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Evaluation of the Activity of Essential Oil and Hydrosol from *Eucalyptus camaldulensis* Against Some Bacterial Species

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Abstract

Nowadays, the use of medicinal plants is being practiced at a wide range as a result of antibiotics resistant for the vast majority of microorganisms. *Eucalyptus camaldulensis* essential oil and hydrosol were used in this study against planktonic forms and biofilms of some species of Gram negative and Gram positive bacteria. The antibacterial and antibiofilm activities of this plant were detected using the microtiter plate and MIC approaches. The results proposed that the oil and hydrosol preparations have antibacterial activities against planktonic cells in different concentrations depending on the type of isolate. For antibiofilm activity, the results showed that *E. camaldulensis* oil was highly effective against bacterial biofilms of different bacterial species in comparison with the hydrosol. In conclusions, the studied plant can be used as an alternative treatment against bacterial biofilms that cause chronic infections for humans and animals.

Keywords: *Eucalyptus camaldulensis*, Biofilm, MIC, Hydrosol, essential oil

تقييم فعالية الزيت العطري والهيدروسول لليوكالبتوس *Eucalyptus camaldulensis* ضد بعض الانواع البكتيرية

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الخلاصة:

اصبحت النباتات الطبية في وقتنا الحالي واسعة الاستخدام نتيجة لمقاومة العديد من الكائنات الدقيقة للمضادات الحيوية. تناولت الدراسة الحالية استخدام الزيت العطري وماء التقطير لنبات اليوكالبتوس كمضاد لنمو البكتريا وتكوين الغشاء الحيوي لبعض الانواع البكتيرية. استخدمت طريقة microtiter plate و MIC لتحديد الفعالية المضادة لنمو البكتريا وتكوين الغشاء. اظهرت النتائج ان الزيت العطري والهيدروسول لنبات اليوكالبتوس يمتلكان الخاصية المضادة لخلايا انواع البكتريا المدروسة وكذلك اظهرت النتائج ان للزيت العطري فعالية كبيرة على الغشاء الحيوي البكتيري مقارنة بالهيدروسول، لذا يمكن استخدام الزيت العطري لنبات اليوكالبتوس كعلاج بديل ضد تكوين الغشاء الحيوي للانواع البكتيرية التي تسبب العدوى المزمنة في الانسان والحيوانات.

Introduction

Eucalyptus camaldulensis Dehnh. (River red gum) is a native tree in Australia which has been broadly planted around the world. It belongs to a genus from the Myrtaceae family which also includes about 800 species [1]. The medicinal properties of *Eucalyptus* reside in its oil which is secreted and stored in the sub-dermal cavities [2]. This oil has different biological effects, including antioxidants, antibacterial, antiviral and antifungal [3]. The essential oil in association with hydrosol

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can be obtained through steam distillation process for the leaves of this aromatic plant. Hydrosol derives from the Latin “hydro” which means water, while “sol” stands for solution [4]. It has been used in food, cosmetic industries, biological agriculture against insects, and for fertilization of soil [5]. The recognition of the bacterial resistance problem brings the need to analyze the antimicrobial properties of new substances. The mode of growth, i.e. biofilm, is characterized by bacterial cells embedded in extracellular matrix that they produce [6]. Bacterial biofilms are associated with many kinds of infections such as catheters or prosthetic joints and chronic tissue infections such as cystic fibrosis [7]. Biofilms have different defense mechanisms against attacks from antimicrobial agents [8]. For these reasons, the current study aims to evaluate the antibacterial and antibiofilm activities of the volatile oil and hydrosol of *E. camaldulensis* against some species of bacteria.

Materials and Methods

Plant material preparation

Fresh leaves of *Eucalyptus camaldulensis* were collected from field, washed with tap water and dried at room temperature for 3 days. 100 g of leaves were chopped and semi grinded into small pieces, followed by steam distillation according to European Pharmacopeia [9] for 4 hours using a Clevenger apparatus. Hydrosol and oil were stored in a separated, marked glass vials in a refrigerator until required.

Bacterial Isolates

Bacterial isolates were obtained from the microbiology laboratory in the Biology Department/ College of Sciences/ University of Baghdad. In this study, eight species of bacteria were used, four of which are Gram negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus vulgaris*) and four are Gram positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes* and *Streptococcus mutans*).

