


Hydro-Distilled and Supercritical Fluid Extraction of *Eucalyptus camaldulensis* Essential Oil: Characterization of Bioactives Along With Antioxidant, Antimicrobial and Antibiofilm Activities

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Abstract

Present research work evaluates variation in volatile chemicals profile and biological activities of essential oil (EO) obtained from the leaves of eucalyptus (*Eucalyptus camaldulensis* Dehnh.) using hydro-distillation (HD) and supercritical fluid extraction (SFE). The yield (1.32%) of volatile oil by HD was higher than the yield (.52%) of the SFE method ($P < .05$). The results of physical factors like density, color, refractive index, and solubility of the EOs produced by both the methods showed insignificant variations. Gas chromatography - mass spectrometry (GC-MS) compositional analysis showed that eucalyptol (31.10% and 30.43%) and α -pinene (11.02% and 10.35%) were the main constituents detected in SFE and HD extracted *Eucalyptus camaldulensis* EO, respectively. Antioxidant activity-related parameters, such as reducing ability and DPPH free radical scavenging capability exhibited by EO obtained via SFE were noted to be better than hydro-distilled EO. Supercritical fluid extracted and hydro-distilled essential oils demonstrated a considerable but variable antimicrobial potential against selected bacterial and fungal strains. Interestingly, oil extracted by SFE showed relatively higher hemolytic activity and biofilm inhibition potential. The variation in biological activities of tested EOs can be linked to the difference in the volatile bioactives composition due to different isolation techniques. In conclusion, the EO obtained from *Eucalyptus* leaves by the SFE method can be explored as a potential antioxidant and antimicrobial agent in the functional food and nutra-pharmaceutical sector.

Keywords

oil extraction, liquid carbon dioxide, reducing power, hemolytic activity, gas chromatography - mass spectrometry, eucalyptol, α -pinene

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Introduction

A variety of plants are used traditionally as folk medicine to treat various diseases.¹ Such medicinal health functions of plants can be due to the occurrence of certain bioactives that have multiple biological and pharmaceutical activities.² Various herbs and spices are also among those plants which have been historically used in the food and flavor industry as well as employed as native medicines in different civilizations.^{3,4} The essential oils (EOs) produced from vegetable materials such as leaves and flowers are responsible for multiple functions in plant's body. Plant EOs are composed of various volatile compounds³ with major part comprising of terpenoids, which are further divided into monoterpene, di-terpene, and sesquiterpene.⁵

The EOs extraction from plant materials depends upon its rate of diffusion to the upper surface from plant tissues, from where it can be collected by hydro-distillation (HD), steam distillation,⁶ use of supercritical CO₂ and high or low pressure distillation, etc.^{4,7} Steam distillation is used as a conventional method for isolation of EO but this method has some limitation especially with regard to the use of accelerated conditions and long preparation time.⁸ Some of the other shortcomings include low extraction efficiency and loss of some heat sensitive volatile components.⁹ Therefore, the use of an alternative eco-friendly method, that is, SFE, is encouraged for the extraction of EOs and CO₂ is highly promising in this regard because of its experimentally affordable critical conditions of pressure and temperature.^{2,10,11} A number of EOs are isolated/obtained from various medicinal plants by using supercritical fluid extraction (SFE) such as jasmine, basil, purslane, etc.^{2,12,13} In SFE, liquid CO₂ is used as a supercritical liquid/fluid to recover the aromatic components of plants because of its non-toxic nature, inert behavior, and mild values of critical points such as temperature (31-40°C) and pressure (7.38 MPa). The use of SFE involving liquid CO₂ is an impressive green extraction technique applicable for the isolation of high-quality and functional food/nutra-pharmaceutical grade extracts and EOs. The SFE process variables such as critical temperature and pressure and concentration of co-solvent can be optimized to improve the efficiency of the method offering better quality and optimal yield of EOs and extract.^{7,10,11} Furthermore, this technique is also quite applicable to selectively extract high-value vegetable oils from different seeds.¹³ However, SFE is especially superior method to extract EOs compared to other conventional techniques because it is more selective, protects the components from thermal decomposition, and offers contamination free healthier and pure volatile mixtures and natural extracts.¹⁴

Essential oils are composed of biologically active components, and thus, they possess antioxidant, bactericidal and fungicidal properties, and can be used for pharmaceutical purposes.¹⁵ Because of their natural antioxidant and biological

attributes, they are much popular in the food and preventive medicines. Recent studies reveal that EOs can be used for curing infectious diseases and as therapeutic agents.^{5,16} The extensive use of EOs in the field of pharmacy, cosmetics, foods, and beverages industries prompts the need to explore and characterize new plant sources of EOs.¹⁷

