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Original Article

Comparison of Essential Oil Yield, Chemical Composition and Biological Activities of *Eucalyptus camaldulensis* Leaf: Conventional Distillation versus Emerging Superheated Steam Distillation

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Abstract

Eucalyptus essential oil (EO) has significance economically since it is used in the medicinal and fragrance industries. The main objective of this study was to investigate the differences in yield, composition, antioxidant capacity, and antibacterial effectiveness of the EO extracted from *Eucalyptus camaldulensis* leaves. To achieve this, three distinct extraction techniques, namely steam distillation (SD), hydro distillation (HD), and superheated steam distillation (SHSD), were utilized to isolate the EO. The study aimed to analyze and compare these parameters among the three extraction methods. Based on the findings from the experiments, it has been found that using SHSD resulted in a higher EO yield than conventional techniques, and this SHSD produced a greater amount of EO in a shorter time. The EOs extracted using all three techniques have 1,8 cineole as the main constituent, according to gas chromatography-mass spectrometry (GC-MS) analysis results. All of the EOs demonstrated significant antioxidant capacities when tested *in vitro* using a variety of antioxidant assays. The EOs extracted through HD showed greater antibacterial activity among the other extraction processes, as evaluated by agar well diffusion and resazurin microtitre-plate assays. In conclusion, SHSD is more efficient for extracting EOs and antioxidant activity than traditional HD and SD.

Keywords: Superheated steam distillation; *Eucalyptus camaldulensis*; Essential oil; 1, 8 cineole; Antioxidant activity; Antibacterial activity; Hydro distillation; Steam distillation.

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1. Introduction

The family Myrtacea, which includes more than 700 species, includes the *Eucalyptus* genus [1]. The plant genus *Eucalyptus* is native to Australia and includes the scented and medical *Eucalyptus camaldulensis* [2]. It is a perennial, evergreen plant called long-beaked eucalyptus, Murray red, red gum, river, and river gum tree

[3]. Different types of phytochemicals, which include tannins, terpenoids, steroids, saponins, flavonoids, and alkaloids, are found in *Eucalyptus camaldulensis,* and these phytochemicals are the basis for many of the traditional uses for treating improper alignment [4]. The eucalyptus leaves contain EO and other compounds that have been used by humans as a means of natural remedies since prehistoric eras. Apart from the vast number of plant species, around 2000 in total, that are known to produce EOs, approximately 300 of them hold industrial significance. These species have EOs with notable biological properties, including antifungal, anticancer, antiviral, antimutagenic, antidiabetic, anti-inflammatory, and antibacterial activities [5]. As antiviral, insecticide, antibacterial, and antifungal, EOs are essential to plants' defense mechanisms due to these properties. They also shield plants from herbivores by decreasing their hunger [6].

One of the most significant health problems triggered by eating contaminated food products is food-borne disease (FBD). Preservatives have been used to prevent the development of germs and fungus since ancient times, and they are crucial to the preservation and delivery of food [7]. The *P. aeruginosus*, *S. aureus*, *P. mirabilis, K. aerogenes,* and *Escherichia coli* (*E. coli*) are the food-borne pathogenic bacteria that cause poisoning and contamination; thus, they are prevented via the addition of chemical preservation agents to food [8]. Additionally, chemical deterioration or rancidity driven by the autoxidation of fat and fat-containing food items diminishes their nutrient content and sensory qualities. As antioxidant and antibacterial agents, synthetic chemical preservatives have been employed in food items that might cause intoxication, allergies, and degenerative disorders [9]. As a result, researchers are looking for novel natural antibacterial agents. Because EOs from plants are more resistant to food-borne microbes than chemical preservatives, EOs from plants can be used as a substitute [10]. Microbes and lipid oxidation are the two main causes of food loss; hence, adding EO improves the nutritional value and preservation time by reducing lipid oxidation and microbial activity[11]. According to prior research, EO can be employed in food products as a microbial inhibiting agent [12].

Antioxidants are chemicals that prevent the free radical's formation. The body's metabolism and external factors produce free radicals, unstable molecules, and reactive oxygen species, including smoke from cigarettes, pollutants in the air, X-rays, chemical residues from factories, and ozone [13]. Antioxidants are vital since they protect against damage from oxidation, which is the main source of many disorders such as atherosclerosis, AIDS, aging, arthritic conditions, cancer, Parkinson's disease, liver diseases, inflammatory conditions, Alzheimer's disease, and diabetes [14]. Different forms of reactive free radicals that cause major health issues such as diabetes, cardiovascular illnesses, aging, and cancer are formed during the autoxidation mechanism [14, 15]. Therefore, Antioxidants are crucial in treating various diseases and avoiding oxidative damage. Plants contain a variety of antioxidants [16]. EO and other volatile chemicals have free radical scavenging properties [17].

Furthermore, phenolic substances are in the driver's seat for antioxidant function [18]. Due to its tumoricidal and antioxidant properties, *Melissa officinalis L*. EO can be used to prevent cancer [19]. Studies showed that natural antioxidants like EO help prevent degenerative illnesses [14].