Detection of Minimal Inhibitory Concentration (MIC) for Hydrosol and Volatile Oil of *Eucalyptus camaldulensis* leaves.

The experiment was performed using the microtiter plate method. Different concentrations of oil and hydrosol were prepared. Overnight cultures of Bacterial isolates on Brain Heart Infusion broth (BHI) were used. Each isolate was compared with McFarland tube ($0.5 = 1 \times 10^8$ cell/ml). After that, 100 μ l of Muller- Hinton broth (MHB), followed by an equal volume of the oil or hydrosol, and finally 10 μ l of each isolate were placed in the wells of a microtiter plate (experiments were done in triplicate). The microtiter plate was incubated at 37 °C overnight and the results were read by naked eye.

Biofilm formation

Detection of biofilm formation was performed essentially according to the following method with some modifications [10]. Bacterial isolates were grown in Trypticase soya broth medium (Himedia/ India) overnight at 37 °C. Then the cultures of bacterial isolate suspensions were diluted to a volume ratio of 1/100 by adding 10 μ l of bacterial suspension to 1 ml of Trypticase soy broth supplemented with 0.1 % glucose. 200 μ l of from his suspension was added into each well of a sterile 96-well, flat-bottom, polystyrene tissue culture-treated microtiter plate. The column which represented control was filled only with the Trypticase soya broth, then all plates were incubated for 18-24 hrs at 37 °C. The biomass was discarded and the microtiter plates were washed for two times with D.W. to remove the non-adherent cells and dried at room temperature for several minutes. After that, crystal violet (0.1%) was added in each well at a volume of 200 μ l and left for 20 min. In the next step, the pigment was washed two times by D.W and the plates were dried out. Ethyl alcohol was added at the same volume and, after 10 min, the optical density (O.D) was read using an ELISA reader at 490nm.

Antibiofilm activity of oil and hydrosol extracts of *Eucalyptus*

The antibiofilm activity was achieved by the same previous method, except that the bacterial suspension was added at a volume of 100 μ l for both oil and hydrosol at different concentrations (half serial dilutions were made with equal volume). The microtiter plates were incubated for 24 hrs at 37 c and then all the remaining steps in the above described method were followed. The O.D was measured at 490 nm using an ELISA reader, while the following equation was used to calculate biofilm inhibition percentage [11].

Biofilm inhibition (%) = (Control OD- Test OD / Control OD) \times 100

Results and Discussion

Detection of Minimal Inhibitory Concentration (MIC) for Hydrosol and Oil of *Eucalyptus camaldulensis*

The results showed that the oil extract had effects against all bacterial isolates, but in different degrees. The higher effect was achieved against *P.vulgaris* using the concentration of 25% and against *S.epidermidis* and *S.pyogenes* using the concentration of 50 %, while the activities against all the other isolates was achieved using the concentration of 100 % .Table-1.

Table 1-Antibacterial activity of *Eucalyptus camaldulensis* oil against some bacterial species (microtiter plate)

Bacterial species	MIC
<i>S.aureus</i>	100%
<i>S.epidermidis</i>	50%
<i>S.pyogenes</i>	50%
<i>S.mutans</i>	100%
<i>E.coli</i>	100%
<i>K.pneumoniae</i>	100%
<i>P.aeruginosa</i>	100%
<i>P.vulgaris</i>	25%

The results in Table-2 reveal that *S.aureus* was not affected by the hydrosol, whereas *S.pyogenes* was inhibited when 25% hydrosol was applied.. However, the other isolates were inhibited using the concentration of 100%.Hence, the results obviously demonstrate that the effects of oil have a wider antibacterial scale than those of hydrosol; for example, oil, but not, hydrosol, exerted inhibitory activities against the growth of *S.aureus* isolate.

Table 2-Antibacterial activity of *Eucalyptus* hydrosol against some bacterial species

Bacterial species	MIC
<i>S.aureus</i>	No effect
<i>S.epidermidis</i>	100
<i>S.pyogenes</i>	25
<i>S.mutans</i>	100
<i>E.coli</i>	100
<i>K.pneumoniae</i>	100
<i>P.aeruginosa</i>	100
<i>P.vulgaris</i>	100

