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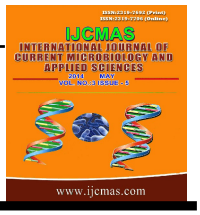

Original Research Article

Anti-biofilm activity and bioactive component analysis of eucalyptus oil against urinary tract pathogen

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Keywords

Eucalyptus oil, biofilm, *Proteus mirabilis*, GC/MS, TLC, Eucalyptol, SEM

A B S T R A C T

The aim of the present study was to determine the anti biofilm effect the of Eucalyptus essential oils along with the evaluation of bioactive components contributing to the biofilm inhibition properties of Eucalyptus oil. *Proteus mirabilis* is an uropathogen and forms a thick biofilm on the urinary catheters in patients with Urinary Tract Infections (UTIs). The anti biofilm activity of the oil was checked against *Proteus mirabilis* ATCC 7002 strain which forms biofilm on urinary catheters, as reported in our earlier studies. The same anti biofilm activity was confirmed by Scanning Electron Microscopy (SEM) under the present investigation. Thin Layer Chromatography (TLC) and Gas Chromatography/Mass Spectroscopy (GC/MS) analysis of the oil was performed to find out the major chemical component present in the oil. The TLC and GC/MS test confirmed that the oil contained high amount of terpenes mainly 1, 8- cineole (44.22 %) and α-pinene (13.6%) and other components in smaller fractions. The results of the SEM analysis showed that eucalyptus essential oil had very high inhibitory effect on the biofilm formation of the bacteria on the urinary catheter. The major fractions of the essential oil were found to be terpenes and related components, which seems to be responsible for anti biofilm properties of eucalyptus essential oil. The results of this study confirmed the possibility of using Eucalyptus essential oils or some of their components against the formation of biofilm on urinary catheters.

Introduction

Eucalyptus plant is native of Australia, but now there are many species of eucalyptus plant found all over the world. In India, the species found are, *E. globulus, E. camaldulensis, E. citriodora, E. crebra, E. major, E. intermedia*, and so on (R.M. Palanna 1996). Different parts of the tree, such as, leaves, bark, seeds, etc, have been used widely for varied medical purposes. The essential oil is usually obtained by aqueous distillation of the fresh leaves which occurs as light yellow, mobile liquid, with a pleasant, refreshing camphoraceous odor (Henriette Kress 2002).

The essential oil of the leaves of this tree has many medicinal properties such as anti-inflammatory, reducing pain and blood pressure (Yang Suk Jun, et.al.

2013), anti-oxidant, anti-bacterial (Mehdi Rahimi -Nasrabadi, et al 2013), anti-fungal (Agarwal V, et al 2008), etc. In recent studies it has been found that eucalyptus oil also has anti-biofilm properties. It has been shown to reduce biofilm formed by *Staphylococcus epidermidis* on skin (T. J. Karpanen, et al 2008). The effect of this essential oil on urinary tract pathogen, *Klebsiella pneumonia* and *Proteus mirabilis* has also been studied and is found to be inhibiting the biofilm growing on urinary catheters, *in-vitro* (Mathur, et al 2013; Mathur, et al. 2013).

It has been established in recent studies that the essential oils obtained from *Eucalyptus* leaves from different species belonging to different regions in the world, mostly differ in their relative content of active ingredient. (Slavenko Grbović, et al 2010**)** Hence, it becomes imperative to determine the specific composition of essential oil which has been procured commercially, in order to further analyze which major component is present. Also, by specifically targeting the active ingredient present in the oil, those compounds can be further tested for their antibiofilm properties. The aim of this study was to assess and confirm the anti biofilm activity of commercially obtained eucalyptus oil on catheter associated urinary tract (CAUTI) pathogen, *Proteus mirabilis* ATCC 7002 strain. The microscopic study was done on longitudinal section of catheter surface. Also, the objective of this study was to access the chemical composition of the essential oil used in this study by GC/MS analysis and TLC profiling of pigments.

Materials and Methods

Bacterial culture

P. mirabilis ATCC 7002 culture was obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, and Chandigarh, India. The MTCC number for the strain is 425. The bacterial culture was further subcultured and stored on Tryptone Soy Agar (TSA). Tryptone Soy Broth (TSB) media was used for conducting further experiments.

