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Original Article

Chemical Composition and Antimicrobial Potential of *Eucalyptus camaldulensis* Essential Oil from Shushtar, Iran

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ABSTRACT

Here, to assess the chemical composition and antibacterial/antioxidant effects of the essential oils of eucalyptus, the young leaves of eucalyptus trees (*Eucalyptus camaldulensis* Dehnh.) cultivated in Shushtar city (Khuzestan Province, Iran) were utilized in the late spring season of 2024. The essential oil extraction was carried out using the water distillation method, resulting in a 2% yield based on the dry weight of the leaves. Gas chromatography-mass spectrometry (GC-MS) analysis of the essential oil indicated the presence of (S,E)-2,5-Dimethyl-4-vinylhexa-2,5-dien-1-yl acetate, alpha-terpinene, and (-)-Globulol as the major components, accounting for 17.63%, 9.97%, and 6.23% of the total composition, respectively. Further DPPH assay testing showed a concentration-dependent inhibitory effect, with the highest concentration (0.80 mg/ml) exhibiting an impressive 93.62% inhibition. According to the ANOVA results, a significant difference was observed among all nine treatments (multiplying by three different essential oil concentrations of 0.2, 0.4, and 0.8 mg/mL and three different bacterial agents of *S. aureus*, *B. cereus*, and *E. coli*) in terms of inhibition zone recorded using the agar well diffusion method. The largest inhibition zones were observed for three treatments of "0.8 mg/mL + *B. cereus*", "0.4 mg/mL + *S. aureus*", and "0.8 mg/mL + *S. aureus*" with the inhibition zone values of 39.62 mm, 35.37 mm, and 39.11 mm, respectively. On the other hand, the minimum inhibition zone value of 14.02 mm was observed for the "0.2 mg/mL + *E. coli*" treatment. According to the results, both gram-positive bacteria of *S. aureus* and *S. cereus* were more sensitive to the essential oil of *E. camaldulensis* essential oil than the gram-negative bacteria of *E. coli*. These current promising results demonstrated the effectiveness of *E. camaldulensis* essential oil as a natural and eco-friendly alternative for antimicrobial studies.

INTRODUCTION

Essential oils are natural mixtures of organic compounds that are extracted from various parts of plants, such as flowers, leaves, stems, and roots [1]. These plant-derived oils are known for their distinct aromas and have been used for centuries in traditional medicine and for their therapeutic properties [2]. Composed of an intricate blend of volatile molecules, essential oils are produced by plants as secondary metabolites, which are responsible for protection against pathogens and predators [3, 4]. Due to their unique chemical composition, essential oils have a wide range of applications, including but not limited to aromatherapy, skincare, and natural cleaning [5, 6]. With growing interest in alternative and complementary medicine, essential oils have become increasingly popular as a natural and holistic approach to healing and enhancing well-being. However, it is important to note that not all essential oils are safe for internal use, and careful research and proper guidance should be sought before using them for medicinal purposes [5]. As with any natural product, it is essential to handle and use essential oils with caution and respect [5].

The chemical compositions and concentrations of essential oil can significantly differ among plant species and even within similar species due to a

variety of internal and external factors [7]. These factors include variances in plant parts, harvest time, drying and storage methods, distillation processes, climatic conditions, and so on [7]. To accurately identify and analyze the various components of essential oil, gas chromatography-mass spectrometry (GC-MS) is commonly utilized [8]. The MS component of this system utilizes the ionized state and unique fragmentation patterns of each separated component to determine its chemical structure [9]. With increasing rates of drug-resistant pathogenic bacteria that are responsible for many fatal infections, finding effective treatments has become crucial. In fact, in 2019 alone, 1.27 million individuals worldwide lost their lives due to these drug-resistant infections [10, 11]. The bacteria *S. aureus*, in particular, has shown high levels of resistance to commonly used antibacterial drugs. Furthermore, the potential for adverse side effects on human health is a concern with chemically synthesized antibacterial drugs. As a result, there has been a growing focus on searching for natural alternatives, such as herbal medicines, that can effectively combat these drug-resistant pathogens. Research in this area has led to the exploration of essential oils as potential agents against these threats [12, 13].

Eucalyptus is a genus of the Myrtaceae family, which comprises 900 species and subspecies [14]. This evergreen woody perennial ranges in height from shrubs to tall trees and grows rapidly to gigantic size [15]. The *Eucalyptus camaldulensis* Dehnh. (*E. camaldulensis*), also known as long beak Eucalyptus, murray red gum, red gum, river gum, and red river gum, is assumed as one the most important and widely distributed *Eucalyptus* species throughout the world [16]. Like all other species within the same genus, its essential oil extracted normally from the leaves is of the utmost importance [14, 16]. This is mainly rooted in the incredible advantages of many chemical constituents available in its leaf essential oils extracted normally using steam distillation- [17] and/or hydro-distillation-based methods [18]. The essential oil of Eucalyptus has demonstrated therapeutic potential and is utilized in medicine for its diverse therapeutic activities, as reviewed comprehensively by [14]. Previous studies on *E. camaldulensis* have demonstrated its essential oil's potential analgesic, anti-inflammatory [19], anticancer, antioxidant [20], antiviral [21], and anti-diabetic activities through mechanisms such as inhibiting α-glucosidase and α-amylase via non-competitive inhibition [22].