Ghalem and Mohamed [12] reported that *E. camaldulensis* showed significant inhibition effects against *S. aureus* and *E. coli*, which are difficult to treat as they have multiple resistances to a range of antibiotics. Traoré *et al.* [13] tested the antimicrobial activity of essential oils of *E. citriodora* and *E. houseana* by agar diffusion method. They found that they have some activity against *Staphylococcus aureus* and *Escherichia coli*; in addition they are active against *Candida albicans*. The results of the current study is in agreement with the recently published studies, which reported that *Eucalyptus* essential oil at different concentrations affected the strains of *E. coli* and *S. aureus*. On the other hand, Rasooli *et al.*[14] emonstrated that the essential oil from *Eucalyptus* spp. leaves has a high antibacterial activity against Gram positive and Gram negative bacteria, with a similar effects achieved using the leaves alcoholic extract [15]. However, the antibacterial activity of *E. camaldulensis* oils seems to vary from one isolate to another depending on the concentration used and the characteristics of each isolate. The essential oil constituents of the genus *Eucalyptus* were well characterized [16]. This plant produces numerous volatile compounds in large amounts, especially terpenoids which are accumulated in glands throughout the leaf parenchyma and bark [17]. On the other hand, Elaagib *et al.*

[18] reported that cymene in *Eucalyptus* essential oil may be responsible for its antibacterial activities against *S. aureus*, *Bacillus cereus* and *E. coli*.

Biofilm formation

The results of biofilm formation shown in Table- 3 demonstrate that all bacterial isolates have the ability to produce biofilm, but in a different ranges, when compared with the control. Two bacterial isolates (*S. aureus* and *P. aeruginosa*) showed moderate biofilms, while the other isolates produce stronger ones. Salman *et al.* [19] showed that *Pseudomonas aeruginosa* isolates produce hemolysin (100%), biofilm (52%) and protease (73.33%). The type of isolates and the sample type from which this isolate was collected affected the results of biofilm formation.

Table 3-Biofilm formation by different isolates

Bacterial Isolates	O.D	Biofilm type
<i>S. aureus</i>	0.22	Moderate
<i>S. epidermidis</i>	0.254	Strong
<i>S. pyogenes</i>	0.273	Strong
<i>S. mutans</i>	0.454	Strong
<i>E. coli</i>	0.382	Strong
<i>K. pneumoniae</i>	0.44	Strong
<i>P. aeruginosa</i>	0.214	Moderate
<i>P. vulgaris</i>	0.460	Strong

Antibiofilm activity of *Eucalyptus camaldulensis* oil

The results in Table-4 show that *Eucalyptus camaldulensis* oil had a high biofilm inhibition capacity against all bacterial isolates, but in different percentages. For *S. aureus*, there was no effect by first concentration, but the inhibition percentage increased with the other concentrations (50%, 63%, and 72%). *S. epidermidis* was affected by all concentrations, which was the same for *S. mutans*, except that it had a lower effect when the concentration of 12.5% was applied. *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *P. vulgaris* were also inhibited by all concentrations of the oil extract. However the highest effects were achieved by last concentration in *E. coli*, 25% in *K. pneumoniae* and

Table 4-Percentage of biofilm inhibition by *Eucalyptus camaldulensis* oil

Isolates	Control	C1	C2	C3	C4
<i>S. aureus</i>	0.22	0.22	0.11	0.08	0.06
Percentage of inhibition		No	50%	63%	72%
<i>S. epidermidis</i>	0.254	0.07	0.06	0.065	0.064
Percentage of inhibition		72%	76%	74%	74%
<i>S. pyogenes</i>	0.273	0.204	0.270	0.240	0.245
Percentage of inhibition		25%	1%	12%	10%
<i>S. mutans</i>	0.454	0.099	0.082	0.060	0.22
Percentage of inhibition		78%	81%	86%	51.5%
<i>E. coli</i>	0.382	0.09	0.08	0.07	0.01
Percentage of inhibition		76%	79%	81%	97%
<i>K. pneumoniae</i>	0.44	0.197	0.10	0.066	0.099
Percentage of inhibition		55%	77%	85%	77%
<i>P. aeruginosa</i>	0.214	0.10	0.133	0.1	0.112
Percentage of inhibition		53%	37%	53%	47%
<i>P. vulgaris</i>	0.460	0.119	0.29	0.069	0.071
Percentage of inhibition		74%	36%	85%	84%

P. vulgaris, and 100% and 25% in *P. aeruginosa*). *S. pyogenes* showed the lowest percentage of inhibition in comparison with the other isolates.

