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Chemical Composition and Antimicrobial Activity of the Essential Oil of the Leaves of *Cupressus macrocarpa* Hartweg. ex Gordon

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ABSTRACT

Qualitative (GC-MS) and quantitative (GC-FID) analyses of the essential oil (EO) isolated from the leaves of *Cupressus macrocarpa* revealed the presence of fifteen components, and representing 86.43% of the total oil composition. Their identification was based on their retention times and mass spectral fragmentation patterns. *α*-terpineol (19.01%), camphenilone (9.78%), elemol (8.92%), 2-tridecanone (8.75%), *α*-terpinyl acetate (8.62%), 2-pentyl-2-cyclopenten-1-one (6.90%) and *β*-bisabolol (5.83%) represent major components. Agar diffusion method was conducted to test the antimicrobial activity of the EO against eight pathogenic strains, including gram positive (+ve) and negative (-ve) bacteria and fungi. The obtained results showed inhibition zones with 10.5-21mm diameters, compared to Streptomycin and Nystatin.

INTRODUCTION

Cupressus macrocarpa (Hartweg. ex Gordon) as a medicinal plant belongs to the family Cupressaceae and commonly known as Monterey Cypress (Cool, 2005; Thukral *et al*., 2014). It is widely distributed throughout the tropical and temperate regions around the world *i.e.,* Mexico, North America Asia, and North Africa (El-Ghorab *et al*., 2007). *C. macrocarpa* was used traditionally for decades for the treatment of various ailments, *e.g.,* styptic problem, eliminates fluid retention, whooping cough and rheumatism (Thukral *et al*., 2014). *C. macrocarpa* received little concern regarding its phytochemical constituents; Cool (2005) reported the isolation of ten sesquiterpenes from its foliage. Furthermore, Al-Sayed and Abdel-Daim (2014) investigated the protective role of the isolated cupressuflavone from *C. macrocarpa* against CCl_4 induced hepato- and nephrotoxicity in mice.

Several *Cupressus* species have been investigated for their essential oil content and evaluated for biological activity. El-Ghorab *et al.* (2007) identified 43 components from the fresh and dried leaves of the Egyptian *C. macrocarpa* that showed remarkable antimicrobial and antioxidant activities. In addition, thirteen components from the Iranian *C. sempervirens* L. were identified **(**Emami *et al*., 2004) and investigated for activity as fungistatic and bacteriostatic activity. Allo-ocimene along with other fourteen components were identified from the Egyptian *C. sempervirens* (Ibrahim *et al*., 2009), which exhibited promising antimicrobial and antiviral activities.

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Furthermore, Boukhris *et al*. (2012) identified 24 components from the Tunisian *C. sempervirens* essential oil and evaluated their antioxidant and antimicrobial activities. The aims of the current study were to analyze the essential oil composition of the Egyptian *C. macrocarpa* fresh leaves qualitatively by GC-MS and quantitatively via GC-FID, as well as the evaluation of the antimicrobial activity against eight pathogenic microbial strains.

MATERIAL AND METHODS

Plant material

Fresh leaves of *Cupressus macrocarpa* Hartweg. ex Gordon (Cupressaceae) were collected from the Zoo Garden, Giza, Egypt in June 2014. The plant was identified by Mrs. Threase Labib, consultant of plant taxonomy at the Ministry of Agriculture; formerly, the head of taxonomist specialists at the garden, a voucher specimen (No.C10/1/4) was kept at the herbarium of the garden.

Essential oil isolation

The fresh leaves of *C. macrocarpa* (2 kg) were fragmented into small pieces and subjected to hydrodistillation using Clavenger apparatus (Ibrahim *et al*., 2015) to extract the essential oil which was determined as mean of triplicate. The chemical composition of the oil was determined quantitatively via GC-FID and qualitatively via GC-MS by comparing their retention times and mass spectral fragmentation patterns with previously reported data (Adams, 1989, 2001, 2012).

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis was performed using a Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS and TGSMS Fused Silica Capillary Column (30m, 0.25mm, 0.1mm Film thickness). For GC-MS detection, an electron ionization system with ionization energy for 70 ev was used as the carrier gas at a constant flow rate of 1mL/min. The injector and MS transfer line temperature was set at 280°C. The oven temperature was programmed at an initial temperature 40°C (hold 3 min.) to 280°C was a final temperature at an increasing rate of 5°C/min (hold 5 min). The identified components were investigated using a percent relative peak area. A tentative identification of the volatile compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST08s, WILLY8, Adams and Library data of the GC/MS system (Adams, 1989, 2001, 2012; Ghareeb *et al*., 2016).

Gas Chromatography-Flame Ionization Detector (GC-FID) analysis

The GC-FID analyses were carried out with a Varian 3400 apparatus (Varian GmbH, Darmstadt, Germany) equipped with a FID detector and a DB-5 fused-bonded capillary column (30m x 0.25mm i.d., film thickness 0.25μm; Ohio Valley, Ohio, USA). The oven temperature was programmed isothermal at 45°C for 2 min., then rising from 45°C to 300°C (4°C/min.), and finally held isothermal at 300°C for 20 min.; injector temperature was 250°C; detector temp. was 300°C; Helium gas was used as carrier gas (2.0mL/min.); split ratio, 1:20. Peak Simple® 2000 chromatography software (SRI Instruments, California, USA) was used for recording and integrating the chromatograms. Average areas under the peaks of three independent chromatographic runs were used for calculating the % composition of each component.