Chemicals

The Eucalyptus oil was purchased from commercial market. Toluene and Ethyl acetate used was of HPLC grade from S D Fine -chem. limited, Mumbai.

Catheter

Sterile urethral catheter was used to test the biofilm formation, which was manufactured by Romsons Scientific & Surgical Industries Pvt.ltd.

Method

TLC analysis of pigments from Eucalyptus oil

Silica Gel-G was used for TLC analysis. Solvent System employed consisted of Toluene and ethyl acetate. The spraying agent, alcoholic vanillin–sulphuric acid solution was applied of the silica plate after complete evaporation of the solvents used in solvent system. Silica plate was further heated at 100^0C for 5min. Different spots developed were documented. (Wagner H, Bladt S., 1999)

GC/MS Analysis

GC/MS analysis was performed using a Shimadzu Corporation, GCMS-QP2010 Plus chromatograph mass spectrometer equipped with a powerful Dual Inlet turbo pumping system with a Split Injection

mode. It had a Wide Mass Range of 1.5 to 1090 and the independently heated ion source in a range of 100 to 300 °C. The column used was Rxi-5HT which is a highly non-polar column of 30m length and filling diameter of 0.25µm. 0.5µl of sample was injected. The injector and interface were operated at 280 °C. The oven temperature was programmed as follows: 80 °C and raised to 300 °C (8 °C/min), and held for 3 min. The total run time was 30.5 min. By comparing the mass spectra with those of NIST02 library data for the GC-MS system, the major individual constituents were identified.

Scanning Electron Microscopy

To observe the nature of the surfaces of the biofilms, longitudinal sections of each catheter were prepared and incubated in TSB media with *Proteus mirabilis* ATCC 7002 culture (100µl/ml), with and without the eucalyptus oil at sub-MIC concentration (200µl/ml) (Mathur S., et al 2013) and were incubated for 96hrs. The catheters were pretreated as per methods previously followed (Anisio Storti, et al 2005; L. Ganderton, et al 1992) and the sample was dried in a vacuum centrifuge and mounted onto aluminum stubs sputter-coated with gold. The inner and outer surfaces of the catheters tips were examined by SEM (JEOL-JSM-5400 SEM) at 10-15kV and were photographed.

Results and Discussion

TLC analysis of Eucalyptus oil

Eucalyptus oil was spotted in preparative TLC plates coated with silica gel G. The plates were developed in TLC chamber previously saturated with toluene-ethyl acetate (97:3) as the solvent system. The plates were developed was dipped into alcoholic vanillin–sulphuric acid reagent and heated for 5min at 100°C. The two components separated were observed with blue zone and *R*f values as (*R*f = 0.42, *R*f = 0.62) respectively.

GC/MS Analysis

The total ion chromatogram (TIC) of the essential oil is displayed in Fig 1. The amount of the components from the essential oil was determined by the peak area normalization method. The presence of several overlapping peaks shows the complexity of the mixture.

The chemical constituents of the essential oil and the Relative content (%) of the each component were enlisted in Table 1. Identification of the total twenty one components was completed by comparison of their mass spectra with those of NIST02 library data for the GC-MS system. The peaks at Rt 9.038 and 6.601 were the major ones identified as α-pinene and 1, 8-cineole respectively.

Scanning Electron Microscopy

Scanning electron microscopy of the biofilm formed on the surface of the catheter was carried out with and without the eucalyptus oil treatment, of which latter showed very high anti biofilm effect. Scanning electron microscopy of the biofilm formed in the presence of eucalyptus oil revealed almost complete inhibition of biofilm on the catheter surface [Fig. 2(b)]. While in the control, which was not exposed to any eucalyptus oil, showed compact tightly packed cell aggregates [Fig. 2(a)].