In Pakistan, a variety of medicinal plants are grown in various areas of the country, including deserts, plains, and hilly areas.² Soon valley is famous as a rich source of medicinal plants with wide array of bioactives such as phenolics and other antioxidant components. This valley's average temperature is in the range of 1°C (January) to 36°C (June), with low annual precipitation. In view of explicit agro-climatic conditions and soil composition, plants in this region are supposed to consist of a unique profile of phytonutrients.¹⁸ The *Eucalyptus* spp. EO is mainly used in cosmetics, nutraceutical, and medicinal applications. Because of different medicinal and pharmaceutical applications, various species of *Eucalyptus* are employed in folk medicines.¹⁹ Some reports suggest that *Eucalyptus* EO contains bioactive ingredients and thus is capable of showing multiple biological activities, including antiradical and antioxidant activities,²⁰ antifungal activity,²¹ bactericidal activity,²² and anti-larva activity.²³

Very little research work is reported for the comparative study of oil obtained by HD and SFE from wild eucalyptus plants. So the current research work is planned to appraise and compare the yield variations, physico-chemical and biological attributes of EOs obtained by two different techniques (HD and SFE) from wild *Eucalyptus* plant leaves grown in the Soon Valley, District Khusab, Pakistan.

Material and Methods

Collection of Samples

The samples of leaves were obtained from the mature eucalyptus (*Eucalyptus camaldulensis* L.) plants wildly grown in the Soon Valley, Dist. Khusab, Pakistan. These samples were harvested in spring season and further verified and authenticated by a botanist from Dept. of Botany, University of Sargodha. The leaves sample was then rinsed using deionized water to eradicate dirt and then dried under shade to minimize the moisture content (6.5% ± .10). These dried and powdered samples (particle size 7-12 mm) were used for the isolation of EO.

Extraction of EO

For obtaining the essential oil from the sample material, two different methods were used, including HD and SFE.

Hydro-distillation. In this method, a Clevenger-type apparatus was employed for isolation/extraction of EO. The plant material was hydro-distilled for 3 hours, and EO obtained was

dried with Na_2SO_4 (anhydrous). After filtration, the filtered EO was kept at -4°C before used for further analyses.^{6,20}

Supercritical Fluid Extraction. A commercial-scale supercritical fluid extractor (DEVEN supercritical Pvt. Limited, India) was used (Figure 1). A sample of 5 kg was placed in the extractor, and before extraction, it was heated for 60 minutes. The extraction conditions of the instrument were maintained at the temperature of 45°C with 10 mL/min flow rate of liquid carbon dioxide (density = 1.101 g/mL) at a pressure of 100 bar. The time of the extraction for the static method was 90 minutes, and the dynamic method was 30 minutes. The collection of EO was done in a glass vial collector.²⁴

Physical Analysis

The EOs were obtained by both the techniques were subjected to solubility test in 70% alcohol, refractive index and density (at 25°C) measurement.²⁵ A densimeter (digital) (DMA-602, Austria) was used for density determination, while an automatic refractometer (Model RX-7000, Atago, Tokyo, Japan) was used for refractive index determination. Three replicate readings for each analysis were taken, and results averaged.

Essential Oil Composition

Gas Chromatography. A gas chromatographic instrument (PerkinElmer, 8700, USA) coupled with a FID/flame

ionization detector was employed for studying the composition of chemical components of eucalyptus EOs. An HP-5 MS capillary column ($30\text{ m} \times .25\text{ mm}$) was used for separation of chemical volatiles. The temperature of the column was programmed from 80°C to 220°C by the rate of $4^\circ\text{C}/\text{minute}$ while maintaining the initial temperature for 3 minutes and final temperature for 10 minutes constant, total time taken in the column was 48 minutes. Helium (He), as carrier gas/mobile phase, was used with a 1.5 mL/min flow rate. The injector temperature was maintained at 220°C , while the detector temperature at 290°C . The sample ($1.0\ \mu\text{L}$) was injected in split mode (1:100, split ratio). Helium (He), as carrier gas/mobile phase, was used with a 1.5 mL/min flow rate. All the final calculations were performed by using software attached to the chromatographic instruments.

GC-MS Analysis. The volatiles profiling of EOs was also checked and authenticated by using GC-MS technique. A gas chromatographic equipment (Agilent, 6890 N) combined with a mass selective detector (MS-5975) and injector (autosampler, 7683-B) was used. An HP-5 MS capillary column ($30\text{ m} \times .25\text{ mm}$) was utilized. The column chromatographic conditions and mobile phase were the same as described above for GC-FID. For MS detection, 70 eV energy was used for electron ionization mode, and the temperature of the MS transfer line was maintained at 290°C . A 50 and 600 m/z the mass scanning range was used.



Figure 1. Supercritical fluid extractor.