The literature regarding the essential oil *of E. camaldulensis* Dehnh. revealed the dominance of 1,8-cineole (eucalyptol), trans-pinocarveol and terpinen4-ol, all of which belong to the chemical class of oxygenated monoterpenes [16]. Additionally, significant amounts of monoterpene hydrocarbons (β-pinene, α-thujene, γ-terpinene, p-cymene) have been found in the oils, while the presence of sesquiterpene hydrocarbons and oxygenated sesquiterpenes are minimal [16]. Of particular significance is the primary active ingredient, 1,8-cineole, which has been clinically approved for use in the drug Soledum® [23]. This drug is widely utilized for maintaining sinus and respiratory health [23]. However, histopathological examinations on mice administered a high dose of 1,8-cineole revealed the presence of granular degeneration and vacuolar degeneration in liver and kidney tissue [24, 25]. Similar to this, it has been observed that the administration of atropine, a tropane alkaloid medication commonly used for a variety of medical purposes, may result in a number of potential side effects. These can include but are not limited to blurred vision, dryness of the mouth and eyes, confusion, sensitivity to light, dizziness, rapid heart rate, fatigue, flushing of the skin, palpitations, and difficulties with urination such as hesitance or retention [26].

In recent years, there has been a growing interest in plant essential oils, and plant extracts followed by the nanoformulations of different natural produces as potential sources of alternative, bioactive compounds [27-29]. Notably, their potential antimicrobial properties have been widely studied [28, 30, 31]. The mechanisms by which essential oils combat disease-causing microbes include inhibiting protein synthesis, preventing cell wall synthesis and nucleic acid replication, and penetrating the lipid membrane of pathogenic microbes, ultimately leading to apoptosis [32]. As a result, the investigation of the antimicrobial activities of essential oils derived from medicinal plants has become increasingly important. These oils are known to possess unique chemical structures, and numerous studies have explored the relationship between the chemical structure of oxygen-containing compounds and their

antimicrobial mechanisms [33]. The essential oils of *E. camaldulensis* plant have been tested against a wide range of bacteria [16]. The Gram-positive bacterium in antibacterial screening of the essential oil of *E. camaldulensis* plant is *Staphylococcus aureus* [34-36], which is a methicillin-resistant bacterial agent [16]. Besides, other bacteria including *Bacillus cereus* [37], *Escherichia coli* [34-37], *Bacillus subtilis* [36], and so on [16]. Various studies have identified different mechanisms by which essential oils exert their antibacterial effects. For instance, cumin essential oil has been found to decrease the permeability of bacterial cell membranes, leading to the release of cellular components and ultimately, cell death [38]. *Trachyspermum copticum* essential oil has been demonstrated to disrupt bacterial cell membrane integrity, leading to the formation of excessive pores and eventual cell lysis [39]. Similarly, exposure to fenugreek essential oil has been found to increase the permeability of the bacterial cytoplasmic membrane, leading to the leakage of proteins and K^+ ions and ultimately, cell death [40]. These findings demonstrated the significant potential of essential oils as a natural and effective means of combating bacterial infections.

As mentioned above, numerous investigations have been conducted to assess the variability of chemical composition and antibacterial effects of essential oils from different ecotypes of Eucalyptus. However, the essential oil of the ecotype collected from the Shushtar region in Khuzestan Province, Iran has not yet been studied for its antibacterial effects against both Gram-positive (including *S. aureus* and *B. cereus*) and Gram-negative (such as *E. coli*) bacterial agents. In light of this, the current project has undertaken the task of determining the chemical composition of the essential oil extracted from the leaves of *E. camaldulensis* using the GC-MS method. Moreover, the antioxidant and antibacterial activities of the resulting essential oils were also thoroughly investigated in order to gain a better understanding of their potential applications in the field of natural remedies and therapeutics. Through this research, we hope to shed light on the potential benefits of this ecotype of Eucalyptus in combatting bacterial infections and promoting overall health and well-being.

MATERIALS AND METHODS

Collection of Plant Materials and Extraction of Essential Oil

The eucalyptus plants cultivated in Shushtar City (Khuzestan, Iran) were used for this purpose. The *Eucalyptus* species identification process was validated by the esteemed Herbarium of Razi University Agriculture and Natural Resources Campus, Iran. Once the leaves of the *Eucalyptus* plants were carefully collected, they were meticulously dried in the shade to preserve their quality. A precise approach was taken to extract the precious essential oils from the plant, with 50 g of dried leaves being ground into a fine powder using an electric mill. The resulting powder was then placed into a flask of a distillation apparatus, specifically the Clevenger apparatus, and 500 ml of distilled water was added to it. A cold-water flow was established and the flask was then placed on an electric heater for 90 minutes, allowing for the essential oil to be extracted. Once collected, the pure and potent essential oil was stored in sealed glass bottles in the refrigerator, ensuring its longevity and maintaining its properties for future use. This careful and precise process ensures the quality and effectiveness of the essential oil of the Eucalyptus plant for any desired purpose. To accurately measure the yield of the essential oil, the following formula was used.

$R (\%) = (ME/MS) * 100$

This measurement, represented by the symbol R, is calculated by dividing the weight of the extracted essential oil, represented as ME, by the weight of the dry plant material used, symbolized by MS.