EOs are physically isolated, volatile secondary metabolites of plants with molecular weights≤300 [20]. The terpenes family comprises the bulk of the ingredients in EOs [21]. Minor components also play a crucial part in the bioactivities of EO [22]. Various techniques may be used to isolate the various plant EOs, such as solvent extraction [23], SD and HD [23, 24], microwave-assisted extraction [24], ultrasound-assisted extraction [24], cold pressing [24, 25], microwave hydro diffusion and gravity extraction [26, 27], enfleurage [24], superheated water extraction [28], supercritical fluid CO_2 extraction [24, 29]. The simplest and most traditional method for extracting EO from plant biomass is HD and SD [23]. Conventional HD and SD drawbacks include extended extraction times, high fuel consumption, poor EO yields, and esters' hydrolysis to alcohol and acids [24, 30, 31]. Supercritical fluid CO2 extraction has garnered significant attention in recent years due to its multitude of advantages compared to traditional extraction methods such as its low running temperature, compatibility for isolating both polar and non-polar compounds, ecologically friendly and free of solvents nature [24, 32, 33]. The main disadvantages of this supercritical fluid CO_2 extraction process are the equipment expenses, costly maintenance, requirements and the extraction of wax, coloring agents, resin, and fatty acids in along with EO [29]. After supercritical fluid CO_2 extraction, the another technique called the subcritical water extraction has attracted a lot of attention recently. It is an easy and inexpensive method that has no negative impacts on the environment, a low working temperature, a quick extraction process, and prevents the deterioration of volatile and heat-sensitive EO components [34, 35]. The disadvantage of this method is that vital oil components degrade at a certain temperature [36].

Superheated steam is an alternate extraction source that may be utilized to extract EO from aromatic plants. In contrast to saturated steam, superheated steam has a temperature above the saturation point under constant pressure and quickly releases heat through condensation [37]. When steam is heated over the critical point, the saturated steam, referred to as steam at a temperature where the water and gas phases may coexist, transforms into superheated steam. At the same pressure, superheated steam stores more heat than saturated steam. Because of the increased enthalpy of the superheated steam, the material is heated quickly, producing more EO with fewer losses from oxidation and with more efficiency [38]. In addition, because superheated steam has a higher heat transfer rate than regular steam, it creates an atmosphere devoid of oxygen and quickly raises the temperature of the food's surface [37]. According to literature, EOs decompose at temperatures exceeding 230 ºC, biomass pyrolyzes, and carcinogenic chemicals are produced [39, 40]

Rare research uses superheated steam to extract EO from plant biomass. To the best

extent of our information, no preliminary study has been published associating *Eucalyptus camaldulensis* EO extracted by HD, SD, and SHSD. One of the primary objectives of the present study was to examine the variations in the chemical composition, yield, antioxidant capacity, and antibacterial efficacy of EO obtained through SHSD in comparison to the conventional HD and SD techniques. The aim was to evaluate and compare these parameters among the different extraction methods used in the study.

2. Materials and Methods

2.1. Plant sample

The identification of the fully grown *Eucalyptus camaldulensis* tree, from which plant samples (leaves) were collected, was carried out by Dr. Fahim Arshad, an associate professor in the Department of Botany at the University of Okara, Punjab, Pakistan. A voucher specimen (OK-880) was deposited at the herbarium department of the University of Okara for future reference. The plant material was cleaned three times with distilled water and dried out under a shelter till it reached a consistent weight. The material was grounded and stored in plastic zipper bags until processed.

2.2 Hydro and Steam distillation

The leaves sample underwent three rounds of washing with double-distilled water, three weeks of air drying beneath the shed, grinding, and storage in polyethylene bags until further processing. The HD components were the heating mantle, flask, condenser, and Dean Stark. Except for employing a steam flask, the SD equipment is the same. The 5 L container had 300 g of dried *Eucalyptus camaldulensis* leaves and distilled water. The EO and water combination flowed through a condenser after evaporating from the flask and collecting in the dean stark. Processes for steam and HD were carried out for three hours. The water and EO created the two levels and divided by a funnel. The EOs were collected, dried over anhydrous sodium sulfate, filtered, and kept at +4 ºC until more investigation. Five times were used to distill using steam and water. The % yield of EOs was evaluated using the following formula:

$$EO\ yield\ (\%) = \frac{EO\ in\ grams}{Dry\ sample\ weight\ in\ grams} \times 100$$

2.3. Superheated Steam Distillation

The SHSD process involved utilizing an extraction vessel of stainless steel with a volume capacity of 10 L. This extraction vessel was linked to a superheated steam generator, which had a volume of 100 L. The setup included a condenser and a hydrosol collection vessel with a volume of 5 L, equipped with a glass separating funnel. The superheated steam temperature range of the generator is 100-220ºC and is equipped with an automatic temperature control system. 1kg of dried leaves that had been finely powdered (60 mesh) was put in a stainless-steel extraction vessel and heated to 150 ºC for one hour with superheated steam. Anhydrous sodium sulfate was used to extract the moisture from the EO, which was then filtered using filter paper no. 1 and kept in glass vials of black color until further examination. Five repeats were run through the extraction procedure to verify repeatability [41].