Identification of Components. The compounds separated were identified by comparing their RI (retention indices) value relative to *n*-alkanes (C₉-C₂₄), with the data already published and some previous spectra of compounds already authenticated. Further identification and authentication were performed by their mass spectrometric data comparison with the library of NIST mass spectra and previously reported spectra.²⁶

Antioxidant Activity Assays

DPPH Scavenging Activity. For DPPH assay, a previously reported method²⁷ was used. In this test, .5-100 µg/L of oil sample was added into 1 mL of 90 µM solution of DPPH free radical. The solution volume was adjusted up to 4.0 mL by the addition of 95% methanol. The contents were kept for 60 minutes and noted the optical density at 515 nm. A positive control (BHT) was used separately along with investigated sample. The DPPH[•] inhibition (%) was estimated as given below

$$I(\%) = 100 \times (A_b - A_s / A_b)$$

Here, A_s represents the absorbance of tested sample EO, while A_b is the absorbance of control mixture. For calculation of IC₅₀ value (oil concentration that caused 50% of radical scavenging), a plot, that is, obtained equation, between percentage inhibition and sample concentration was used.

Reducing power. The reducing power assay was also used to determine potential antioxidant potential.² According to this method, 2.5-10.0 mg of EO was added with 5.0 mL of .2 mol/L Na-phosphate buffer of pH 6.6 along with 5.0 mL of 1.0% K-ferricyanide solution. Incubation was for 20 minutes at 50°C, then 10% trichloroacetic acid solution (5 mL) was added. Centrifugation of the above-prepared content was done at 980 × *g* for 10 minutes using a refrigerated centrifuge machine (H-200-NR, Japan) at 5°C. Then, 5.0 mL of the clear upper layer was mixed with 5.0 mL of H₂O, followed by 1.0 mL of .1% freshly prepared solution of ferric chloride and finally, the absorbance was measured at 700 nm.

Antimicrobial Activity

A group of microorganisms which included four strains of bacteria (*S aureus*, *E coli*, *P multocida*, and *B subtilis*) and four fungi strains (*A alternata*, *G lucidum*, *A niger*, and *A flavus*) were used for the evolution of antimicrobial activity. A culture of nutrient agar (Oxoid) for optimum growth of bacteria and potato dextrose agar (Oxoid) for optimum fungal growth was used at 30°C.

Inhibition Zone. The isolated EOs were subjected to the disc diffusion method²⁸ for assessing the antimicrobial activity. For this purpose, 6 mm diameter discs containing 50 µL of

obtained EOs were put on an agar plate where microorganisms were inoculated previously. For positive reference, Streptomycin (30 µg/disc) was used for bacterial and Fluconazole (30 µg/disc) for fungal strain as a standard drug. The same experimental setup was also performed but without a sample or standard drug as a negative control treatment and incubated these Petri dishes for 24 h at 37°C for bacteria and 3 days at 25°C for fungi. The inhibition zone (millimeter) was measured for the calculation of antimicrobial activity.

Minimum inhibitory concentration. The microdilution broth assay was adopted for MIC analysis.²⁸ In a 96 well microtiter plate, a series of essential oil dilutions were placed along with one sterility control and one growth control. For bacteria, 160 µL of NB and fungi 160 µL of SDB were mixed into plates and a tested solution (20 µL). The plates were inoculated with standard microorganism suspension (20 µL of 5 × 10⁵ CFU/mL). The plates were kept for 48 hours at 28°C for fungal and 24 hours at 37°C for bacterial growth. The standard drugs were the same as the disc diffusion method used as a positive control. A white pellet that appeared at the bottom indicated the growth of the microorganisms.

Biofilm Inhibition

Analysis of biofilm inhibition was done by a previously reported spectrophotometric method² using 96 well microtiter plate reader. In this method, the essential oil was added to the microtiter plate with 100 µL of *E coli* and *S aureus* cell suspension. The plates were kept for 3 days at 37°C and then the liquid suspension was removed and 100 µL of 1.0% crystal violet aqueous solution was added. After staining for 30 minutes, wells were washed after removing the dye and again incubated for 15 minutes after adding a 95.0% ethanol. Absorbance was measured at 570 nm spectrophotometrically, and the formula given below was used to calculate the percentage biofilm inhibition.

$$\text{Biofilm inhibition}(\%) = \frac{OD_{\text{in control}} - OD_{\text{in treatment}}}{OD_{\text{in control}}} \times 100$$

Hemolytic Activity

Hemolysis of the tested EOs was checked by a method previously reported by Ref. 29 on human erythrocytes of O blood group. This activity was performed under the *in-vitro* condition. The blood samples were obtained from the healthy volunteers. Initially, the blood sample was centrifuged at 5000 rpm for 5 minutes and prepared the erythrocyte solution (2.0%) in phosphate buffer for analysis. The essential oil, concentration ranging from 50 to 500 µg/mL, were assorted with sodium chloride solution (.85%) followed by erythrocytes suspension (2.0%). The mixture was incubated at 25°C for 30 minutes and centrifuged and absorbance was recorded

at 540 nm for liberated hemoglobin in the supernatant. A negative control without essential oil but only sterile phosphate buffer solution and a positive control using Triton X-100 (.1%) were used for comparison. The percentage hemolysis was calculated by multiplying the ratio of absorbance of sample and positive control with 100.