The Chemical Composition of the Essential Oil

The component analysis and identification of the essential oils were conducted using gas chromatography-mass spectrometry (GC-MS) equipment from Agilent, consisting of 6890N GC and 5973N MS systems with HP5-ms columns. Using the split mode in the inlet, a defined portion of the sample was introduced into the column, ensuring a continuous flow of Helium as the carrier gas. The columns operated in constant flow mode, maintaining a steady flow rate of 1 ml/min consistently throughout the analysis. Consistent conditions were utilized for each injection of the essential oil to identify the constituent compounds accurately. The compounds in the essential oil were

identified and quantified using mass spectra analysis. The Wiley library data was consulted to determine the input material type for the mass spectrometer analysis.

Preparation and Culturing of the Bacteria

In the current study, three different bacterial agents of *Bacillus cereus*, *Escherichia coli*, and *Staphylococcus aureus* were utilized. The *E. coli* strain ATCC 25922 and the *S. aureus* strain ATCC 25923 were purchased from the Iranian Scientific and Industrial Research Company (IROST), Iran. The *B. cereus* strain of KCCM 40935 was obtained from the bacterial archive of Razi University, Kermanshah, Iran. To initiate the experiment, these bacteria were lyophilized and then incubated in 20 ml of LB culture medium at a temperature of 37 ºC for 24 h. Following this step, the bacteria were transferred to a solid culture medium to facilitate their growth. To prepare a microbial suspension, multiple colonies from the 24-hour bacterial cultures were transferred into tubes filled with sterile distilled water. The concentration of bacteria per milliliter of distilled water was meticulously determined using a spectrophotometer, and the turbidity was standardized to the 0.5 McFarland standard. This process yielded an average optical density ranging from 0.133 to 0.080 at a wavelength of 600 nm, suggesting an estimated concentration of approximately 1.5×10^8 bacteria per milliliter.

Agar well Diffusion Method

By now, various antimicrobial testing methods have been proposed for the *in vitro* investigation of extracts and pure drugs as potential antimicrobial agents, as reviewed comprehensively by [41]. Among these, the agar well diffusion method, a widely used technique for evaluating the antimicrobial properties of substances [42, 43], was employed to investigate the effectiveness of eucalyptus essential oil against both Gram-positive (*S. aureus* and *B. cereus*) and Gram-negative (*E. coli*) bacteria. In accordance with the established protocols, bacterial suspensions with a concentration of 0.5 McFarland were prepared and 1.0 ml of each suspension was used in the experiment. These bacterial strains were then inoculated onto plates containing eucalyptus essential oil. In order to evaluate the potential inhibitory effects of the essential oil, wells with a diameter of 6 mm were created on Muller-Hinton agar plates using an agar gel punch. Subsequently, three different essential oil concentrations of eucalyptus (i.e., 2.0, 4.0, and 8.0 mg/ml) together with DMSO as a control were carefully dispensed into the wells using a micropipette. After 24 hours of incubation at 37 °C, the zones of inhibition were measured with a caliper to determine the antibacterial activities of the samples. To ensure accuracy, this experiment was repeated three times.

Free Radical Scavenging DPPH

A meticulous experiment was conducted to investigate the potential antioxidant activity of various extracts by measuring their ability to scavenge free radicals. A solution of 1.0 mM of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol was first prepared and then one milliliter of the DPPH purple solution was added to individual vials containing different concentrations of the extract in methanol. The vials without samples served as the control group in the study. To ensure complete mixing of the samples, all vials were thoroughly shaken and then incubated in darkness for one hour. Subsequently, the absorbance of all samples was measured at 517 nm using a spectrophotometer. The percentage of DPPH radical inhibition was then calculated using the established formula proposed by [44]. Afterward, the absorbance of all samples was measured at 517 nm. The percentage of DPPH radical inhibition was calculated using the following formula [44]:

$$\text{Inhibition (\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

Statistical Analyses

A factorial design with two independent factors (three types of bacteria (i.e., *B. cereus*, *E. coli*, and *S. aureus*) and three different concentrations of eucalyptus essential oil (i.e., 0.2, 0.4, and 0.8 mg/ml)) based on a completely randomized design (CRD) with three replicates was employed. Then, to determine possible differences among the resultant nine treatments in terms of inhibition zone (mm) a two-way ANOVA was conducted. In addition, a completely randomized design (CRD) with three replicates was applied for the DPPH assay. Statistical analyses were carried out using SPSS software, and the mean values were compared via Duncan's multiple range test at $p < 0.05$. Finally, to visually classify the gram-negative and gram-positive bacteria studied here, hierarchical cluster analysis (HCA) combined with heatmap

visualization was applied using the ClustVis web tool [45].