2.4. Antioxidant activity

2.4.1. DPPH free radical scavenging activity (DPPH-FRSA)

The DPPH free radical scavenging activity (DPPH-FRSA) of the EOs isolated through HD, SD, and SHSD was assessed using a modified version of the DPPH assay previously described in the literature [42]. To conduct the DPPH assay, 1 mL of a 90 µM DPPH solution was prepared. Subsequently, 2.5 mL of the EO sample mixture (containing 100 mg/mL of EO in ethanol) or a standard butylated hydroxytoluene (100 mg/L) was added to the DPPH solution. Furthermore, the mixture was supplemented with an additional 0.5 mL of methanol. After preparing the mixture, it was placed in a dark environment and allowed to stand undisturbed for one hour. Following the incubation period, the absorbance of the control and reaction mixture was measured at a specific wavelength of 515 nm using a double-beam spectrophotometer. The EOs were evaluated for % inhibition using this formula:

$I\ (\%) = 100 \times (A_{blank} - A_{sample}/A_{blank})$

A_{blank} = control reaction mixture absorbance value

A_{sample} = reaction mixture absorbance value

2.4.2. Ferric reducing antioxidant power (FRAP) assay

The total antioxidant contents (TAC) of the EOs were assessed using the FRAP test, which has been widely used in previous studies for this purpose [43]. A mixture was prepared by combining 1 mL of the sample mixture (100 mg of EO per mL of ethanol) with 2.5 mL of phosphate buffer (0.2 M; pH 6.6) and 2.5 mL of a 1% potassium ferricyanide solution. Each test tube was then immersed in a water bath heated to a temperature of fifty degrees Celsius for 10 minutes. After allowing the mixture to cool to room temperature for five minutes, 10% trichloroacetic acid was added. A fresh test tube was filled with 2.5 mL of the reaction mixture to quantify the TAC, diluted with 2.5 mL of deionized distilled water and 0.5 mL of a 0.1% ferric chloride solution. After thirty minutes of incubation at the ambient temperature, the absorbance of the solution at 700 nm was taken with a UV-visible spectrophotometer. These absorbance values were then used along with a calibration curve ($y=0.021x+0.0151$, $R^2=0.99$) to determine the TAC, expressed in milligrams per liter of Gallic Acid Equivalent.

2.4.3. Percentage Inhibition in the linoleic acid system

A previously documented procedure from the literature was employed to determine the inhibition percentage of the EOs on the production of linoleic acid peroxide [44]. 50 mg of EO was dissolved in 1 mL of ethanol to prepare the sample. Subsequently, 4 mL of a 2.5% linoleic acid solution was added to the mixture. Then, 4 mL of a pH=7 sodium phosphate buffer with a 0.05 molar concentration was added. All test tubes were in an oven at 40 degrees Celsius for one week. By using the spectroscopic approach, the peroxide values were used to evaluate the oxidation levels [45]. Add 200 mL of the reaction mixture, 200 mL of a 30 percent ammonium thiocyanate solution, 200 µL of ferrous chloride solution, and 10 milliliters of ethanol to the mixture (20 mM in 3.5 percent HCl). After five

minutes of stirring in each test tube, the absorbance at 500 nm was measured using a spectrophotometer. The positive control (100 mg/L) butylated hydroxytoluene and the control (reaction mixture without EOs) followed a similar technique. The percentage inhibition of linoleic acid oxidation was determined using the following method:

% Inhibition of linoleic acid oxidation = 100 − [(Abs. Increase of sample at 175 h/Abs. Increase of control at 175 h) × 100].

2.5 Antimicrobial activity

2.5.1. Microbial strains

Bacillus subtilis (*B. subtilis*) ATCC 10707 and *E. coli* ATCC 25922 bacterial strains selected for individual evaluation of each EO sample. These strains were provided by the Institute of Microbiology at the University of Agriculture Faisalabad in Punjab, Pakistan.

2.5.2. Agar well diffusion method

The antibacterial activity of the EOs was assessed using the agar well diffusion technique, a well-established method described in previous literature [46]. The microbial strains' night culture, including 10^8 colony-forming cells per milliliter, was injected into a 25 mL medium for growth solution. The flask contents were then shifted to medium-sized petri dishes. After the agar was set at room temperature, a sterile cork borer made wells for further tests. To test for antibacterial activity, each of these wells contained 10 L of pure EOs and conventional prescription drugs (1 mg of ampicillin per mL). For 24 hours, the Petri plates were cultured for bacteria at 37°C. After the incubation, a digital Vernier caliper was used to measure the width of the inhibitory zones (mm).

2.5.3. Resazurin microtitre-plate assay

As reported in earlier research, the EOs' minimum inhibitory concentration (MIC) against various bacterial strains was measured using a modified resazurin microtitre-plate assay [47]. Ten µL of essential oil (EO) was dissolved in 1 mL of 10% dimethyl sulfoxide (DMSO) to prepare the sample solution. Similarly, 27 mg of resazurin was dissolved in 4 mL of sterilized distilled water for the resazurin indicator solution. In the first row of the ninety-six well plates, 100 µL of the sample solution and the reference antibiotic Ampicillin (1 mg/mL in 10% DMSO) were pipetted. Then, except for the first row of wells, 50 mL of nutritional broth was added to each well, and two-fold serial dilutions were carried out such that 50 μL of the sample combination was present in each well. Then, in all wells, 30 μL of 3.3x strength sensitized broth, 10μL of resazurin solution and 10 μL (5×10^5 colony forming units per mL) were added. The plates were incubated for twenty-four hours at 37°C. The values of the MIC were visually evaluated after incubation. The MIC was determined as the lowest concentration at which a color change from purple to colorless or pinkish was observed in the resazurin microtitre-plate test. This color change indicates the growth of bacteria in the presence of different concentrations of the tested substance. The MIC value is an essential indicator of the effectiveness of the substance in inhibiting bacterial growth.