Statistical Study

Three samples of eucalyptus plant leaves were obtained to extract essential oil, and each was separately analyzed. Experiments were done in triplicate, and data is statistically analyzed by ANOVA (STATISTICA, Stat Soft Inc. USA). The significant difference was considered at $P \leq .05$, and data are given as mean \pm SD for triplicate values.³⁰

Results and Discussion

Yield and Physicochemical Parameters

The data regarding the yield and some physical parameters of the isolated EOs are presented in Table 1. The SF extracted EO yield (.52%) with the specified conditions given in this research work was notably ($P < .05$) lower than hydro-distilled EO yield (1.32%). The essential oil yield of plant material using SFE depends upon the nature and type of plant material, along with the conditions of SFE process. A lower yield in SFE can be explained by the specificity and selective nature of the technique. The higher yield in the later technique can be due to the effectual volatilization of terpenoid compounds.³¹ The present result of percentage yield is well in line with the previous study² that shows high yield of hydro-distillation as compared to SCFE. Such low yield of SCF extracted essential oil was also reported in previous research studies.^{48,51}

A slight difference was found in color intensity of EOs obtained by two different techniques. The color of hydro-distilled EO is slightly lighter than SCF extracted oil, possibly due to the main component's lower concentration in the EO by HD.² The refractive index, at 25°C, of oil extracted by the SFE method (1.4280) is higher than EO obtained by HD (1.3930); however, this difference is not significant. Similarly, at 25°C, the density of EO extracted by SFE is slightly higher (.942 g/mL) than EO obtained by HD (.891 g/mL). Obviously, this difference in the physical parameters might be due to the varying composition of EO obtained by two different techniques.³²

Chemical Compositional Analysis

GC-MS chromatograms relating to the analysis of the chemical constituents of *Eucalyptus camaldulensis* EO extracted by SFE and HD methods are depicted in Figures 2 and 3, whereas the percentage of each component is given in Table 2. A total of 31 components were detected in the EO extracted by SFE, while 43 components were identified from the EO extracted by HD. Eucalyptol was detected to be the principal component in *E camaldulensis* EO obtained by both the techniques; SFE gave a slightly higher percentage of eucalyptol (31.10%) as compared to HD (30.43%). The other important chemical components were α -pinene [SFE (11.02%) and HD (10.35%)], Spathulenol [SFE (5.59%) and HD (10.15%)], Globulol [SFE (4.19%) and HD (4.44%)], Aromadendrene [SFE (4.44%) and HD (4.40%)], menthol [SFE (6.34%) and HD (.42%)], *trans*-Pinocarveol [SFE (–) and HD (4.17%)], and Epiglobulol [SFE (1.21%) and HD (1.58%)].

The composition of eucalyptus leaves EO has been reported previously by some researchers^{23,33,34} who, in well agreement with our present data, reported 1,8-cineole (eucalyptol) as the principal member/component in the oil. Meanwhile, the present results varied compared to a few previous studies that found a different profile for key compounds of essential oil.³⁵ The difference in the chemical composition can be attributed to various features such as geographical location, the agro-climatic condition of the plant, soil nature, physical state of plant material, that is, fresh or dried, time of collection, age of the plant, and extraction method.^{2,19} The chemotype and genotype of the plant species might also affect the chemical composition of EOs, as previously investigated by Zouari et al.³⁶ SCFE method was previously used as extraction technique to obtain volatile components/essential oil from different plant materials, which showed that this technique is a good eco-friendly alternative to conventional methods.^{13,14}

Antioxidant Activities

DPPH radical scavenging activity. DPPH free radical gives maximum absorption at the range of 515-528 nm in visible spectrum because of its deep violet color. When it receives a proton from an antioxidant compound, it becomes yellow due to the change of the chromophore group. In terms of DPPH free radical potential, the antioxidant activity is reported to be

Table 1. Percent Yield and Some Physical Attributes of Eucalyptus Essential Oils Recovered by Different Extraction Methods.

Technique	Yield (%)	Color	Solubility	Density g/mL	Refractive Index
Supercritical fluid extraction	.52 \pm .05 ^b	Bright yellow	In alcohol	.94 \pm .03 ^a	1.482 \pm .04 ^a
Hydro-distillation	1.32 \pm .09 ^a	Light yellow	In alcohol	.89 \pm .03 ^a	1.393 \pm .03 ^a

The tabulated data is mean of replicate (triplicate measurements) of tested oil. Significant ($P < .05$) difference of values between the extraction methods are portrayed by different superscripts inside the same column.