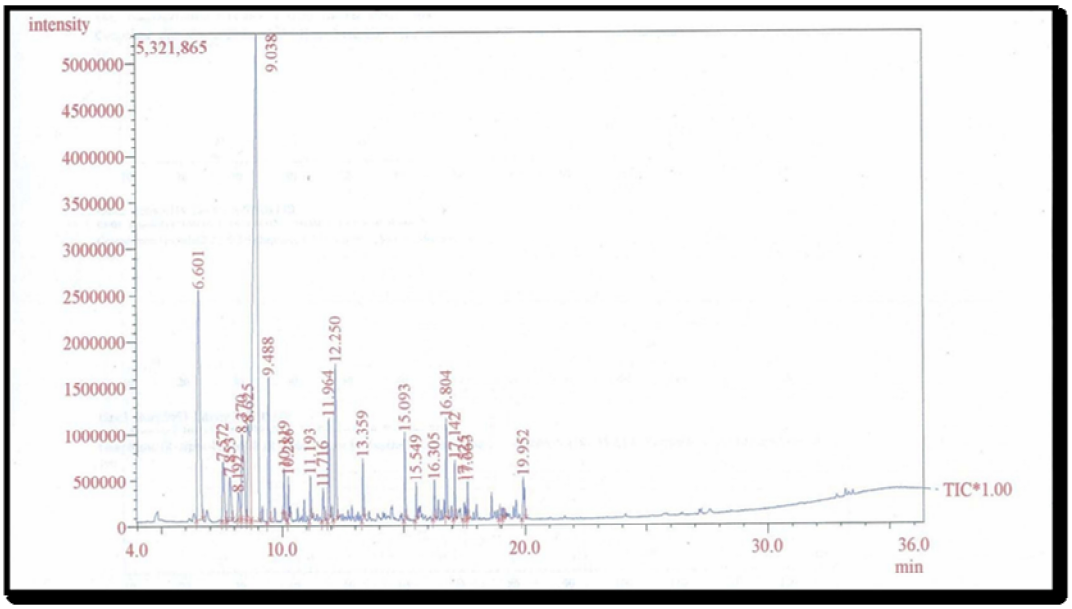

Fig.1 The total ion chromatogram (TIC) of the eucalyptus essential oil

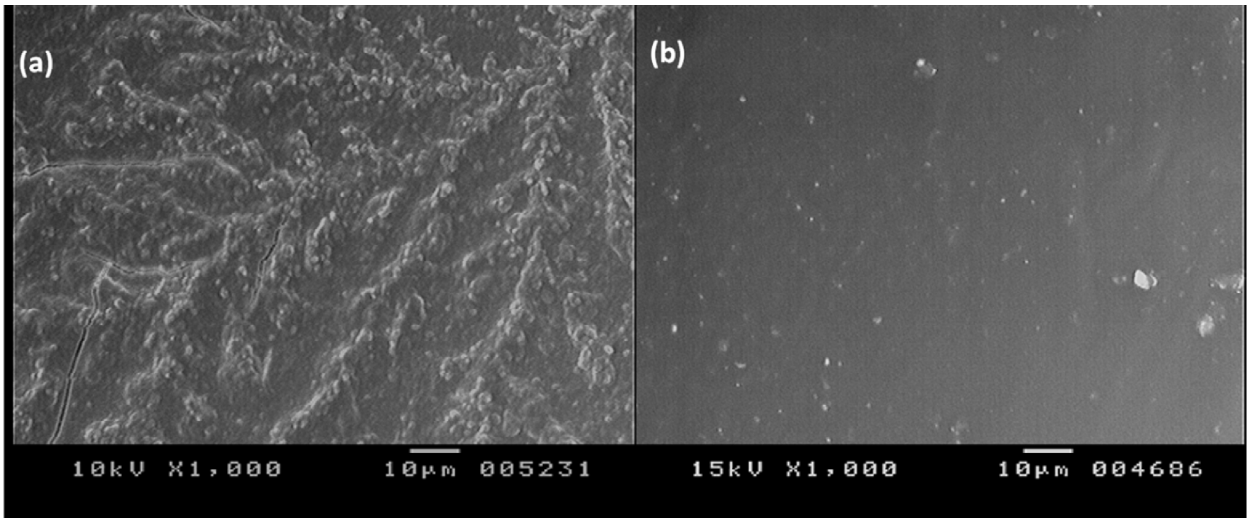

Fig.2 The scanning electron micrographs of catheter sections, (a) normal biofilm growth can be seen on the surface of the catheter, (b) complete inhibition of biofilm due to the effect of eucalyptus oil can be seen on the surface of the catheter.

