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Evaluation of Antimicrobial Activity of *Eucalyptus camaldulensis* Essential Oil Against the Growth of Drug-Resistant Bacteria

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Abstract

Background: Due to the increasing resistance of pathogenic bacteria to common antibiotics, researchers are seeking alternative antimicrobial agents with plant origins.
Objectives: The purpose of this study was to evaluate the activity of essential oil, extracted from the leaves of *Eucalyptus camaldulensis*, the major *Eucalyptus* species cultivated in Khuzestan, South of Iran, against the growth of drug-resistant bacteria.
Methods: Essential oil was extracted from the leaves using the hydrodistillation method in a Clevenger apparatus. The constituents of the essential oil were determined by gas chromatography-mass spectrometry (GC-MS). Moreover, the antimicrobial activity of essential oil was assayed using the disk diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also determined using the macrodilution method.
Results: Isolation and identification of the main components of essential oil identified 1,8-cineole (55.2%) as the main component. The essential oil could control resistant pathogenic bacteria. The greatest effect of essential oil was reported against *Klebsiella pneumoniae* with an inhibition zone diameter of 35 mm and MIC and MBC of 500 and 1500 ppm, respectively. On the other hand, the lowest effect was reported against *Salmonella infantis* and *Salmonella enteritidis* with an inhibition zone diameter of 11 mm and MIC and MBC of 6,000 and 8,000 ppm, respectively.
Conclusions: The essential oil of *E. camaldulensis* (Myrtaceae family) grown in Iran exhibited significant activities against some Gram-positive and Gram-negative bacteria. Therefore, *E. camaldulensis* is an effective antibacterial and bactericidal agent in the treatment of infectious diseases.

Keywords: Antimicrobial Agents, Essential Oil, GC-MS, *Klebsiella pneumoniae*, *Salmonella infantis*, *Salmonella enteritidis*, *Eucalyptus camaldulensis*

1. Background

Due to bacterial resistance to common antibiotics, surreptitious use of antimicrobial drugs by the general population, and high rates of allergies and side effects in chemical treatments, it is essential to find antimicrobial compounds with minimum side effects. Use of herbs for their medicinal properties dates back to long ago. Researchers are now seeking alternative antimicrobial agents with plant origins (1-3). Herbal medicine as a traditional health approach is popular among 80% of the world's population in Latin America, Asia, and Africa, and it has been shown to have fewer side effects (3, 4). According to previous research, there are various compounds and substances in different plants, including essential oils (EO), peptides, water, ethanol, phenol, methanol, soluble butanol compounds, and chloroform (4, 5).

It has been shown that EOs have insecticidal, antifungal, antiviral, antibacterial, and antioxidant properties. Moreover, some EOs have been used in cancer treatment (6-8), while others are used in food preservation, fragrance, and aromatherapy industries. EOs are valuable sources of biologically active compounds. Accordingly, there has been a growing interest in the antimicrobial effects of extracts from aromatic plants, particularly EOs (9).

Eucalyptus species are commonly used in traditional medicine. *Eucalyptus* is a large native genus from Australia, which belongs to the Myrtaceae family and includes nearly 900 species and subspecies (10). *Eucalyptus* species are well-known for their rapid growth. In fact, some species have exceptional growth and are among the tallest trees

in the world (20 - 50 m) (11). *Eucalyptus* species are important sources of gum, tannins, polyphenols, terpinenes, proteins, and dyes.

EO is the most important product, easily distilled from *Eucalyptus* leaves. The EO of several *Eucalyptus* species, such as *Eucalyptus maidenii*, includes 83.59% 1,8-cineole (eucalyptol) (12, 13). *Eucalyptus* EO is commonly used in deodorizing and cleaning products, as well as cough suppressants and decongestants (14). It has been used for the treatment of many diseases, such as influenza, dysentery, and skin disorders (1). Today, it is a common over-the-counter drug for cold treatment and has been long used to treat pneumonia, common colds, bronchitis, sore throat, and headache (15).

2. Objectives

The purpose of this study was to evaluate the antibacterial activity of EOs from the leaves of *Eucalyptus camaldulensis*, the major *Eucalyptus* species cultivated in Khuzestan, South of Iran, against the growth of drug-resistant bacteria.

3. Methods

3.1. Plant Collection and Identification

Eucalyptus was collected from botanical gardens, and leaf samples were identified at the herbarium of the faculty of pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran in September 2014.