RESULTS AND DISCUSSION

GC-MS Analysis of Eucalyptus Essential Oil

The GC-MS analysis conducted on the eucalyptus essential oil revealed the presence of forty major compounds, as depicted in Fig. 1. A thorough examination of the results confirmed that the primary constituents of the eucalyptus essential oil were (S,E)-2,5-Dimethyl-4-vinylhexa-2,5-dien-1-yl acetate, alpha-terpinene, and (-)-Globulol, accounting for 17.63%, 9.97%, and 6.23% of the total composition, respectively (Table 1). These results indicated a significant concentration of these two compounds, suggesting their potential role in determining the overall properties and potential uses of eucalyptus essential oil. In the study of [46], the yield of essential oils extracted from seven *Eucalyptus* species developed in Tunisia ranged from 1.2% to 3% (w/w). Three compounds of α-pinene, 1,8-cineol and pinocarveol-trans were detected for the entire essential oils extracted from seven *Eucalyptus* species. Among different constituents, the major compound in all species was 1,8-cineol, ranging from 49.07 to 83.59% [46]. The essential oils composition of twenty *Eucalyptus* species originating from Zerniza and Souinet arboreta (North West and North of Tunisia) were analyzed by GC and GC/MS. From the results, 18 major compounds were overall identified, of which, the major constituents included 1,8-cineole followed by *α*-pinene, *p*-cymene, borneol, cryptone, spathulenol, viridiflorol and limonene [47]. Recently, the chemical composition of the essential oils extracted from leaves of four *E. urophylla* clones and one *E. urophylla* × *E. camaldulensis* hybrid clone grown in Thailand were studied using GC-MS [48]. Based on the C-MS results, 1,8-cineole, *α*-terpinyl acetate, *β*-caryophyllene, and spathulenol were superior in the *E. urophylla* oils, while 1,8-cineole, *α*-terpinyl acetate, *p*-cymene, and *γ*-terpinene were the major compounds in essential oils extracted from hybrid clone [48]. More recently, the chemical composition of the essential oils extracted from leaves of two different *Eucalyptus* species of *E. camaldulensis* and *E. tetragona* were assessed via GC-MS method. Overall, 17 and 14 components were identified in the essential oils of *E. camaldulensis* and *E. tetragona*, respectively, with the major components of 1,8-cineole, β-cymene and α–pinene recognized in both *Eucalyptus* species [49].

Antioxidant Activity (DPPH Scavenging Efficiency) of Eucalyptus Essential Oil

The DPPH assay was conducted to assess the potential antioxidant activity of eucalyptus essential oil. Analysis of the ANOVA results revealed a significant difference among the three tested concentrations (0.20, 0.40, and 0.80 mg/ml) of eucalyptus essential oil in terms of DPPH scavenging efficiency (p<0.01).

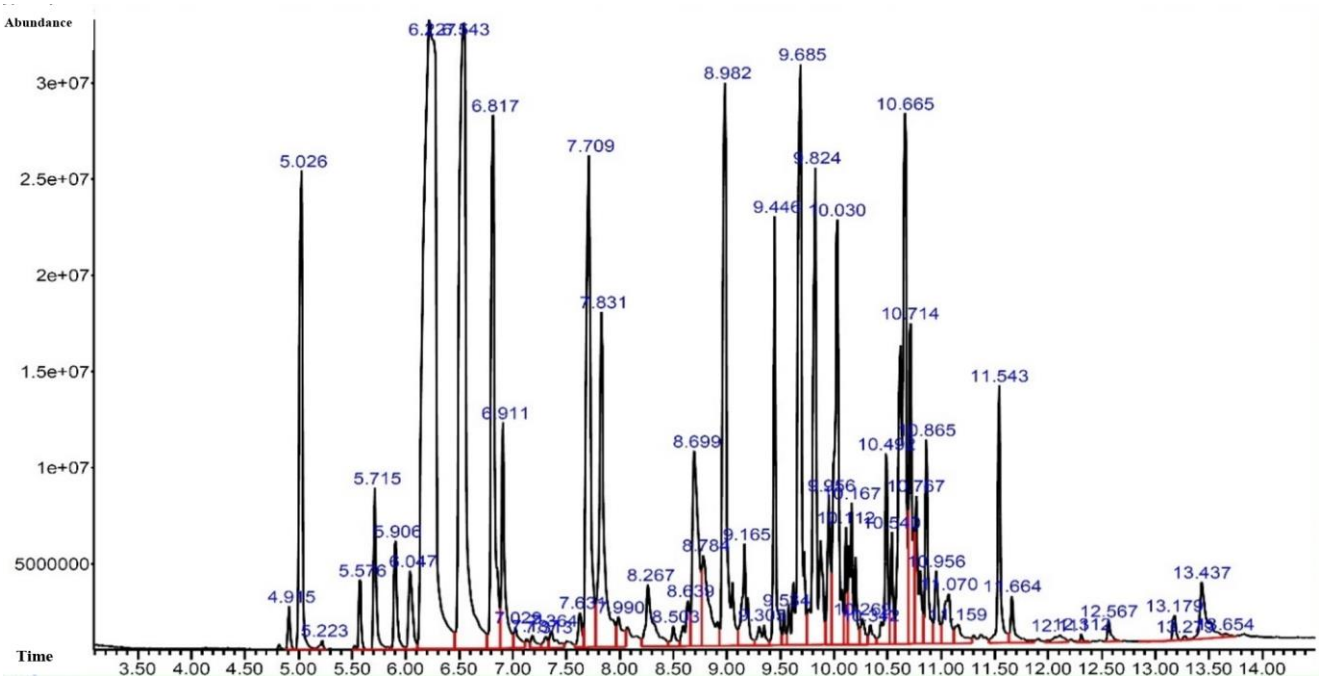

Fig. 1 The GC-MS chromatogram of eucalyptus essential oil extracted from leaves.