Table.1 Chemical constituents of the Eucalyptus essential oil

No.	Molecular formula	Compound	Relative content (%)
1	$C_{10}H_{16}$	α-pinene	13.6
2	$C_{10}H_{16}$	β-pinene	2.45
3	$C_{10}H_{16}$	β-Myrcene	1.77
4	$C_{10}H_{16}$	α-phellandrene	1.16
5	$C_{10}H_{16}$	3-Carene	5.05
6	$C_{10}H_{14}$	o-Cymene	6.2
7	$C_{10}H_{18}O$	1,8-cineole	44.22
8	$C_{10}H_{16}$	γ-terpinene	3.45
9	$C_{10}H_{16}$	Terpinolene	0.88
10	$C_{10}H_{18}O$	Linalool	0.73
11	$C_{10}H_{16}O$	2(10)Pinen-3-ol	0.8
12	$C_{10}H_{18}O$	(-)-4-Terpineol	2.32
13	$C_{10}H_{18}O$	α-Terpineol	5.01
14	$C_{10}H_{18}O$	Geraniol	1.11
15	$C_{12}H_{20}O_2$	α-Terpineol acetate	1.62
16	$C_{12}H_{20}O_2$	Geranyl acetate	0.6
17	$C_{15}H_{24}$	α-Gurgujene	4.23
18	$C_{15}H_{24}$	Bicyclo[5.3.0]decane, 2-methylene-5-(1-methylvinyl)-8-methyl-	0.5
19	$C_{15}H_{24}$	(+)-Ledene	0.67
20	$C_{15}H_{26}O$	(-)-Globulol	1.16
21	$C_{15}H_{26}O$	Eudesm-4(14)-en-11-ol	1.25

The review study conducted at University of Maryland Medical centre, also confirmed that in 19th century, Eucalyptus oil had been used to clean urinary catheters in hospitals as a precautionary measure to kill bacteria (UMMC Overview 2011). The potency of eucalyptus oil has been evaluated and confirmed in our initial study, in which the anti-biofilm activity of the Eucalyptus oil against the urinary tract pathogen, especially *Proteus mirabilis* was ascertained (Mathur S., et al 2013). As continuation of the previous findings, the present study confirms that there is more than 90% reduction in the biofilm formation by *Proteus mirabilis*, based on the SEM analysis. This strongly supports the anti-biofilm activity of the Eucalyptus oil. Also, in order to allocate the bioactive component underlying anti-biofilm mechanism of eucalyptus oil, further analysis such as TLC and GCMS was conducted. These analysis identified the eucalyptus essential oil consisted mainly of various types of Terpenes such as oxygenated monoterpenes, acyclic and cyclic monoterpenes and terpene alcohol. Of these, 1, 8- cineole (44.22 %), was the main oxygenated monoterpenes, while α-pinene (13.6%) was the main cyclic monoterpenes that was obtained. The yield of essential oil and the content of 1, 8-cineole and α-pinene are within the values reported in the earlier studies (Aihua Song, et al 2009; Sen-Sung Cheng, et al 2009).

Thus, from this study we can conclude that 1, 8- cineole and α-pinene, present in the eucalyptus oil sample used, are the main components that are affecting the normal biofilm formation process of *P. mirabilis* ATCC 7002. Further molecular analysis is essential which might reveal the underlying mechanism of biofilm inhibition process induced by these bioactive components on the surface of urinary catheter. Synergistic activity of 1, 8-cineole along with certain other antimicrobial drug, such as, 2% chlorhexidine gluconate, has been already confirmed against biofilm of other bacterial pathogens like *S. aureus*, MRSA, *E. coli* and *C. albicans* (E. R. Hendry, et al 2009). Similarly, further specific studies can be carried out to find out the synergistic effect of 1, 8-cineole and α-pinene, in isolation as well as with other conventional antimicrobial agents.

References

Agarwal V, Lal P, Pruthi V.2008.Prevention of *Candida albicans* biofilm by plant oils. Mycopathol 165: 3–19.