3.2. EO Preparation

The identified plant leaves were weighed accurately and washed with distilled water. Then, the plant leaves were dried in shade at room temperature for 3 days. The dried leaves were chopped into small pieces, and EO was extracted by a Clevenger device. Extraction was performed by mixing 150 g of *Eucalyptus* leaves after 4 hours of maceration in 500 mL of distilled water. EOs were kept in dark glass bottles at -12°C until further use. The EO yield was 1% (16, 17).

3.3. Bacterial Strains

The microorganisms used in the present study were *Escherichia coli*, *Acinetobacter baumannii*, *Proteus vulgaris*, *Shigella sonnei*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Salmonella para typhi*, *Salmonella infantis*, *Salmonella enteritidis* (Gram-negative), and *Staphylococcus aureus* (Gram-positive). The resistant bacteria were obtained from the department of biology, faculty of basic sciences, University of Shahed, Bu-Ali hospital, Tehran, Iran.

3.4. Gas chromatography-Mass Spectrometry (GC-MS)

The most potent antibacterial EO from *E. camaldulensis* was analyzed by GC-MS. GC analysis was performed in a Shimadzu-9A gas chromatograph with a flame ionization detector. Quantitative analysis was performed with EuroChrom 2000 (Knauer), using the area normalization method, regardless of response factors. The analysis was performed by a DB-5 fused-silica column (30 m $\times$ 0.25 mm; film thickness, 0.25 μm; J&W Scientific Inc., Rancho Cordova, CA, USA).

The operating conditions were as follows: detector temperature, 250°C; injector temperature, 265°C (helium as the carrier gas). The oven temperature was set at 40 - 250°C, changing at a rate of 4°C per minute. The GC-MS unit was a gas chromatograph (Varian Model 3400), coupled with a Saturn-II ion trap detector. The column was the same as that of GC, and the GC conditions were the same as described above. The MS conditions were as follows: ionization potential, 70 EV; electron multiplier energy, 2000 V. The characteristics of EO components were derived from GC retention indices related to C7-C25 n-alkanes.

3.5. Determination of Antimicrobial Activity of EO

3.5.1. Inoculum Preparation

All bacterial strains were prepared freshly. The plates were cultured on a nutrient agar medium (NA, Que Lab, Canada) in several directions and parallel lines. Then, the plates were incubated for 24 hours at 37°C. For each test, to evaluate the antimicrobial effects, fresh bacteria were used, and fresh medium was prepared.

3.5.2. Preparation of Microbial Suspension

Preparation of microbial suspension requires a 24-hour culture from each microorganism. Accordingly, 24 hours before the experiments, the microorganisms were inoculated from stock medium to nutrient agar slant. In each sterile tube, 5 mL of Mueller-Hinton Broth (MHB; Que Lab, Canada) was poured and incubated with different strains of bacteria. The tubes were placed in a shaker-incubator at 37°C for 24 hours at 200 rpm (IKA KS 4000I Control, Germany). Afterwards, 100 μL of each suspension was added to sterile tubes, containing 4 mL of MHB and incubated again. Finally, absorbance of the samples was determined by a spectrophotometer (UNIC-UV-2100, USA) at 620 nm. The time needed to achieve the target concentration in the range of 0.08 - 0.13 nm was considered as the optimal interval and varied for each microbial strain (18-20).

3.5.3. Determination of Inhibition Zone

In vitro antibacterial activities were tested against 8 bacterial isolates with agar well diffusion assay. The antimicrobials in the plant EOs diffused into the medium and interacted with organisms which were seeded freshly in the

plate. The test was performed in sterile petri dishes (diameter, 80 mm), containing MHB agar medium (25 mL; pH, 7). Four sterile blank Whatman discs (diameter, 6 mm; Teb Padtan, Iran) were also placed in each plate.

Under aseptic conditions, different quantities of the extracted EO (5, 10, and 20 μL) were placed on paper discs. Blank discs were used as the positive controls. All the plates were incubated for 24 hours at 37°C, and diameters of microbial inhibition zones were measured with a ruler. Ultimately, antibacterial activity was evaluated as the average inhibition zone diameter in millimeters from triplicate samples. The final mean diameter was recorded, and the total inhibition zone diameter was scaled, including discs with 6 mm-diameter filters (16, 21, 22).

3.6. Measurement of MIC

The susceptibility of resistant bacteria to *E. camaldulensis* EO was assessed using the macrodilution method. Briefly, before preparing the microbial suspension, 20 sterile tubes, each containing MHB, were prepared. Different concentrations of *E. camaldulensis* EO (0.5 - 18 μL) were placed in the tubes, while in the 20th tube, as the negative culture, no amount of EO was used. Then, 100 μL of fresh bacterial suspension was added to each tube and incubated in a shaker-incubator for 24 hours at 37°C. The tubes were assayed for microorganism growth, and MIC was specified. MIC was defined as the lowest concentration of EO, which inhibited the visible growth of microorganisms. The concentration related to the first clear culture tube was considered as the MIC (16).