The results in this study showed that *Eucalyptus camaldulensis* volatile oil has the ability to inhibit the formation of biofilm in all strains. This oil, when applied before the formation of the biofilm, could interact with the proteins of the bacterial surface and interfere with the quorum sensing systems. The

inhibition of cellular links is the initial stage in the formation of the biofilm, with the previous conditioning of the surface which represents a favorable environment for bacterial attachment [20-22].

Merghni *et al.* [23], in their study with other essential oils, demonstrated that the antibiofilm activity was observed in half MIC, reaching inhibition percentages of 50-70% in *S. aureus* biofilms. On the other hand, Knezevic *et al.* [24] revealed that *Eucalyptus* oil had significantly stronger effects than those of chlorhexidine in terms of antibacterial activity and *in vivo* biofilm prevention. Nowadays, there is no biofilm targeting therapy in the market so the best strategy is to avoid the training instead of trying to eliminate them after their graduation [18]. The antibiofilm activity of the genus *Eucalyptus* was also observed by Mathur *et al.* [11] against the urinary tract pathogen *Proteus mirabilis*, presenting approximately 90% inhibition of biofilm formation, whereas *Eucalyptus* oil showed the maximum reduction in biofilm formed by *Proteus mirabilis* on urinary catheter.

Antibiofilm activity of hydrosol

The results of the current study, shown in Table-5, demonstrate a lower biofilm inhibition activity for the plant hydrosol as compared with the oil extract. The highest inhibition effect was about 27% for the higher concentration when used against *K.pneumoniae* biofilm. The percentage of inhibition decreased with the decreased of concentration for *P.aeruginosa*, reaching 11% using 25% concentration. Moreover, the concentrations of 100% and 50% had the same inhibition effect (1.8%). For *S.aureus*, the highest inhibition activity of about 13% was achieved by 3rd concentration, while the other concentrations caused lower percentages of inhibition. Other bacterial species (*S.epidermidis*, *S.pyogenes*, *S.mutans*, *E.coli* and *P.vulgaris*) showed no biofilm inhibition abilities when compared with the positive control.

Several studies illustrated the antimicrobial and antibiofilm properties of herbs, spices and their derivatives, such as essential oils and other extracts. Nevertheless, no attention has been focused on plant hydrosols. However, a previous report showed that the higher concentrations of the hydrosol were most effective to improve the inhibition of bacterial growth [25].

The volatile components in hydrosols were demonstrated to be of extremely low concentrations as compared to the those in oils [26].

Table 5-Percentage of biofilm inhibition by *Eucalyptus camaldulensis* hydrosol

Isolates	Control	C1	C2	C3	C4
<i>S.aureus</i>	0.22	0.20	0.21	0.19	0.20
Percentage of inhibition		9%	4.5%	13%	9%
<i>S.epidermidis</i>	0.254	0.260	0.266	0.254	0.255
Percentage of inhibition		No	No	No	No
<i>S.pyogenes</i>	0.273	0.275	0.275	0.270	0.290
Percentage of inhibition		No	No	No	No
<i>S.mutans</i>	0.454	0.440	0.450	0.1452	0.460
Percentage of inhibition		No	No	No	No
<i>E.coli</i>	0.382	0.350	0.345	0.351	0.355
Percentage of inhibition		No	No	No	No
<i>K.pneumoniae</i>	0.440	0.32	0.442	0.350	0.410
Percentage of inhibition		27%	20%	20%	6.8%
<i>P.aeruginosa</i>	0.214	0.210	0.210	0.190	0.250
Percentage of inhibition		1.8%	1.8%	11%	No
<i>P.vulgaris</i>	0.460	0.461	0.462	0.461	0.466
Percentage of inhibition		No	No	No	No

This difference could suggest that the intensity of antimicrobial activity of hydrosols is not always the same as that of the essential oil. Thus, the nonvolatile chemical group reported to occur in hydrosols, which is primarily made of hydrophilic acidic constituents, could contribute in the inhibitory effect possibly in synergy with the trace essential oil components.

Essential oils contain different compounds such as aldehydes, ketones and alcohols which have been reported to show stronger antimicrobial effects than those of the terpenes [27,28].

Conclusions

The susceptibility of Gram-positive and Gram negative bacteria to *E. camaldulensis* essential oil is an important result of the current study, especially in relation to the effect of this oil against bacterial biofilm. This oil can be used as a natural antibacterial agent.

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