Aihua Song, Ying Wang, Yanmei Liu.2009. Study on the chemical constituents of the essential oil of the leaves of *Eucalyptus globulus* Labill from China, Asian Journal of Traditional Medicines, 4 (4)

Anisio Storti, Antonio Carlos Pizzolitto, Elisabeth Loshchagin Pizzolitto.2005.Detection of Mixed Microbial Biofilms On Central Venous Catheters Removed From Intensive Care Unit Patients, Brazilian Journal Of Microbiology 36:275-280.

Ganderton, L., J. Chawla, C. Winters, J. Wimpenny, D. Stickler.1992. Scanning Electron Microscopy of Bacterial Biofilms on Indwelling Bladder Catheters, Eur. J. Clin. Microbiol Infect. Dis., September 1992, p. 789-796.

Hendry, E. R., T. Worthington, B. R. Conway and P. A. Lambert.2009. Antimicrobial efficacy of eucalyptus oil and 1,8-Cineole alone and in combination with chlorhexidine digluconate against microorganisms grown in planktonic and biofilm cultures, Journal of Antimicrobial Chemotherapy, 64, 1219–1225, doi:10.1093/jac/dkp362

Henriette Kress.2002. The British Pharmaceutical Codex, Oleum Eucalypti, B.P. Oil of Eucalyptus,http://www.henriettesherbal.com/eclectic/bpc1911/eucalyptus_oleu.html, accessed on 8 August 2013

Karpanen, T. J., T. Worthington, E. R. Hendry, B. R. Conway and P. A. Lambert, (2008) Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of *Staphylococcus epidermidis*, Journal of Antimicrobial Chemotherapy, 62, 1031–1036, doi:10.1093/jac/dkn325.

Mathur S., Udgire M., Khambhapati A. (2013), Effect of essential oils on biofilm formation by *Proteus mirabilis*, Int J Pharm Bio Sci 2013 Oct; 4(4): (B) 1282 – 1289

Mehdi Rahimi -Nasrabadi, Seied Mahdi Pourmortazavi, Shahram Nazarian, Farhad Ahmadi, Hosein Batool.2013. Chemical Composition, Antioxidant, and Antibacterial Activities of the Essential Oil and Methanol Extracts of *Eucalyptus oleosa* Leaves, International Journal of Food

Properties, 16:1080-1091, doi: 10.1080/10942912.2011.558227.

Palanna,R.M.1996. Eucalyptus in India. In: Reports submitted to the regional expert consultation on eucalyptus. Volume II, RAP Publication: 1996/44, Bangladesh, p 1-280.

S. Mathur, M. Gutte, D. Paul, M. Udgire (2013), Study the effect of essential oils on microbial biofilm formation by *Klebsiella pneumonia*, Sch. Acad. J. Biosci., 1(3):76-79.

Sen-Sung Cheng, Chin-Gi Huang, Ying-Ju Chen, Jane-Jane Yu, Wei-June Chen, Shang-Tzen Chang.2009. Chemical compositions and larvicidal activities of leaf essential oils from two eucalyptus species, Bioresource Technology 100 452–456.

Slavenko Grbović, Dejan Orčić, Maria Couladis, Emilija Jovin, Dušan Bugarin, Kristina Balog Neda Mimica-Dukić.2010. Variation of essential oil composition of *Eucalyptus camaldulensis* (Myrtacease) from the Montenegro coastline, APTEFF, 41, 1-203, DOI: 10.2298/APT1041151G

UMMC Overview.2011. Eucalyptus, University of Maryland Medical Center, http://umm.edu/health/medical/altmed/herb/eucalyptus, accessed on 8 July 2013.

Wagner H, Bladt S. (1996), Plant Drug Analysis: A Thin Layer Chromatography Atlas, 2nd ed. Berlin: Springer-Verlag, 121.

Yang Suk Jun, Purum Kang, Sun Seek Min, Jeong-Min Lee, Hyo-Keun Kim, and Geun Hee Seol.2013. “Effect of Eucalyptus Oil Inhalation on Pain and Inflammatory Responses after Total Knee Replacement: A Randomized Clinical Trial,” Evidence-Based Complementary and Alternative Medicine, vol. 2013, doi:10.1155/2013/502727