2.6. GC-MS analysis

GC–MS analysis evaluated the volatile compounds in the EOs obtained through SHSD, HD, and SD. The analysis was performed using a Shimadzu GC-2010 system. The system was outfitted with a QP-2010 plus mass detector and a DB-5 capillary column (50 m × 0.25 mm, with a film thickness of 0.25 µm). For the analysis, a 1 µL volume of EO, diluted with n-hexane at a ratio of 1:10, was injected into the injection port using an injection syringe. The column was initially heated to 60 ºC for 3 minutes and then increased to 240 ºC at a heating rate of 24ºC/min, which was maintained for the succeeding 10 minutes. Nitrogen gas served as the carrier gas, flowing through the system at a 1.5 mL/min rate. The MS transfer line temperature was set at 240 ºC. MS detection was performed using an electron ionization mode (70 eV) [48]. n-alkanes (C_9-C_{24}) standards were analyzed under identical chromatographic conditions to determine the retention indices of the identified compounds.

Additionally, these retention indices, and mass spectrum data, were matched to available data and the NIST and probate mass spectral database library [48, 49]. Substantiation of certain compounds was achieved by co-injecting authentic standards. The quantification of the EO constituents was conducted following the methodology outlined in reference [50]. The response factors (RFs) were calculated by comparing the peak areas of the essential oil (EO) component to that of the internal standard (undecane) using the equation:

RFc = (Ac / Ais) / (Cc / Cis)

Here, RFc represents the response factor for the EO component, Ac and Ais represent the peak areas of the EO component and internal standard, respectively, and Cc and Cis represent their corresponding concentrations. For minor unidentified peaks, the expected RF was set to 1.0.

Subsequently, the RFs were utilized to determine the proportion (%) of each EO ingredient using the following equations:

Corrected area = peak area for the component/response factor for the same component

Percentage (%) = (corrected area for the component/total of corrected areas) x 100

2.7. Statistical analysis

The tests were performed in triple quantities, and the statistical analysis was carried out using STATISTICA 5.5 software from Stat Soft Inc., which is based in Tulsa, Oklahoma, USA. The variance analysis (ANOVA) method and the Post-Hoc Tukey HSD test were applied. A $p \leq 0.05$ significance level was judged statistically significant. The data was provided as mean values with standard deviations based on three measurements.

3. Results and Discussion

3.1. Essential oil Yield

Figure 1 indicates the results of the extraction of EO from *Eucalyptus camaldulensis* leaves using HD, SD, and SHSD 150 ˚C. The distillation methods significantly influenced the yield of EO from Eucalyptus camaldulensis leaves. SHSD resulted in a significantly higher yield of EO with a content of 1.12 g per 100 g

of dry plant material. In comparison, SD produced a yield of 0.65 g per 100 g of dry plant material, while HD yielded 0.59 g per 100 g. It shows that the SHSD process extracts EO from plant material more efficiently, producing a higher yield. This remarkable output can be due to the fascinating interaction between superheated steam's low viscosity, polarity, improved penetration ability, and increased kinetic energy [51]. The steam that has been heated above the boiling point of water is said to be superheated steam. As a result, the steam is more volatile and can liberate more EO components from the plant matter [52]. Notably, superheated steam's energetic composition and penetrative powers are superior to ordinary steam, significantly increasing the possibility of extraction [51]. These results are consistent with earlier literature studies, which showed that HD produced less thyme EO yield than SHSD [53]. The same phenomenon also resulted in a greater yield of *Origanum onites* [35], *Origanum micranthum* [34], fennel seeds [54], and marjoram leaves EO extracted in superheated water extraction [55].

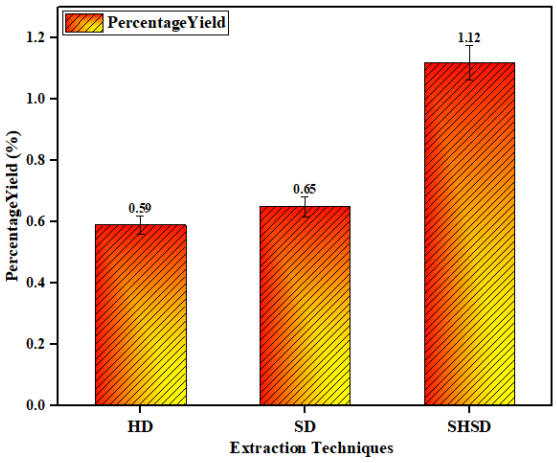

Figure 1. Comparison of the yield of EOs of *Eucalyptus camaldulensis* leaves extracted by HD, SD, and SHSD at 150 ˚C.

The results of this study are also comparable with previous literature research, which showed that *Eucalyptus camaldulensis* leaves produced more EO with SD than HD [56]. There is no literature available to compare the yield of *Eucalyptus camaldulensis* leaves EO with EO distilled by SHSD, although SHSD gave the highest yield of thyme EO [53], *Boswella Serrata* oleogum resin EO [51], *Syzgium aromaticium* EO [52] and *Pinus roxburgi* EO [57]. These exciting studies by the SHSD showed that this technique is a more environmentally friendly, economically viable, and time-efficient distillation method for extracting EO from plants. In this study, the EO yield of SHSD was greater than HD and SD, leading one to believe that SHSD is a more efficient method for obtaining the highest production of EO from *Eucalyptus camaldulensis* leaves than HD or SD in a shorter time, and it also consumes less amount of energy which is a benefit to the environment.

3.2. Antioxidant Activity

The antioxidant capacity of the EOs of *Eucalyptus camaldulensis* leaves was evaluated using DPPH-FRSA, FRAP, and % inhibition in linoleic acid assays, and the antioxidant findings are shown in **Table 1**. All of the EOs extracted through different extraction methods demonstrated significant antioxidant activity by preventing the development of linoleic acid peroxide. The different extraction methods show substantial influence on the EOs' antioxidant activity. The EO extracted by SD showed the highest suppression of linoleic acid peroxide production (72.04 ± 0.47 %), whereas the EO extracted by HD showed the lowest inhibition (63.92 ± 0.53 %).