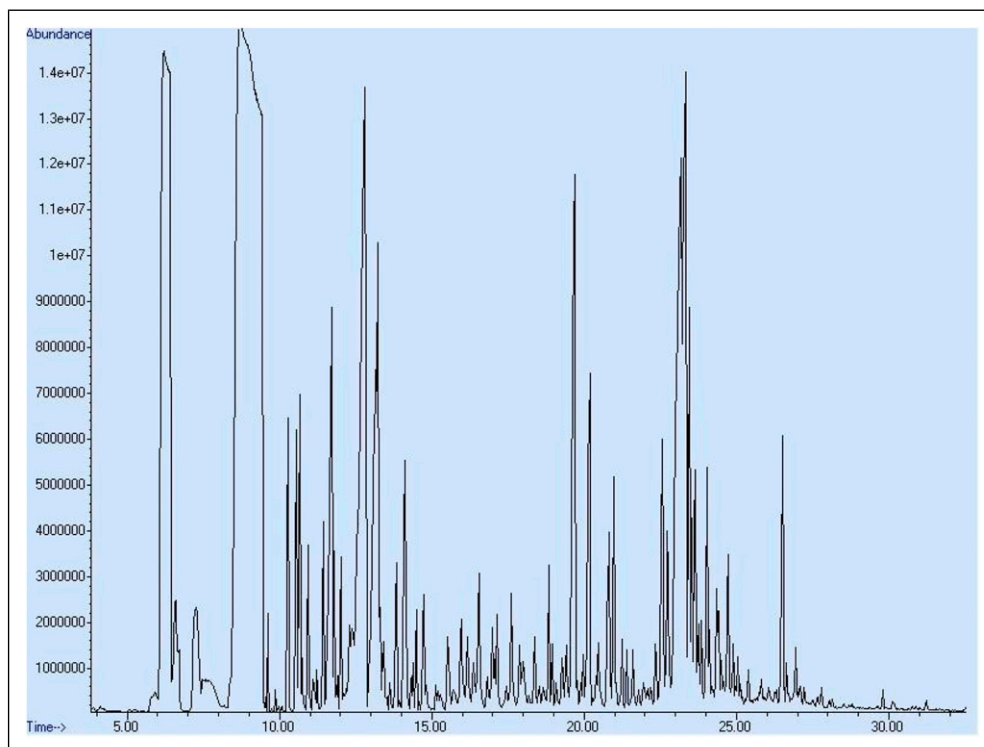


Figure 2. GC chromatogram of supercritical fluid extraction eucalyptus essential oil.

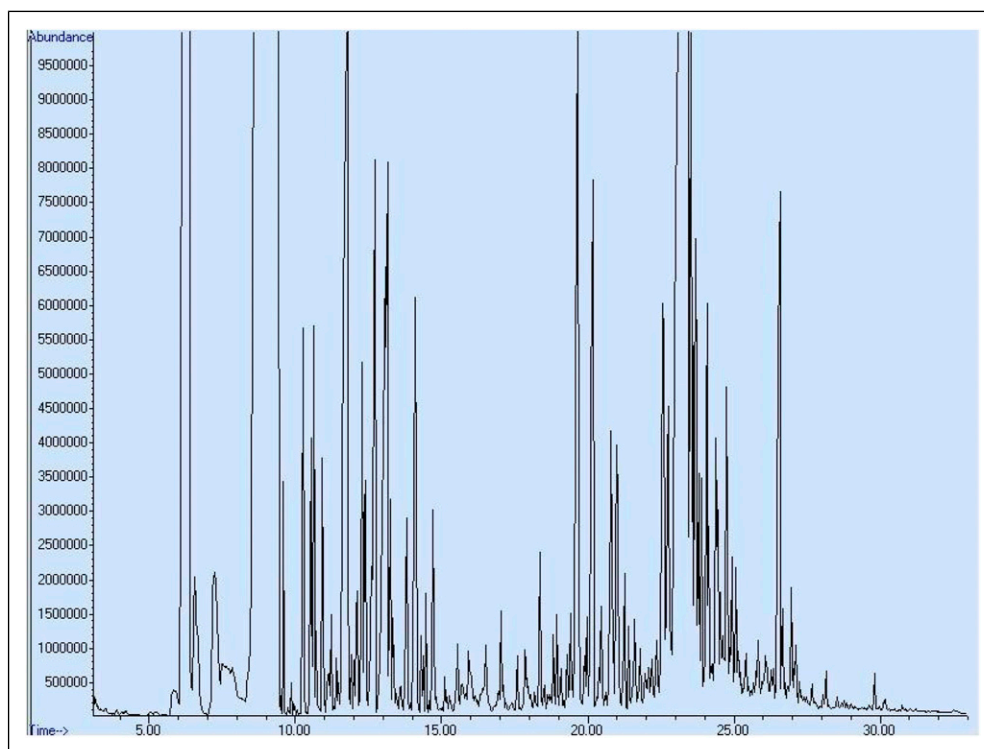


Figure 3. GC chromatogram of hydro-distillation eucalyptus essential oil.

Table 2. Chemical Composition (%) of Eucalyptus Essential Oils Recovered by Different Extraction Methods.

