentration of 50%. This prevention capacity decreased in a proportional way with its concentration. It is about 77%, 53%, 36%, 28%, 37%, 45%, 25%, 32% and 37% at 25, 12.5, 6.25, 3.12, 1.56, 0.8, 0.4, 0.2 and 0.1 % of oil concentration respectively (figure 2).

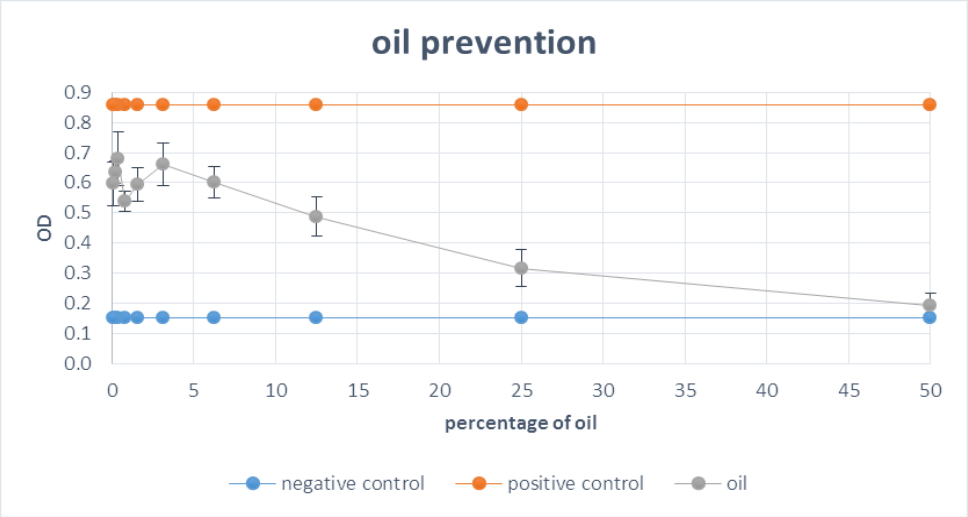

Figure 2: Preventive capacity of essential oil extracted from *Eucalyptus Globulus* against S.*epidermidis* biofilm formation at different concentrations.

DISCUSSION

Bacteria embedded in biofilms are known to exhibit a significantly greater tolerance to antibiotics (often up to 1000 fold greater) and other antimicrobial agents than their planktonic counterparts.[37] In fact, Biofilm formation enables bacterial pathogens to and persist in harsh environments, making their eradication particularly difficult. As the biofilm matures, there is a reduced entry and activity of antimicrobial agents making biofilm-forming pathogens progressively more resistant to antibiotic regimens.[38] Thus, novel strategies, designed to block a specific biofilm step, such as the use of antiadhesion agents, are exciting avenues for exploration and ultimately the development of fast-acting and potent treatment strategies.

In this context, we investigate in this study the antibacterial and antibiofilm activities of essential oil extracted from Eucalyptus Globulus, which is the principal source of EO in the world.[14] Our results showed that EO exhibited bacteriostatic and bactericidal activities against the difference strains of Gram positive and Gram negative bacteria used. This important antibacterial effect of EO are in concordance with previous studies showing antimicrobial activity of EO on different bacterial strains.[30,39,40] Another study has also shown that Eucalyptus Globulus has more pronounced activity against methicillin-resistant *Staphylococcus aureus* compared to oils extracted from other Eucalyptus species *(E. radiata* and *E. citriodora).*[41]

The most potent antibacterial effect of EO was found against the Gram positive *S. epidermidis*. The difference in bacterial sensitivity toward EO 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.[38]

S. epidermidis is the most frequently isolated species from human epithelia. It colonizes predominantly the axillae, head, and nares.[42] Particularly, *S.epidermidis* represents the most frequent causative agent involved with infections of any type of indwelling medical devices, such as peripheral or central intravenous catheters (CVCs).[43] Furthermore, *S. epidermidis* may be involved in prosthetic joint, vascular graft, surgical site, central nervous system shunt, and cardiac device infections.[43] Moreover, *S.epidermidis* shows genome-wide adaptation to the biofilm mode that may explain limited activity of many antibiotics such as penicillins[44], aminoglycosides[45], and quinolones[46], against *S. epidermidis* biofilms. Together, these considerations highlight the need for preventive therapeutic agents against *S.epidermidis* biofilm formation.

This study found that EO was very effective in preventing *S. epidermidis* biofilm formation without having an eradicating capacity. The exact mechanisms behind the tolerance of *S. epidermidis* within biofilms to EO treatment is complex and no single factor can fully account for this specific trait. In fact, it has been previously proposed the existence of three cell

subpopulations residing within *S. epidermidis* biofilms[47]; normal cells that are rapidly killed by antibiotics, tolerant-but-killable (TBK) cells that only respond to high concentrations of antibiotics, and dormant cells that resist very high concentrations of antibiotics; the latter two comprise persister cells[48] and play important roles in biofilm drug tolerance.[49]

The nature of biofilms, the ability of *S. epidermidis* to adhere to surfaces and the problems associated with their treatment and removal indicate that *S. epidermidis biofilm* prevention is preferable to biofilm disruption and removal. Moreover, the *S. epidermidis*biofilm prevention capacity of EO found in this study appear to have an important impact on preventing infections since *S. epidermidis provides* a "reservoir" function for the transfer of genetic elements to enhance pathogenic success of *S. aureus*.[50,51]

The mechanism by which EO inhibited *S. epidermidis* biofilm formation is most probably related to the inhibition of the initial attachment of bacteria to the solid surface which constitutes the first step in biofilm formation.[38] In fact, the development of a biofilm requires adhesive forces for the colonization of surfaces and the interaction of cells among each other.[52] Thus numerous studies have focused on ablating bacterial adherence. Adhesion to abiotic surfaces such as catheters is mainly governed by bacterial cell surface Hydrophobicity.[53] The hydrophobic character of *S. epidermidis* cell surface is mainly governed by the abundant surface protein AtlE.[54] Whether or not EO affected AtlE protein or another attachment protein for the biofilm prevention capacity showed in this study remains to be established.

In conclusion, this study showed for the first time, to our knowledge, an *S. epidermidis* biofilm prevention activity of Eucalyptus oil in a dose-dependent manner. These results indicate that EO is promising to be an antibacterial agent and a preventive agent against *S. epidermidis* biofilm related infections. EO could also be used as a coating agent on medical devices to prevent staphylococcal adherence and biofilm formation as previously demonstrated with catheters using phages mixture.[55] Future works studying the chemical composition of eucalyptus oil to establish a relationship between the chemical composition and the corresponding antimicrobial properties as well as the attachment proteins affected by EO could better explained the mechanisms underlying the relevant results found in this study.

Conflict of interest statement

We declare that we have no conflict of interest.

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