3.7. Measurement of MBC

MBC was described as the highest dilution (the lowest concentration) with no growth on the plates. On MHB medium plates, 100 μL of clear tubes was cultured using the spreader method, and all the plates were incubated for 24 hours at 37°C. If there was no growth in the MHB medium plate, MBC was determined. To ensure the test results, each episode was repeated 3 times (16).

4. Results

The output of the extraction process was estimated at 1.33%. The results of GC-MS analysis of *E. camaldulensis* EO are presented in Table 1. The GC-MS analysis indicated 17 compounds, constituting 99.96% of total EO. The major component was 1,8-cineole (55.2%). The other compounds included β-selinene (6.88%), hexadecanoic acid (5.5%), allo-aromadendrene (4.62%), 3-carene-δ (4.04%), γ-terpinene (3.94%), 8-octadecenoic acid (3.8%), β-gurjunene (3.3%), 9,10-dehydro-isolongifolene (3.1%), limonene (2.31%), α–pinene (2.07%), valencene (1.46%), aromadendrene (1.32%), β-elemene (1.02%), longifolene (0.5%), and ledene (0.5%). The results of antibacterial activity assay of *E. camaldulensis* EO against 8 resistant bacteria are presented in Table 2.

Table 1. The Chemical Composition of the Essential Oil (EO) of *Eucalyptus camaldulensis*

No.	Oil Compounds	%	RI
1	A-pinene	2.07	941
2	3-Carene-δ	4.04	1008
3	Limonene	2.31	1025
4	1,8-Cineole	55.2	1030
5	γ-Terpinene	3.94	1062
6	Clovene	0.4	1359
7	β-Elemene	1.02	1388
8	β-Gurjunene	3.3	1430
9	allo-aromadendrene	4.62	1439
10	Longifolene	0.5	1404
11	Aromadendrene	1.32	1448
12	Valencene	1.46	1482
13	β-selinene	6.88	1484
14	Ledene	0.5	1485
15	9,10-Dehydro-isolongifolene	3.1	1913
16	Hexadecanoic acid	5.5	1921
17	8-Octadecenoic acid	3.8	1939
	Total compounds	99.96	

Abbreviation: RI, retention indices.

The results showed that *E. camaldulensis* EO was effective against the tested organisms. The highest antibacterial activity of *E. camaldulensis* EO was reported against *K. pneumoniae* I1, which showed significant susceptibility to EO at concentrations of 5, 10, and 20 μL per disc per petri plate, based on the large growth inhibition diameters (18, 27, and 35 mm, respectively). Following *K. pneumoniae* strains, the largest inhibition zone was observed for A. *baumannii*, ranging from 14 to 30 mm.

The *S. aureus* strains showed minor difference in the inhibition zone diameter, compared to *A. baumannii* strains and susceptible bacteria. The activity of 5, 10, and 20 μL of *Eucalyptus* EO against *E. coli* F3 was significant (8, 19, and 26 mm, respectively). *E. camaldulensis* EO also showed a relatively good activity against *P. vulgaris* 1 (14, 16, and 20 mm). Moreover, it showed relatively moderate inhibition against *P. aeruginosa* P2 (8, 13, and 20 mm).

The antibacterial activity of 5, 10, and 20 μL of *Eucalyptus* EO against *S. sonnei* 3 and 5 strains indicated inhibition zone diameters of 8, 12, and 15 mm and 8, 11, and 14 mm, re-

Table 2. The Antibacterial Activitya and MIC and MBCb of *Eucalyptus camaldulensis* EO Against Resistant Bacteria[a]