Sr. #	Compound Name	RI*	Supercritical Fluid Extraction	Hydro-Distillation
1	α -Pinene	941	11.02 \pm .33 ^a	10.35 \pm .38 ^a
2	Camphene	950	.83 \pm .03 ^a	.54 \pm .06 ^b
3	β -Pinene	976	1.04 \pm .04 ^a	.91 \pm .08 ^b
4	β -Myrcene	985	.14 \pm .02	—
5	Eucalyptol	1033	31.10 \pm 1.81 ^a	30.43 \pm 1.73 ^a
6	γ -Terpinene	1058	.14 \pm .03 ^b	.24 \pm .04 ^a
7	<i>p</i> -Cymenene	1112	.89 \pm .04 ^a	.84 \pm .09 ^a
8	Isovaleric acid	1117	—	.72 \pm .07
9	Fenchol	1121	.40 \pm .06 ^a	.47 \pm .04 ^a
10	α -Campholenal	1133	.09 \pm .01 ^b	.15 \pm .02 ^a
11	<i>cis</i> -Limonene oxide	1138	.48 \pm .03 ^a	.09 \pm .01 ^b
12	<i>trans</i> -Pinocarveol	1144	—	4.17 \pm .32
13	Menthol	1170	6.34 \pm .32 ^a	.42 \pm .03 ^b
14	α -Terpineol	1195	1.66 \pm .09 ^a	1.21 \pm .13 ^b
15	Myrtenol	1203	.21 \pm .02 ^b	.39 \pm .05 ^a
16	(-)-Verbenone	1208	—	.04 \pm .00
17	<i>cis</i> -Carveol	1226	.50 \pm .03 ^a	.50 \pm .05 ^a
18	Carvone	1245	.23 \pm .02 ^a	.17 \pm .02 ^b
19	Geraniol	1250	.45 \pm .02 ^a	.42 \pm .03 ^a
20	Estragole	1192	—	.14 \pm .02
21	Carvacrol	1302	—	.11 \pm .01
22	exo-2-Hydroxycineole acetate	1340	—	.16 \pm .02
23	Isolatedene	1377	.23 \pm .02 ^a	.15 \pm .03 ^b
24	Copaene	1380	.07 \pm .01 ^a	.05 \pm .00 ^a
25	β -Bourbonene	1383	—	.04 \pm .01
26	Jasmone	1389	—	.06 \pm .01
27	α -Gurjunene	1406	.39 \pm .06 ^a	.14 \pm .02 ^b
28	Caryophyllene	1425	.37 \pm .01 ^a	.09 \pm .01 ^b
29	γ -Maaliene	1431	—	.12 \pm .01
30	β -Gurjunene	1436	.23 \pm .04 ^a	.19 \pm .03 ^b
31	Aromadendrene	1442	4.44 \pm .12 ^a	4.40 \pm .37 ^a
32	δ -Guaiene	1501	.09 \pm .01 ^a	.08 \pm .01 ^a
33	Virdiflorene	1496	—	.87 \pm .09
34	β -Cadinene	1510	.20 \pm .01 ^a	.14 \pm .02 ^b
35	δ -Cadinene	1520	—	.19 \pm .02
36	Epiglobulol	1552	1.21 \pm .08 ^b	1.58 \pm 0.11 ^a
37	Aromadendr-I-ene	1454	—	1.02 \pm .12
38	Spathulenol	1577	5.59 \pm .25 ^b	10.15 \pm .42 ^a
39	Globulol	1585	4.19 \pm .19 ^a	4.44 \pm .25 ^a
40	Valencene	1523	.26 \pm .03 ^b	.44 \pm .04 ^a
41	<i>tau</i> -Muurolol	1640	—	.32 \pm .04
42	α -Cadinol	1650	.57 \pm .04 ^b	.77 \pm .06 ^a
43	Cadalene	1674	.06 \pm .00 ^a	.08 \pm .01 ^a
44	Farnesol	1723	—	.10 \pm .02
45	Geranyl linalool	2003	.10 \pm .01	—

The tabulated data is mean of replicate (triplicate measurements) of tested oil. Significant ($P < .05$) difference of values between the extraction methods are portrayed by different superscripts inside the same row.

*Retention index on HP-5MS column.

generally increased due to higher amount of bioactive in the extracts.³⁷ The data/result regarding the free radical scavenging ability (IC_{50}) of EO is presented in Table 3. The scavenging activity of EO obtained by SFE is slightly higher

(19.89 μ g/mL) than EO obtained by HD (16.21 μ g/mL). Many plants derived EOs are known to exhibit antioxidant activity in terms of radical scavenging capability, hence these can be employed for treating some degenerative and cardiovascular

disorders and infections.³⁸ The eucalyptus essential oil revealed a potent activity that can be linked to its volatiles chemical composition.