Table 1: Antioxidant Activity of *Eucalyptus camaldulensis* Leaves Essential Oils (EOs) Extracted by HD, SD, and SHSD.

Extraction Methods	DPPH FRSA (%)	Total antioxidant contents/ FRPA (mg/100g)	Inhibition in linoleic acid system (%)
HD	73.27 ± 0.63^{d}	78.19 ± 0.43^{c}	63.92 ± 0.53^{d}
SD	83.40 ± 0.25^{b}	96.21 ± 0.77^{a}	72.04 ± 0.47^{b}
SHSD	82.14 ± 0.29^{c}	92.47 ± 0.63^{b}	70.27 ± 0.32^{c}
BHT (Standard)	96.63 ± 0.85^{a}	---------	92.35 ± 0.97^{a}

The values represent the mean ± standard deviations obtained from three independent determinations. The total antioxidant contents/FRAP (mg/L of EO, measured as Gallic acid equivalent) were determined in this study. Different letters in superscripts indicate significant differences among the *Eucalyptus camaldulensis* essential oils extracted using HD, SD, and SHSD

Results are consistent with existing research where *Eucalyptus camaldulensis* EO extracted using the SD technique showed up to 83.2% suppression of the generation of linoleic acid peroxide [58]. The most popular and widely used method for determining the FRSA of plant material is the DPPH assay, which involves adding an antioxidant's proton or electron to a DPPH free radical to alter the solution's color from purple to yellow [59]. The DPPH test was used to measure the FRSA of *Eucalyptus camaldulensis* leaves, and the findings are shown in **Table 1**.

All EOs were found to have reasonable amounts of FRSA, which ranged from 73.27 ± 0.63 to 83.40 ± 0.25 %. The EO extracted using SD had the highest FRSA (83.40 ± 0.25 %), whereas EO extracted using HD had the lowest FRSA (73.27 ± 0.63 %). The FRSA of the EO extracted by SHSD and the EO extracted by SD were almost identical. The FRSA of the leaves of *Eucalyptus camaldulensis* obtained using various extraction techniques is comparable to that reported in the work [60]. FRSA of EO from SHSD was notably correlated with FRSA of *Eucalyptus camaldulensis* EO extracted by HD, which demonstrated 82% FRSA by DPPH test in the existing literature study [60]. In the FRAP assay, the spectrophotometer was used to assess the antioxidant potential of plant EO by reducing ferric to blue ferrous complex under acidic circumstances [61]. Using various extraction techniques, the total antioxidant content of EO ranged from 78.19 ± 0.43 to 96.21 ± 0.77 mg/L of gallic acid equivalent. The EO extracted by SD had the highest antioxidant content (96.21 ± 0.77 mg/L of gallic acid equivalent), whereas the EO extracted by HD had the lowest (78.19 ± 0.43 mg/L of gallic acid equivalent). Overall, the antioxidant level of the EO was good. The observed fluctuations in the antioxidant activity of the EOs of *Eucalyptus camaldulensis* leaves can be related to various variables, including variances in the chemical makeup and extraction procedures used during the distillation process. According to research, the chemical makeup, secondary metabolites, and phenolic components with the double bond in conjugation all affect the antioxidant activities of EO [62]. According to previous studies, the antioxidant activity of *Eucalyptus camaldulensis* EO was caused by1,8-cineol, α-terpineol, borneol, cis-sabinene hydrate, trans-caryophyllene, camphor, α-pinene, and α-

thujene [63-65]. These EO constituents, some of which are present in *Eucalyptus camaldulensis* EO as major or minor components produced using various techniques, may be responsible for antioxidant action (see the GC-MS **Table 2**).

Table 2: GC-MS analysis of *Eucalyptus camaldulensis* leaves EOs extracted by HD, SD, and SHSD.