Table 1 Components of *Eucalyptus* leaf essential oil. Concentrations are given as percentages determined based on peak area

No.	Compound	[a]RT	[b]RI	[c]RI	Formula	[d]%
1	1R-á-Pinene	5.026	929	1013	C10H16	3.365
2	(S,E)-2,5-Dimethyl-4-vinylhexa-2,5-dien-1-yl acetate	6.227	1451	-	C12H18O2	17.631
3	ã-Terpinene	6.543	1050	1246	C10H16	9.974
4	á-Terpinolene	6.817	1079	1283	C10H16	4.497
5	Isoamyl valerianate	6.911	1089	1290	C10H20O2	1.575
6	Terpinen-4-ol	7.709	1164	1602	C10H18O	4.782
7	á-Terpineol	7.831	1175	1697	C10H18O	3.534
8	Thymol	8.699	1270	2189	C10H14O	2.540
9	á-Terpinyl acetate	8.982	1333	1692	C12H20O2	5.293
10	isoledene	9.165	375	-	C15H24	1.040
11	á-Gurjunene	9.446	1406	1528	C15H24	2.140
12	Aromandendrene	9.685	1447	1635	C15H24	5.520
13	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-, (1R,4E,9R)-	9.824	1464	1572	C15H24	4.381
14	Ledene	10.030	1492	1697	C15H24	3.876
15	â-Cadinene	10.167	1522	1720	C15H24	1.422
16	Epiglobulol	10.494	1554	2025	C15H26O	1.274
17	(-)-Globulol	10.665	580	-	C15H26O	6.231
18	Viridiflorol	10.714	1582	2095	C15H26O	2.277
19	Ledol	10.767	1586	2035	C15H26O	1.257
20	2-Naphthalenemethanol, decahydro-á,á,4a-trimethyl-8-methylene-, [2R-(2á,4aá,8aâ)]-	10.865	1636	2239	C15H26O	1.324
Total						78.64

[a]RT: Retention time (min); [b]RI: Retention index about n-alkanes (C7–C20) (Standard non-polar); [c]RI: Retention index about n-alkanes (C7–C20) (Polar); [d]%, Relative proportions of the essential oil components expressed as percentages obtained by GC-MS.

Further examination through Duncan-based mean comparison (Fig. 2) revealed no significant difference between the 0.40 and 0.80 mg/ml concentrations, indicating similar DPPH scavenging efficiency at these levels ($p > 0.05$). However, both were found to be significantly more effective than the 0.20 mg/ml concentration. This suggested that the antioxidant activity of eucalyptus essential oil is influenced by concentration. The results also showed a clear relationship between concentration levels and inhibitory activity, with a moderate inhibitory effect of 59.85% seen at the lowest concentration of 0.20 mg/ml, and a substantial increase in inhibition percentage (93.62%) observed at the highest concentration of 0.80 mg/ml. This demonstrated the concentration-dependent nature of the antioxidant efficacy of eucalyptus essential oil, with the highest level of inhibition being achieved at the highest concentration. These findings highlighted the potential of eucalyptus essential oil as a natural and effective source of antioxidants. These findings were consistent with the prior research conducted by [50], further strengthening the validity and significance of our results.

Agar well diffusion-based Inhibition for Antibacterial Activity of Eucalyptus Essential Oil

The results displayed in Figures 3-5 served as a compelling illustration of the effectiveness of different concentrations of eucalyptus essential oil in inhibiting the growth of either Gram-positive (*S. aureus* and *B. cereus*) or Gram-negative (*E. coli*) bacterial agents. This is evidenced by the distinct and well-defined zones of inhibition surrounding the agar well diffusion containing the essential oil. In order to obtain verifiable data, precise measurements were taken to determine the diameter of the inhibition zones at varying concentrations of the essential oil. Through this quantitative analysis, it was determined that the potency of eucalyptus essential oil increases with higher concentrations, thereby reinforcing its potential as a natural antibacterial agent. These findings served to further support the growing body of research on the therapeutic properties of essential oils, and the potential for their use in combating bacterial infections.

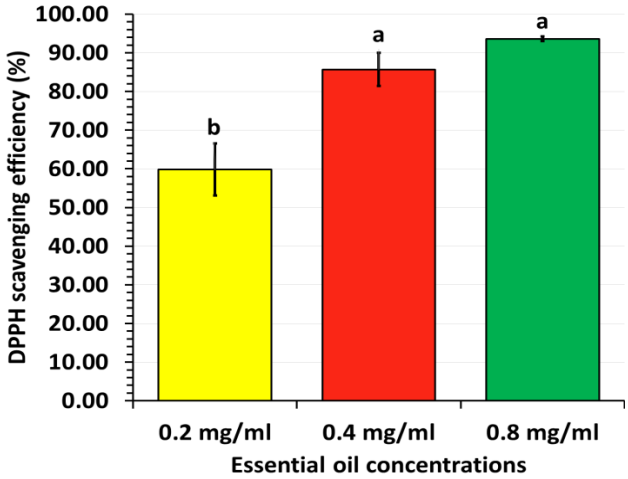

Fig. 2 Antioxidant activity (DPPH scavenging efficiency) of three different concentrations of eucalyptus essential oil determined by DPPH assay. Data represented the mean of three independent replicates ± SD. Values with different letters differed significantly by Duncan's multiple range test (p< 0.05).