Reducing Power. The antioxidant activity of different plant EOs can also be assessed by finding out its reducing ability.³⁹ For this purpose, the reduction of ferric ions into ferrous ion is made and this process changes the yellow color of the test solution to bluish-green. The intensity of color gives us information regarding the reducing ability of tested EOs. The reducing power of plant EO can be directly correlated to color intensity of the solution, which can easily be assessed colorimetrically.² Hence, higher absorption revealed higher reducing power. The results regarding the reducing power of eucalyptus EO are depicted in Table 3, which show a regular increase in the reducing power with an increase in the concentration of EO. At a 10 mg/mL concentration, the maximum absorbance was observed for SF extracted EO (1.84) compared with the EO obtained by HD (1.18). The antioxidant assays outcome furnished that EO from SFE has better antioxidant activity as compared to the EO by HD, so the SFE

method is superior for the extraction of high-value bioactives/active constituents in the oil.

Antimicrobial Activity

The results regarding the antimicrobial efficacy of eucalyptus EOs using the disc diffusion method against tested bacteria and fungi are given in Table 4. The results showed that SFE extracted EO showed potent antibacterial activity (16 mm, inhibition zone) against *E coli* while EO by HD showed higher activity (14 mm, inhibition zone) against *P multocida*. The result of the antifungal activity described that the stronger activity was shown by SFE extracted essential oil (14 mm inhibition zone) and hydro-distilled essential oil (16 mm inhibition zone) against *A alternata*. The EOs obtained by both techniques exhibited lower antifungal and antibacterial action relative to the standard drugs, fluconazole, and streptomycin, used in this experiment. In general, it can be supposed that the SFE extracted EO is a good antibacterial agent, while the hydro-distilled essential oil is more effective as an antifungal agent.

Table 3. Antioxidant Potential of Eucalyptus Essential Oils Recovered by Different Extraction Methods.

Extraction Technique	DPPH Radical Scavenging Assay		Reducing Potential			
			Concentration (mg/mL)			
	Inhibition (%)	IC ₅₀ (µg/mL)	2.5	5.0	7.5	10.0
Supercritical fluid extraction	65 ± 3 ^a	19.89 ± .79 ^a	.83 ± .04 ^a	.97 ± .09 ^a	1.24 ± .17 ^a	1.84 ± .18 ^a
Hydro-distillation	53 ± 2 ^a	16.21 ± .97 ^b	.64 ± .04 ^b	.78 ± .07 ^b	.87 ± .09 ^b	1.18 ± .17 ^b
BHT	—	7.83 ± .25 ^b	—	—	—	—

The tabulated data is mean of replicate (triplicate measurements) of tested oil. Significant ($P < .05$) difference of values between the extraction methods are portrayed by different superscripts inside the same column.

Table 4. Antimicrobial Attributes of Eucalyptus Essential Oils Recovered by Different Extraction Methods.

Microorganisms	Zone of Inhibition (mm)			MIC (µg/mL)		
	SFE	Hydro Distillation	Standard Drug*	SFE	Hydro Distillation	Standard Drug*
Bacteria						
<i>S aureus</i>	12.00 ± .24 ^a	10.00 ± .18 ^b	28.00 ± 1.12 ^a	129 ± 5 ^a	134 ± 5 ^a	62 ± 2 ^c
<i>B subtilis</i>	14.00 ± .28 ^a	13.00 ± .23 ^b	31.00 ± 1.18 ^a	112 ± 4 ^a	119 ± 5 ^a	73 ± 3 ^b
<i>P multocida</i>	10.00 ± .19 ^b	14.00 ± .26 ^a	30.00 ± 1.12 ^a	131 ± 5 ^a	106 ± 4 ^b	54 ± 2 ^b
<i>E coli</i>	16.00 ± .32 ^a	12.00 ± .21 ^b	29.00 ± 1.21 ^a	83 ± 3 ^b	121 ± 4 ^a	63 ± 3 ^b
Fungus						
<i>G lucidum</i>	12.00 ± .21 ^b	13.00 ± .26 ^a	26.00 ± 1.08 ^a	131 ± 5 ^a	124 ± 5 ^b	73 ± 2 ^c
<i>A flavus</i>	11.00 ± .18 ^a	10.00 ± .14 ^b	28.00 ± 1.15 ^a	121 ± 4 ^b	137 ± 5 ^a	78 ± 2 ^c
<i>A niger</i>	13.00 ± .22 ^a	12.00 ± .21 ^b	30.00 ± 1.23 ^a	109 ± 4 ^a	114 ± 5 ^a	53 ± 2 ^b
<i>A alternata</i>	14.00 ± .27 ^b	16.00 ± .31 ^a	24.00 ± 1.02 ^a	119 ± 4 ^a	92 ± 3 ^b	83 ± 3 ^b

Abbreviations: SFE: supercritical fluid extraction.