Sr. No	Components [A]	RT	RI Cal	RI Lit	% Composition of EOs			Method of identification
					HD	SD	SHSD	
	Monoterpene hydrocarbon							
1	α-Thujene	2.29	923	923	7.17 ± 0.24 [a]	3.44 ± 0.14 [c]	4.05 ± 0.19 [b]	a,b
2	α-Pinene	2.35	934	933	2.96 ± 0.03 [a]	1.66 ± 0.02 [c]	2.06 ± 0.06 [b]	a,b
3	β-Myrcene	2.52	992	991	1.50 ± 0.03 [a]	0.75 ± 0.02 [c]	0.98 ± 0.01 [b]	a,b
4	β-Pinene	2.56	989	988	0.75 ± 0.01 [c]	0.39 ± 0.00 [b]	0.92 ± 0.03 [a]	a,b
5	α-Phellandrene	2.64	1004	1005	0.59 ± 0.04 [b]	0.85 ± 0.05 [a]	0.35 ± 0.00 [c]	a,b
6	β-Cymene	2.80	1026	1027	7.24 ± 0.12 [a]	4.77 ± 0.09 [b]	4.98 ± 0.05 [c]	a,b
7	Limonene	2.84	1031	1031	---	1.10 ± 0.05 [b]	1.74 ± 0.08 [a]	a,b
8	γ-Terpinene	2.30	1058	1059	3.78 ± 0.07 [a]	1.70 ± 0.00 [c]	2.10 ± 0.01 [b]	a,b
	Oxygenated Monoterpene hydrocarbon							
9	*trans*-2-Menthenol	3.56	1126	1127	0.94 ± 0.08 [a]	0.62 ± 0.02 [b]	0.54 ± 0.01 [c]	a,b
10	*cis*-2-Menthenol	3.7	1130	1130	0.76 ± 0.06 [a]	0.26 ± 0.00 [c]	0.46 ± 0.04 [b]	a,b
11	*trans*-Pinocarveol	3.78	1139	1139	0.33 ± 0.03[b]	0.12 ± 0.02 [c]	1.23 ± 0.04 [a]	a,b
12	*cis*-Sabinol	3.90	1141	1140	3.46 ± 0.04 [a]	2.42 ± 0.00 [c]	2.47 ± 0.01 [b]	a,b
13	Camphor	3.98	1143	1143	0.80 ± 0.04 [c]	1.15 ± 0.05 [a]	1.02 ± 0.04 [b]	a,b
14	*trans*-3(10)-Caren-2-ol	4.08	1160	1160	0.48 ± 0.02[a]	---	0.38 ± 0.01[b]	a,b
15	Terpinen-4-ol	4.14	1178	1178	5.35 ± 0.12 [a]	2.96 ± 0.00 [b]	1.30 ± 0.01 [c]	a,b
16	α-Terpineol	4.25	1188	1189	1.54 ± 0.12 [b]	1.05 ± 0.09 [c]	2.60 ± 0.05 [a]	a,b
17	1,8-cineol	4.66	1222	1221	17.74 ± 0.12 [c]	21.79 ± 0.05 [a]	21.43 ± 0.06 [b]	a,b
18	Geraniol	4.88	1255	1255	0.39 ± 0.02 [b]	---	0.44 ± 0.01 [a]	a,b
19	Piperitone	4.985	1283	1282	7.98 ± 0.13 [a]	4.23 ± 0.08 [c]	5.56 ± 0.04 [b]	a,b
20	Thymol	5.32	1290	1290	0.26 ± 0.03 [b]	1.03 ± 0.04 [a]	0.17 ± 0.02 [c]	a,b
21	Carvacrol	5.62	1298	1298	0.99 ± 0.00 [c]	1.56 ± 0.06 [a]	1.36 ± 0.03 [b]	a,b
22	Pinanediol	6.08	1314	1313	6.36 ± 0.04 [c]	7.02 ± 0.03 [a]	6.54 ± 0.05 [b]	a,b
	Sesquiterpenes hydrocarbon							
23	Isoledene	6.11	1374	1373	---	0.33 ± 0.02 [b]	1.35 ± 0.03 [a]	a,b
24	Copaene	6.15	1378	1377	---	0.35 ± 0.03 [a]	0.14 ± 0.01[b]	a,b
25	α-Gurjunene	6.597	1409	1409	0.82 ± 0.00 [b]	0.87 ± 0.02 [a]	0.66 ± 0.01[c]	a,b
26	α-Ionene	6.755	1426	1426	---	0.38 ± 0.03 [a]	0.21 ± 0.01[b]	a,b
27	β-Caryophyllene	7.45	1428	1428	0.74 ± 0.01[a]	---	---	a,b
28	γ-Elemene	7.86	1430	1430	8.64 ± 0.05 [b]	8.85 ± 0.02 [a]	8.43 ± 0.04 [c]	a,b
29	Aromandendrene	8.21	1439	1439	3.29 ± 0.03 [b]	3.19 ± 0.02 [c]	3.84 ± 0.03 [a]	a,b
30	Dehydroaromadendrene	8.48	1466	1466	0.62 ± 0.02 [c]	1.46 ± 0.04 [b]	1.94 ± 0.05 [a]	a,b
31	β-Guaiene	8.76	1490	1490	1.10 ± 0.06 [c]	2.14 ± 0.05 [a]	2.02 ± 0.03 [b]	a,b
32	δ-Selinene	9.11	1495	1495	---	0.49 ± 0.01 [b]	0.95 ± 0.03 [a]	a,b
33	δ-Cadinene	9.17	1523	1524	---	0.40 ± 0.01 [a]	0.32 ± 0.03 [b]	a,b
34	Ledene	9.231	1565	1565	1.13 ± 0.08 [b]	1.45 ± 0.05 [a]	1.05 ± 0.03 [c]	a,b
	Oxygenated sesquiterpenes hydrocarbon							
35	Spathulenol	10.22	1575	1575	0.86 ± 0.11 [c]	2.71 ± 0.05 [a]	1.88 ± 0.01 [b]	a,b
36	Globulol	10.43	1576	1576	1.75± 0.01 [c]	1.9± 0.07 [a]	1.24 ± 0.03 [b]	a,b
37	Epiglobulol	10.64	1588	1588	1.97 ± 0.04 [c]	2.67 ± 0.05 [a]	1.92 ± 0.06 [b]	a,b
38	Veridiflorol	11.40	1590	1590	2.67 ± 0.03 [a]	2.43 ± 0.05 [b]	2.34 ± 0.04 [c]	a,b
39	Rosifoliol	11.95	1602	---	1.52 ± 0.05 [a]	1.05 ± 0.03 [c]	1.12 ± 0.04 [b]	a,b
40	δ-Cadinol	12.38	1636	1636	---	0.26 ± 0.01 [a]	---	
41	α-Cadinol	12.58	1653	1653	0.75 ± 0.02 [c]	1.01 ± 0.05 [a]	0.76 ± 0.01 [b]	a,b
42	Alloaromadendrene oxide	13.72	1646	1646	1.5± 0.08 [c]	3.88 ± 0.15 [a]	3.03 ± 0.08 [b]	a,b
43	α-Santalol	14.49	1678	1678	1.59 ± 0.06 [c]	2.71 ± 0.06 [a]	2.52 ± 0.04 [b]	a,b
	Oxygenated Diterpene hydrocarbon							
44	Andrographolide	18.83	2635	2635	0.93 ± 0.01 [b]	1.72 ± 0.06 [a]	0.86 ± 0.01 [c]	a,b