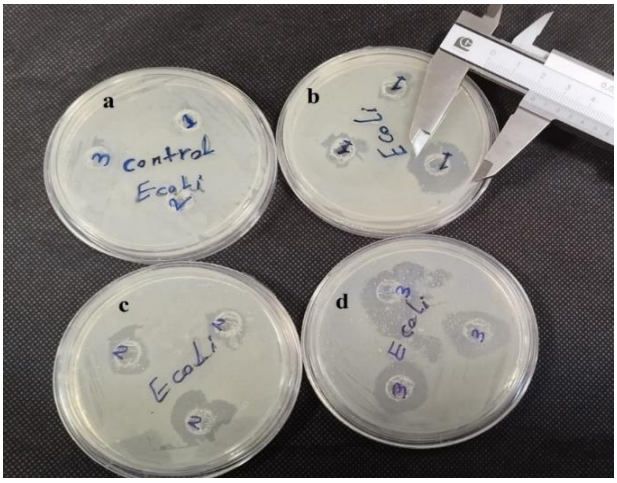

Fig. 3 Antimicrobial activity using the well diffusion method against *E. Coli*

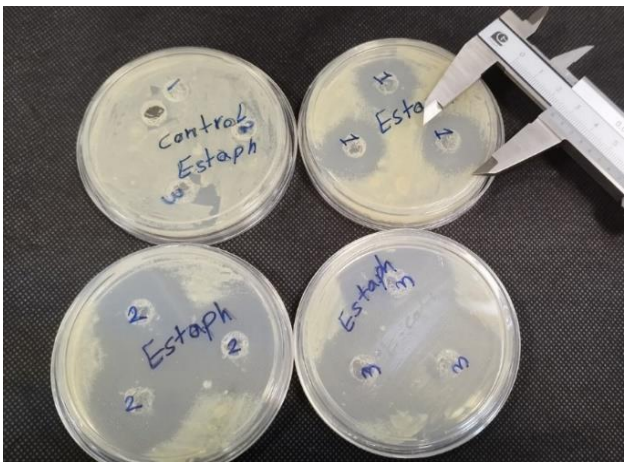

Fig. 4 Antimicrobial activity using the well diffusion method against *S. aureus*.

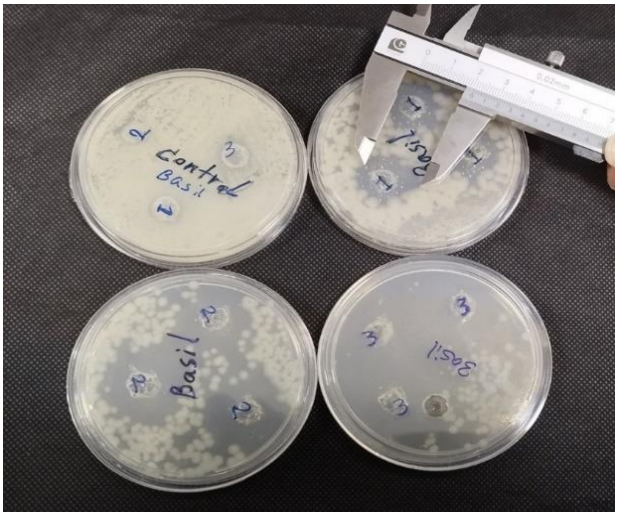

Fig. 5 Antimicrobial activity using the well diffusion method against *B. cereus*.

According to the ANOVA results, the interaction between bacteria and concentrations of eucalyptus essential oil was statistically significant (p < 0.05). In this sense, the effectiveness of three different concentrations (0.2, 0.4, and 0.8 mg/ml) of essential oils on the treatment of three bacterial agents (i.e., *E. coli*, *B. cereus*, and *S. aureus*) was investigated using Duncan's multiple range test. All these nine treatments (multiplied by three different essential oil concentrations and three different bacterial agents; 3 * 3 = 9) were referred to as "0.2 mg/mL + *E. coli*", "0.4 mg/mL + E. *coli*", "0.8 mg/mL + *E. coli*", "0.2 mg/mL + *B. cereus*", "0.4 mg/mL + *B. cereus*", "0.8 mg/mL + *B. cereus*", "0.2 mg/mL + *S. aureus*", "0.4 mg/mL + *S. aureus*", and "0.8 mg/mL + *S. aureus*", representing the combination of each bacterial agent with the respective essential oil concentration. This approach allowed for a comprehensive evaluation of the effects of different concentrations of essential oils on each bacterial agent individually. According to the mean comparison resulting from Duncan's multiple range test, all these nine treatments were categorized into four groups (Fig. 6, a-d;). In this sense, the maximum inhibition zone (mm) was obtained for "0.8 mg/mL + *B. cereus*", "0.4 mg/mL + *S. aureus*", and "0.8 mg/mL + *S. aureus*" treatments with the inhibition zone values of 39.62 mm, 35.37 mm, and 39.11 mm, respectively. On the other hand, the minimum inhibition zone value of 14.02 mm was observed for "0.2 mg/mL + *E. coli*" treatment.

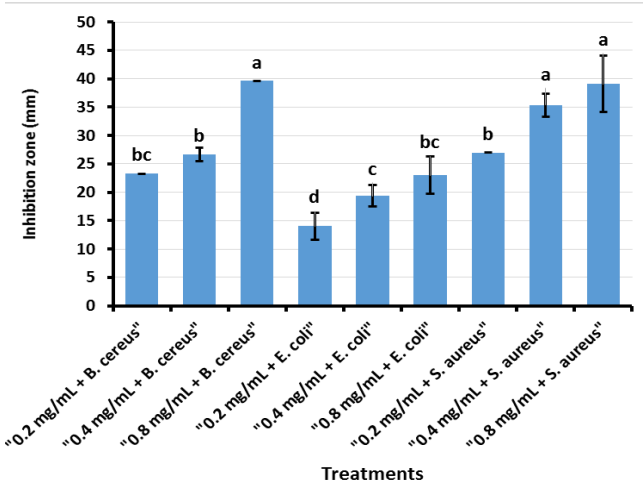

Fig. 6 Duncan's mean comparison test of nine treatments (i.e., interaction effect) resulted from multiplying three different essential oil concentrations (i.e., 0.2, 0.4, and 0.8 mg/ml) and three different bacterial agents (i.e., *B. cereus*, *E. coli*, and *S. aureus*) in terms of the agar well-based inhibition zone (mm) trait. The mean values with different letters differed significantly according to Duncan's multiple range test ($p< 0.05$). Data represented the mean of three independent replicates ± SD. Details of all nine treatments were clarified in the main text.