No.	Bacteria	5 μL	10 μL	20 μL	MIC	MBC
	Gram-Positive					
1	*Staphylococcus aureus* 2	11	15	22	1500	2500
2	*Staphylococcus aureus* D2	10	19	28	1000	2000
3	*Staphylococcus aureus* D3	15	17	28	1000	2000
4	*Staphylococcus aureus* D5	11	16	30	1000	2000
5	*Staphylococcus aureus* D7	12	17	29	1000	2000
6	*Staphylococcus aureus* D8	13	17	30	1000	2000
	Gram-Negative					
7	*Escherichia coli* E1	10	19	24	1500	2500
8	*Escherichia coli* E2	7	18	25	1500	2500
9	*Escherichia coli* F3	8	19	26	1500	2500
10	*Acinetobacter baumannii* A2	14	22	30	1000	2000
11	*Acinetobacter baumannii* A5	15	21	30	1000	2000
12	*Acinetobacter baumannii* A7	14	20	30	1000	2000
13	*Proteus vulgaris*	10	15	20	2500	3500
14	*Proteus vulgaris* 1	14	16	20	2500	3500
15	*Proteus vulgaris* 2	13	15	19	2500	3500
16	*Shigella sonnei* 34	12	15	18	3000	4500
17	*Shigella sonnei* 3	8	12	15	3500	4500
18	*Shigella sonnei* 5	8	11	14	3500	4500
19	*Pseudomonas aeruginosa* P2	8	13	20	2500	4000
20	*Pseudomonas aeruginosa* G2	9	12	20	2500	4000
21	*Klebsiella pneumoniae* I1	18	27	35	500	1500
22	*Klebsiella pneumoniae* K1	17	26	33	500	1500
23	*Salmonella typhi* A1	8	10	13	4000	6000
24	*Salmonella typhi* A2	10	10	13	4000	6000
25	*Salmonella para typhi* B	8	14	17	3500	4500
26	*Salmonella typhi* 146	10	11	14	4000	6000
27	*Salmonella infantis*	-	9	11	6000	8000
28	*Salmonella enteritidis* 119	-	8	11	6000	8000

Abbreviations: MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration.
[a] Disc diameter, 6.2 mm.

spectively. *Eucalyptus* EO exhibited relatively poor activity against *S. typhi* at concentrations of 20 and 30 μL with inhibition zone diameters of 10 and 13 mm, respectively. The lowest activity at 20 μL (inhibition zone diameter, nearly 11 mm) was demonstrated against *S. infantis* and *S. enteritidis* 119. At an EO concentration of 5 μL per disc per petri plate, no activity was found against *S. infantis* and *S. enteritidis*. The MIC and MBC of *E. camaldulensis* EO for 8 resistant bacteria are presented in Table 2.

The lowest MIC and MBC of EO were reported against *K. pneumoniae* (500 and 1500 ppm, respectively), and they were considered as the most sensitive bacteria. Moreover, the MIC and MBC for *A. baumannii* and *S. aureus* strains were 1000 and 2000 ppm, respectively. It was revealed that the MICs for *E. coli* strains (1500 ppm) were lower than those of *P. vulgaris* and *P. aeruginosa* strains (2500 ppm). The MBCs against *P. vulgaris* and *P. aeruginosa* were 3500 and 4000 ppm, respectively.

The MIC of EO against *S. sonnei* was 3500 ppm. Although EO was effective against most tested pathogenic

strains, its effectiveness against *S. infantis* and *S. enteritidis* 119 was significantly lower (6000 and 8000 ppm, respectively). Therefore, higher levels of antibacterial activity are required in the treatment of infections caused by *S. infantis* and *S. enteritidis* 119 if they are not toxic to the tissues.

5. Discussion

Medicinal plants have been used for the treatment of infectious diseases. With respect to ecophysiological differences among plants grown in different geographical areas, research is necessary to discover their pharmaceutical efficacy (23). Recent emergence of drug-resistant bacteria highlights the importance of antimicrobial activity (11, 24). In this study, hydrodistillation of *E. camaldulensis* leaves yielded 1.33% EO (considering the fresh weight of young leaves) with a spicy aromatic odor. These results are in line with reports from the literature, indicating yields of 1.3 - 1.8% (considering the fresh weight of immature *E. globulus* leaves) in Buenos Aires (25) and 1.8% (considering the fresh weight of immature *E. globulus* leaves) in Montenegro (26). Despite limited consistent evidence in the literature, the yield was estimated at 1.9 - 2.7% (considering the fresh weight of immature *E. globulus* leaves) in Morocco (27), 2.68% in Argentina (28), and 3.91% in Brazil (considering the fresh weight of young *E. cinerea* leaves) (29).

Antibacterial activity of EO has been attributed to the presence of some active components. Earlier research has shown that the antibacterial activity of EOs is because of their major components (30). The analysis of *E. camaldulensis* EO indicated 1,8-cineole as the main component. Because of the high content of 1,8-cineole (73.07%), EO is categorized as a medicinal or eucalyptol type (29). Overall, cineole is monoterpenoid cyclic ether, which can affect the cytoplasmic membrane of target bacteria (11). The 1,8-cineole content in *E. globulus* has revealed larvicidal and ovicidal activities against *Haemonchus contortus* (31).