The values represent the mean ± standard deviations obtained from three independent determinations.
Distinct superscript letters indicate significant differences among the essential oils extracted from Eucalyptus camaldulensis leaves using HD, SD, and superheated steam extraction methods.
A Compound listed in order of elution from a DB-5 capillary column. RT= Retention Time. RI Lit= Literature reported retention indices. RI Cal= Retention indices calculated against n-alkanes.

3.3. Antibacterial activity

The antibacterial activity of *Eucalyptus camaldulensis* EOs was assessed using disc diffusion and resazurin microtiter plate tests, and antibacterial findings are shown in **Figure 2**. The essential oil (EO) extracted using different methods demonstrated significant antibacterial activity against gram-negative and gram-positive bacteria. The inhibition zone values of the *Eucalyptus camaldulensis* EO against *E. coli* ranged from 14.46 ± 0.03 mm to 19.34 ± 0.05 mm, while against *B. subtilis*, the range was from 10.77 ± 0.05 mm to 15.81 ± 0.04 mm. These results indicate the potential of *Eucalyptus camaldulensis* EO to inhibit the growth of both bacterial strains, suggesting its antimicrobial efficacy. It was discovered that *E. coli* was more susceptible to the antibacterial effects of *Eucalyptus camaldulensis* EOs than *B. subtilis* bacterium. Additionally, against both bacterial strains, hydro-distilled EO showed greater antibacterial action on both bacterial strains. The antibacterial activity against *E. coli* (14.46 ± 0.03) and *B. subtilis* (10.77 ± 0.05) bacteria was the lowest in the SHSD technique for extracting *Eucalyptus camaldulensis* EO.

The differences observed in the antibacterial activity of Eucalyptus camaldulensis EO against both bacteria, including *E. coli* and *B. subtilis*, could be attributed to the variations in the chemical composition of the EOs obtained through different extraction techniques. The MIC values of *Eucalyptus camaldulensis* EOs obtained through different approaches varied from 79.82±2.12 to 122.23±1.84 *μ*g/ml and 105.90±1.26 to 253.94±1.87 *μ*g/ml against the microorganisms *E. coli* and *B. subtilis*, correspondingly. According to the MIC data, *E. coli* was the bacterial strain that was the most susceptible, whereas *B. subtilis* was less susceptible to EO. It was found that EO yielded by HD exhibited the best antibacterial activity against both bacterial strains (**Figure 2**).

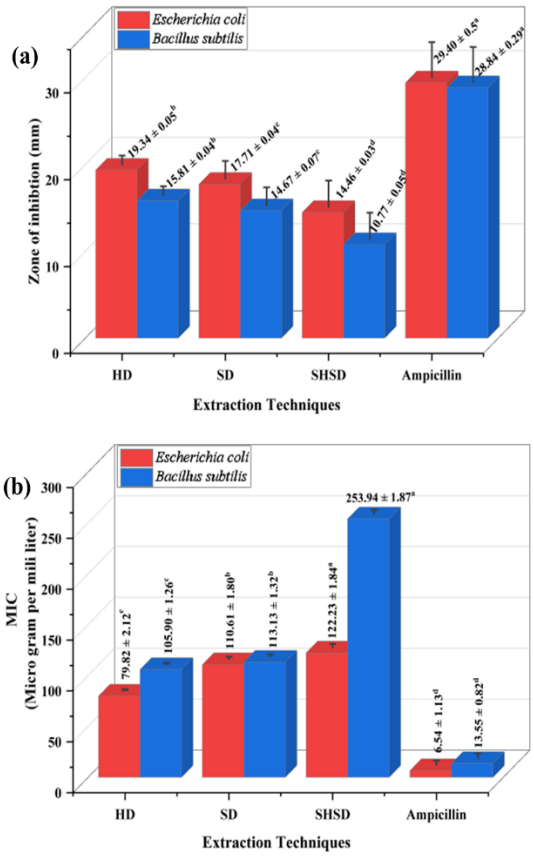

Figure 2. Comparison of the antimicrobial activity of *Eucalyptus camaldulensis leaves* EOs extracted by HD, SD, and superheated steam extraction through **(a)** zone of inhibition and **(b)** MIC values. (The values represent the mean ± standard deviations from three independent experiments. Distinct superscript letters indicate significant differences among the EOs extracted from *Eucalyptus camaldulensis* leaves using HD, SD, and superheated steam extraction methods. Ampicillin (25 µg/disc) was used as a positive control for bacteria in the experiment.).