Heat-map Clustering

A clustered heatmap was used to visually classify the gram-negative (i.e., *E. coli*) and gram-positive bacteria (i.e., *S. aureus* and *S. cereus*) studied here in terms of three different essential oil concentrations of 0.2, 0.4, and 0.8 mg/ml.

As could be seen, the gram-negative bacterial agent of *E. coli* was placed in the first main cluster, while both gram-positive bacteria of *S. aureus* and *S. cereus* were positioned in the second major group. Furthermore, the gram-negative bacterial agent of *E. coli* exhibited the minimum inhibition zone (mm) at all three different concentrations of 0.2, 0.4, and 0.8 mg/ml as compared with the other two gram-positive bacteria of *S. aureus* and *S. cereus*, indicating that *E. coli* was more resistant against eucalyptus essential oil even at higher concentrations of 8.0 mg/ml (Fig. 7). On the other hand, between two gram-positive bacteria of *S. aureus* and *S. cereus*, it seems that *S. aureus* is more sensitive rather than *S. cereus* in response to different essential oil concentrations of *E. camaldulensis*. The results of this study have shown that the bactericidal effects of eucalyptus essential oil vary depending on the concentration used and the specific bacterial strain being targeted. This suggested that eucalyptus essential oil has the potential to be an effective natural antibacterial agent,

with promising implications for its use in medicine and other industries. These findings could warrant further investigation into the specific properties and mechanisms of action of eucalyptus essential oil, as well as its efficacy against a wide range of bacterial strains. By better understanding its potential and limitations, we can fully harness the benefits of this natural substance and potentially develop more effective treatments for bacterial infections. Moreover, this research highlighted the importance of exploring alternative and sustainable options for fighting bacteria, especially in the face of growing antibiotic resistance. Further studies and developments in this area have the potential to greatly affect the field of medicine and contribute to the preservation of public health.

The current study set out to examine the antibacterial properties of eucalyptus plant essential oil through the utilization of the agar well diffusion method. The results revealed that the essential oil had a significant antimicrobial effect, with the concentration of 0.8 g/ml exhibiting superior properties compared to the lower concentration of 0.2 g/ml. Notably, the resultant essential oil of *E. camaldulensis* demonstrated the most potent inhibitory effect on the growth of *S. aureus*, followed by *B. cereus* and *E. coli*, respectively. The diameters of the bacterial inhibition zones around the wells containing the essential oil further confirmed the superior capacity of the oil in comparison to the lower concentrated one, agreeing with [51]. These findings highlighted a direct correlation between the concentration of eucalyptus essential oil and its antibacterial activity, suggesting its potential as a potent alternative to synthetic drugs in the treatment of infections caused by bacteria resistant to chemical agents. Thus, eucalyptus possesses promising benefits as a natural, effective, and safe option for preventing and controlling bacterial infections.

The findings of the current study align closely with those of previous research investigations. The results of a trial that evaluated the antimicrobial effects of thyme essential oil and its combination with nisin on *E. coli* and *S. aureus* showed its more efficiency in targeting gram-negative bacteria compared to gram-positive bacteria [52]. In a separate study, the antimicrobial properties of

eucalyptus, thyme, and summer savory essential oils on *Streptococcus mutans* were studied [53]. The results highlighted that all three oils exhibited effective antibacterial properties against *Streptococcus mutans*, with summer savory essential oil displaying the most potent action in all concentrations tested and exposure durations [53]. Essential oils of seven *Eucalyptus* species developed in Tunisia were individually studied for their antibacterial effects against *S. aureus* (ATCC 6539), *E. coli* (ATCC 25922), *Enterococcus faecalis* (ATCC29212), *Listeria ivanovii* (RBL 30), *B. cereus* (ATCC11778) [46]. The diameter of inhibition zone of essential oils of all seven *Eucalyptus* species varied from 10 to 29 mm [46]. The maximum and minimum zone of inhibition was recorded for *B. cereus* (*E. astrengens*) and *S. aureus (E. cinerea)*, respectively. Meanwhile, the essential oils extracted from four *Eucalyptus* species of *E. maideni, E. astrengens, E. cinerea* and *E. bicostata* presented the maximum antibacterial activity when applied against *L. ivanovii and B. cereus* [46]. Antimicrobial activities of essential oil of the leaves of *Eucalyptus globulus* were studied against gram-negative bacteria (*E. coli*) as well as gram-positive bacteria (*S. aureus*) [51]. The diameter of the inhibition zones ranged from 8 to 26 mm, with the largest inhibition zone observed for *E. coli* (10^3 dilution) and the smallest for *S. aureus* (10^1 dilution) at 100% and 25% concentrations of essential oils, respectively [51]. The antibacterial properties of essential oils extracted from twenty *Eucalyptus* species from Zerniza and Souinet arboreta (North West and North of Tunisia) were evaluated using the agar-disc diffusion method [47].