The tabulated data is mean of replicate (triplicate measurements) of tested oil. Significant ($P < .05$) difference of values between the extraction methods are portrayed by different superscripts inside the same row.

*Streptomycin and Fluconazole were taken as standard drugs against bacterial and fungal strains, respectively.

Table 5. Biological Activities of Eucalyptus Essential Oils Recovered by Different 685 Extraction Methods.

Bioactivity	Microbe	Eucalyptus EO			
		supercritical Fluid Extraction	Hydro-Distillation	Rifamacin	Triton-X-100
Biofilm inhibition %	<i>S aureus</i>	54.59 ± 2.96 ^a	43.64 ± 1.98 ^b	87.43 ± 3.89 ^a	—
	<i>E coli</i>	79.65 ± 2.87 ^a	54.43 ± 2.43 ^b	88.92 ± 3.45 ^a	—
Hemolytic assay %		10.80 ± .32 ^b	63.65 ± 2.34 ^a	—	100

The tabulated data is mean of replicate (triplicate measurements) of tested oil. Significant ($P < .05$) difference of values between the extraction methods are portrayed by different superscripts inside the same row.

Minimum Inhibitory Concentration

The MIC values ($\mu\text{g/mL}$) of the tested EOs using the micro dilution broth method are given in Table 4. The SFE extracted EO was a good antibacterial agent (83 $\mu\text{g/mL}$) against *E coli* as compared to HD EO (106 $\mu\text{g/mL}$) against *P multocida*. The antifungal activity showed that HD based EO was better antifungal agent (92 $\mu\text{g/mL}$) against *A alternata* than SFE extracted EO (119 $\mu\text{g/mL}$) against the same. Overall, the EOs obtained by both the techniques gave a lower antibacterial and antifungal activity as compared to the standard drug, fluconazole, and streptomycin, used in this experiment. In previous research,⁴⁰ it is reported that the EO from many plant leaves, including *E salubris*, *E torquata*, *E woodwardii*, and *E stricklandii* showed an appreciable antimicrobial action against bacterial and fungal strains. Some other reports also showed that eucalyptus is very active against fungal and bacterial strains.⁴¹ Another study⁴² investigates the antimicrobial activity of two eucalyptus species and reveals that eucalyptus essential oil is a natural candidate/source of antibacterial and fungal bioactives.

Biofilm Inhibition

The EO was checked for biofilm inhibition activity using two bacteria, that is, *S aureus* and *E coli*, and the result was matched with Rifamacin (standard drug) and presented in Table 5. The results predicted that the EO obtained by SFE had higher (79.65%) inhibition toward the formation of biofilm as compared to HD EO (54.43%) against *E coli*. Furthermore, the inhibition against biofilm formation by SFE extracted EO was comparable with the percentage inhibition (88.92%) of standard drug used.

EOs can also be used as anti-carries treatments because of their significant inhibition in biofilm formation.⁴³ EOs have been reported to be good antimicrobial agents,⁴⁴ therefore they are very effective against oral microorganisms, thus used in mouthwashes because of their safe nature.⁴³ It has been previously reported that eucalyptus extracts can help treat tooth decay because of its biofilm inhibition property. Nagata

et al,⁴⁵ performed a clinical experiment to evaluate the effect of eucalyptus extracts against periodontal diseases.

Hemolytic Activity

The EOs were subjected to assessment of hemolytic activity because some compounds having biological activity may be harmful for hemoglobin and cannot be properly used in pharmacological preparation. This assay may also help to reveal some information regarding the cytotoxicity mechanism. In this research work, 100% hemolysis was obtained using Triton X-100 while a simple buffer was used to get 0% hemolysis. The hemolytic activity results are given in Table 5, which show that the minimum hemolysis (10.80%) is given by SFE extracted EO while the EO by hydro-distillation has higher hemolytic activity (63.65%). So, it can be concluded that the quality of EO obtained using the SFE method⁴⁶⁻⁵¹ because of its lower hemolysis" as "So, it can be concluded that the EO obtained using the SFE method⁴⁶⁻⁵¹ is safer since no toxicity was observed in hemolytic assay because of its lower hemolysis. No such work is reported on the hemolytic activity of eucalyptus EO to compare our present research data.

Conclusions

Volatiles chemical profiling and biological potentialities of EOs of *E camaldulensis* leaves using hydro-distillation and supercritical fluid extraction were investigated. GC-MS analysis revealed eucalyptol and α -pinene as the major chemical constituents in SFE extracted and HD essential oil, respectively. SFE extracted essential oil presented potent antioxidant activity compared to hydro-distilled counterpart. Both the SFE extracted as well as HD EOs demonstrated a considerable antimicrobial potential against bacterial and fungal strains. Superior hemolytic and anti-biofilm activities were recorded in SFE extracted oil. In conclusion, the EO obtained from eucalyptus leaves by the SFE method might be explored as a prospective antimicrobial and antioxidant agent in the functional food and nutra-pharmaceutical industries.

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