The MIC value for SHSD extracted EO against *B. subtilis* was higher at 253.94 ± 1.87 *μ*g/ml, indicating lower antibacterial activity against the *B. subtilis.*

Additionally, it was shown that EO extracted via SD had less antibacterial action than HD. Because EOs were extracted using various extraction methods, variations in their chemical makeup may be associated with variations in their antibacterial action against both bacterial strains. Extraction techniques considerably impact the antibacterial activity of EOs in prior literature assessments [48, 66, 67]. It is also confirmed in the study that the HD-extracted EOs of *Boswellia serrata* oleogum resin possessed low antibacterial action against bacterial strains *E. coli* and *S. aureus* with inhibition zones of 15.10 ± 0.3 and 12.90 ± 0.17 mm and SD extracted EOs showed the higher antibacterial activity against these pathogens with inhibition zones of 16.80 ± 0.3 and 14.15± 0.27 mm, respectively [48]. GC-MS (**Table 2**). Results indicated that 1,8-cineole, α –pineneγ -terpinene, veridiflorol, cis-sabinol, pinanediol, α-thujene, piperitone, β-cymene, aromandendrene, globulol, and α-terpineol are the chief constituents of *Eucalyptus camaldulensis* EO which are responsible for antibacterial activity against both bacterial strains. The 1,8-cineole was revealed to have more antibacterial action against bacterial pathogens in the previous literature study, but the 1,8-cineole alone is not responsible for the complete antibacterial activity; instead, it is a synergetic effect of all EOs components [68]. Previous studies have reported γ-terpinene, α-pinene, β-cymene, and α-thujene as the main chemical constituents of Eucalyptus camaldulensis essential oil (EO), which may contribute to its antibacterial activity against pathogens [69, 70]. Therefore, the collective impact of the major and minor components present in *Eucalyptus camaldulensis* EOs could be accountable for its antibacterial activity against gram-negative and gram-positive bacteria. Hence, the combined effect of the major and minor components present in *Eucalyptus camaldulensis* EOs could be responsible for its antibacterial activity against both types of bacteria.

3.4. Chemical Composition by GC-MS

GC-MS analysis was employed to determine the chemical composition of *Eucalyptus camaldulensis* EOs extracted using HD, SD, and SHSD techniques. The results of the chemical composition analysis can be found in **Table 2**. A total of 44 compounds were found in *Eucalyptus camaldulensis* leaves EOs, with 1,8-cineole being the most dominant in three different extraction techniques. Additional notable compounds found in the *Eucalyptus camaldulensis* EO included pinanediol, γ-elemene, β-cymene, α-thujene, γ-piperitone, and terpinen-4-ol. The predominant monoterpene constituents across all EOs were α-terpinene α-thujene, β-cymene, and α-pinene. Among the oxygenated monoterpene compounds, pinanediol, α-terpineol, terpinen-4-ol, piperitone, and cis-sabinol exhibited the highest levels of presence. The two main sesquiterpenes were aromandendrene and β-elemene.

Similarly, the two primary oxygenated sesquiterpenes in the EO of *Eucalyptus camaldulensis* were 1,8-cineole and veridiflorol. The sole oxygenated diterpene discovered in EO of *Eucalyptus camaldulensis* leaves were andrographolide. The current results are consistent with a previously published study that examined *Eucalyptus camaldulensis* leaves essential oil from

Pakistani flora. The previous study also reported a substantial presence of the compound 1, 8-cineole in the EO, which aligns with the present study's findings. This agreement further reinforces the observation that *Eucalyptus camaldulensis* EO from the Pakistani flora contains a notable concentration of 1,8-cineole [58].

According to reports, the chemical composition is influenced by various elements such as the environment, plant parts, soil nutrients, harvesting season sunshine, and geological location [71-73]. It was found that the EO components varied greatly depending on the extraction methods. The primary variations in the EO components terpinen-4-ol, piperitone, α-thujene, and β-cymene came from extraction methods (see **Table 2**). Compared to previous procedures, SHSD utilizes a higher temperature, which might cause labile and sensitive chemicals to break down, resulting in lesser concentrations of those compounds in the EO produced by SHSD [74]. However, the SHSD has higher concentrations of terpinene, pinene, terpineol, trans-pinocarveol, isoledene, aromandendrene, dehydroaromadendrene, guaiene, and selinene (see **Table 2**). The concentration of the above substances may have grown due to superheated steam's higher enthalpy, lesser oxidation, and high efficiency [38]. Additionally, compared to ordinary steam, superheated steam had a greater extraction power and produced more chemicals. Additionally, water's polarity changes when the temperature rises, making it easier to remove nonpolar chemicals [75].

4. Conclusion

The study's findings indicated that the SHSD significantly impacted the EO chemical makeup, yield, antioxidant, and antibacterial activity of *Eucalyptus camaldulensis* leaves EOs. With SHSD, EO production potentially increased in a shorter time, and maximal EO yield was achieved in one hour of extraction time compared to HD and SD techniques. The antioxidant activity of the SHSD technique was comparable to HD and SD activity. HD EO had the highest antibacterial efficacy, and SD EO had the antioxidant activity. The differences in the activity observed among the *Eucalyptus camaldulensis* EOs can be attributed to variations in their chemical composition. Several factors can influence the chemical composition, including the plant's location, harvesting season, soil and weather conditions, and extraction techniques. These parameters can impact the profile and concentration of bioactive compounds present in the EO, ultimately affecting its biological activity. The significant biological properties, such as antioxidant and antibacterial effects observed in *Eucalyptus camaldulensis* EO, may be attributed to its major and minor components. Based on biological properties, *Eucalyptus camaldulensis* leaves can be antibacterial and antioxidant agents. It is necessary to conduct more studies on optimizing the experimental settings for EO extraction by SHSD. In conclusion, SHSD is an effective extraction method that maximizes EO recovery while requiring less extraction time than conventional techniques and having stronger antioxidant activity.

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Conflict of interest

The authors declare to have no conflict of interest.

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