Based on the results, the most sensitive bacterial strain was the gram-positive *S. aureus* treated with *E. odorata* oil (16.0 mm), whereas the most resistant bacteria were *Pseudomonas aeruginosa* [47]. Recently, the antibacterial activities of the essential oils extracted from leaves of four *E. urophylla* clones and one *E. urophylla* × *E. camaldulensis* hybrid clone grown in Thailand were studied [48]. Among different essential oils, those extracted only from the hybrid had an effect on all gram-negative bacteria of *Streptococcus pyogenes*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus* followed by gram-negative bacteria of *E. coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Enterobacter aerogenes* [48]. More recently, the antibacterial activity of essential oils from two different *Eucalyptus* species of *E. camaldulensis* and *E. tetragona* have been examined against *S. aureus* and *E. coli* [49]. A moderate to strong antibacterial activity was observed for the essential oils, and those extracted from *E. tetragona* inhibited *S. aureus* at a lower concentration of 0.625 µg/ml, whereas *E. coli* was found to be more resistant against the essential oils of *E. camaldulensis* (MIC of 2.5 µg/ml) [49].

The utilization of essential oils as antibacterial agents has been attributed to their hydrophobic nature, which enables them to increase cell permeability and subsequently cause the leakage of cell constituents [54-59]. This disruption of cell structures can be accompanied by a cascade effect on other cellular processes, as indicated by studies on the effects of essential oils on bacterial cells [60]. While essential oils have been found to have multiple target sites on bacterial cells, it is clear that their main mechanism of action is through disruption of the bacterial envelopes.

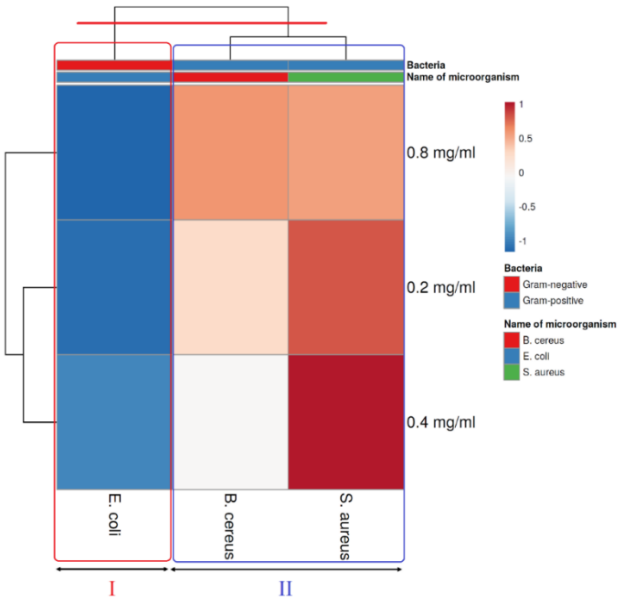

Fig. 7 Heat-map clustered to visually classify the gram-negative (i.e., *E. coli*) and gram-positive bacteria (i.e., *S. aureus* and *S. cereus*) studied here in terms of three different essential oil concentrations of 0.2, 0.4, and 0.8 mg/ml. According to the cutting line, the gram-negative bacterial agent of *E. coli* was placed in the first main cluster (Cluster I; Red box), while both gram-positive bacteria of *S. aureus* and *S. cereus* were positioned in the second major group (Cluster II, Blue box). To create hierarchical cluster analysis (HCA), both rows and columns were clustered using *Euclidean distance* and *average linkage*.

This disruption can lead to a variety of effects such as disturbance of cell wall and membrane [56, 61], depletion of intracellular ATP [55, 61], induction of heat shock proteins [62], changes in pH [55, 61], and alterations in the intracytoplasmic environment (e.g., coagulation, periplasmic space enlargement) [63]. Despite the growing number of studies on the mechanisms of action of essential oils, there is still much to be learned to fully understand their precise mode of action [16]. This is a significant limitation of the widespread use of essential oils and extracts as antimicrobial agents. Therefore, further research is needed to increase our understanding and enable the effective use of essential oils in combating bacterial pathogens and their resistance.

CONCLUSION

The complex chemical makeup of essential oils gives them unique and therapeutic properties, making them valuable in aromatherapy, skincare, and natural medicine. In this sense, the use of plant essential oils in traditional medicine and therapeutic methods has been documented worldwide. The results of the current study indicated that eucalyptus essential oil possessed antioxidant and antibacterial properties. In particular, the study found that eucalyptus essential oil has more ability to inhibit the growth of gram-positive bacteria such as *B. cereus* and *S. aureus* as compared with the gram-negative bacterium of *E. coli* under three different concentrations of 0.2, 0.4, and 0.8 g/ml. This suggested that eucalyptus plant and its essential oil could be considered as natural and effective antibacterial agents, with the potential to reduce the occurrence and severity of bacterial diseases. Furthermore, the results also pointed towards the possible use of eucalyptus essential oil and its bioactive compounds in aromatherapy. However, to confirm these findings, it is recommended to conduct clinical trials on human samples under non-laboratory conditions. Such trials can provide valuable insights into the potential benefits of eucalyptus essential oil and its role in promoting overall health and well-being. Lastly, as our understanding of the inhibitory effects of eucalyptus essential oil on bacterial growth continues to evolve, further investigations may yield new insights and applications for this powerful natural remedy.

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