In a study by Damjanovic-Vratnica et al. the main component, 1,8-cineole (85%), was active against *S. aureus*, *E. coli*, and *A. baumannii* in most *E. globulus* EOs (26). In another study, 1,8-cineole (72.71%) was the most abundant component in *E. globulus* EO, which was active against *Lactococcus garvieae* (32). Identification of these compounds with great biological activities is vital, as it helps determine chemical compositions, which can be helpful in designing novel medications with remedial activities against human pathogens.

It is very difficult to compare the obtained data with the literature, as several parameters can affect the results, such as different chemical structures because of environmental factors (eg, day length, nutrients, temperature, and geography) (33). According to the results, EO of native *E. camaldulensis* leaf grown in Khuzestan is a significant antibacterial agent against both Gram-negative and Gram-positive drug-resistant pathogenic bacteria. The tested bacteria in our study were sensitive to EO, although the extent of antibacterial effect varied, depending on the type of microorganisms. The maximum effect was observed against *K. pneumoniae*, while the lowest effect was reported against *S. infantis* and *S. enteritidis*.

In a study by Cimanga et al. 5 μL of *E. urophylla* and *E. globulus* EO showed an inhibition zone diameter of 18 mm against *K. pneumoniae* strains, which is similar to the results of the present study (34). According to our results, *E. camaldulensis* EO at a concentration of 20 μL displayed major activity against A. *baumannii* with an inhibition zone diameter of 30 mm, while in another study, Damjanovic-Vratnica et al. showed an inhibition zone diameter of 36 mm for *E. globulus* in Montenegro (26).

Inhibition of *S. aureus* is of great importance, as resistant strains from this species appear each year. Treatment can be a major problem in near future, especially in cases with hospital-acquired infections, which are resistant to methicillin and vancomycin to some extent (11). It has been reported that Gram-negative bacteria have lower sensitivity to volatile EOs of *Eucalyptus*, compared to Gram-positive bacteria. This can be due to differences in the cell structure of these bacteria, as Gram-positive bacteria have more mucopeptides in their cell wall structure, while Gram-negative bacteria only have a thin layer of mucopeptides; also, lipoprotein and lipopolysaccharides comprise most of the cell structure; therefore, Gram-negative bacteria are more resistant (1, 35, 36).

Borumand et al. determined the antibacterial activity of *C. sativum* EO against *S. aureus* and reported MIC and MBC of 1000 ppm. Similar inhibitory effects and better bactericidal properties were reported against *Eucalyptus* (37). Moreover, Gandomi Nasrabadi et al. reported the MIC of *Artemisia absinthium* EO against *S. aureus* to be 3000 ppm, which is less effective than *Eucalyptus* in our study (38). Damjanovic-Vratnica et al. also showed the significant antimicrobial activity of *E. globulus* leaf EO against *S. aureus* bacteria (26).

Ghalem and Mohamed showed that the effects of *E. camaldulensis* leaf EO against *S. aureus* bacteria were similar to the present study (35). Furthermore, in a study by Ghaderi et al. *Anethum graveolens* EO with 312.5 ppm, *Coriandrum sativum* EO with 625 ppm, and *Achillea millefolium* EO with 10,000 ppm were effective against the growth of *Escherichia coli*. However, *Achillea millefolium* had weaker effects than *Eucalyptus* in our study (39).

Cimanga et al. showed similar results about the antibacterial activity of EOs from *E. citriodora* and *Monodora myristica* (14 mm) against *P. vulgaris* (14 mm) (34). Since *Pseudomonas* species can metabolize a wide range of or-

ganic compositions (accordingly, it is applied widely in bioremediation), their high level of resistance can be explained. In our experiment, the MIC of *E. camaldulensis* EO against *P. aeruginosa* was 2500 ppm, while in the study by Ghaderi et al. *Coriandrum sativum* and *Anethum graveolens* EO showed MICs of 5000 and 1250 ppm, respectively (39). In the study by Borumand et al. the MBCs for *Coriandrum sativum* and *Anethum graveolens* EO against *Salmonella typhimurium* were found to be more than 4000 ppm (37).

6. Conclusions

The EO of *E. camaldulensis* (Myrtaceae family) grown in Iran exhibited major activities against different pathogenic microorganisms. Treatment can be difficult considering the emergence of strains showing resistance to a wide range of antibiotics. The obtained results confirm the potential use of *E. camaldulensis* EO as an alternative antibacterial agent and a natural drug for the treatment of various infectious diseases.

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Footnotes

Authors' Contribution: This study was carried out in collaboration between the authors. Elia Ostad Asiaei performed the experiments and wrote the first draft of the manuscript. Eskandar Moghimipour and Mohammad Hadi Fakoor designed the study and performed the statistical analyses. All authors read and approved the final manuscript.

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