Antimicrobial activity of the essential oil

Test organisms

The bacteria used in this study were Gram-positive bacterial strains *Bacillus subtilis* NRRL 543 and *Staphylococcus aureus* NRRLB-313 and Gram-negative bacterial strains, *Escherichia coli* NRRL B-210 and *Pseudomonas aeruginosa* NRRL B-23. Fungi, *Aspergillus niger* NRRL 599, *Fusarium oxysporum* NRRL 28184, *Candida albicans* NRRL Y-477 and *Fusarium solani*. These micro-organisms were obtained from Northern Utilization Research and Development Division, United State Department of Agriculture, Peoria, Illinois, USA.

Screening test

The bacterial strains were revived for bioassay by sub-culturing in fresh nutrient broth medium for 24 hours before test, while fungi were cultured on potato dextrose agar (PDA) (2.5% w/v agar) for 7 days at 28°C before the experiment was carried out.

Agar diffusion method

Cup plate agar diffusion method has been employed for the determination of the antimicrobial activity of some essential oils as well as paraffin oil. Several test microbes including G+ve bacteria (*B. subtilis* and *S. aureus*), G-ve bacteria (*P. aeruginosa* and *E. coli*) and fungi (*C. albicans*, *F. oxysporum*, *A. niger* and *F. solani*) were applied. 1 mL of cell suspension of 24h-old bacterial cultures (10^7-10^8 colonies/ml) in sterile distilled water was added to 250 mL of sterile solidified nutrient agar medium. For fungal strains, 1 mL spore suspension of seven days cultures fungal cultures (10^6-10^7colonies/mL) was added to 250 mL sterile solidified PDA (potato dextrose agar) medium. Holes of 9 mm in diameter were made using a cork borer. Aliquots of 0.1mL of the diluted essential oil with paraffin oil were poured inside the holes. A hole filled with paraffin oil only was also used as control. The plates were left for 2h at 4^oC as a period for diffusion. The diameter of each inhibition zone was measured and compared with that of the standard. Plate cultures were kept in an incubator at 28°C for 48h for fungi and at 37°C for 24h for bacteria (Linday, 1962). Streptomycin and Nystatin were used as control antibiotics at a concentration of 50 and 100 μg/mL respectively. Following incubation, the zone of inhibition for each sample was recorded in mm (including the hole).

Microorganisms with inhibition zone diameter ≥ 28 mm were classified as strongly sensitive, while that of < 28–16 mm were moderately sensitive, and with < 16–12 mm assorted as

weakly sensitive and isolates with zone diameter of <12 mm as resistant (Bauer *et al.*, 1996; Elgayyar *et al*., 2001).

The minimum inhibitory concentration (MIC)

Stock solutions of the EO were diluted and transferred into the first tube, and serial dilutions were prepared with concentrations ranged from 0.001-0.02 μL/mL. Spore suspension (10 μL) of each strain was inoculated in nutrient medium and incubated for 24-72 hours at 37°C. The control tubes containing the same medium were inoculated only with bacterial strains suspension. The minimal concentrations at which no visible growth was observed were defined as the MICs and expressed in (v/v %) (Carson *et al*., 1995).

RESULTS AND DISCUSSION

Identification of the essential oil constituents

Analyses of the essential oil of *C. macrocarpa* resulted in the identification of 15 components (**Table 1**) representing 86.43% of the total oil composition, which were identified qualitatively (GC-MS, **Fig. 1**) based on their retention times and mass spectral fragmentation patterns, and quantitatively (GC-FID, **Fig. 2**) based on the average areas under the peaks of three independent chromatographic runs into; α-terpineol (19.01%), camphenilone (9.78%), elemol (8.92%), 2-tridecanone (8.75%), α-terpinyl acetate (8.62%), 2-pentyl-2-cyclopenten-1-one (6.90%) and β-bisabolol (5.83%), and other components. The EOs composition of the genus *Cupressus* can be affected by many factors such as genetics (species or variety) or origin (El-Ghorab *et al*., 2007; Ibrahim *et al*., 2009), nutritional conditions such as fertilization, pesticide use, latitude (Emami *et al*., 2004; El-Ghorab *et al*., 2007; Boukhris *et al*., 2012), harvest time (Briggs and Sutherland, 1942), and drying (El-Ghorab *et al*., 2007). Moreover, the distillation time has been shown to influence the essential oil yield and composition (Zheljazkov *et al*., 2013).

In brief, El-Ghorab *et al*. (2007) studied the essential oil content of *C. macrocarpa* leaves (fresh and dried) and concluded that the EO yield of the dried leaves was more than the fresh one, with neral as the major constituent (35%) together with camphene (0.21%), α-terpinyl acetate (1.48%) and camphenilone (0.32%). In our study, α-terpineol (19.01%) was the major together with camphene (1.49%), α-terpinyl acetate (8.62%), and camphenilone (9.78%).

Briggs and Sutherland (1942) reported that the EO content of *C. macrocarpa* leaves was 20% after one day and was 18% after two days of the harvest time. This can be discussed as follow; α-terpineol in our study recorded 19.01% (June 2014), while it was of 2.7% (Malizia *et al*., 2011; Argentina; October), 2% (Giatropoulos *et al*., 2013; Greece; June) and 0.8% (Adams and Bartel, 2009; USA), according to its origin. In addition, the time of hydrodistillation have strong effect on the EOs constituents *e.g.,* α-terpineol recorded (19.01%) in 5hr, while it recorded 0.69%, 0.11%, 0.0% and 0.47% in 6, 6-12, 24 and 24-48hrs respectively (Xinfeng, 2009).

Table 1: Chemical compositions of the essential oil of *C. macrocarpa* leaves.

No	R_t	Area %	M.W.	M.F.	Main Fragments	Compound name
1	17.65	1.49	136	$C_{10}H_{16}$	(41, 53, 65, 69, 77, 79, 91, 93, 121, 136)	Camphene
2	19.75	1.24	134	$C_{10}H_{14}$	(40, 56, 68, 78, 91, 104, 119, 134)	*p*-Cymene
3	20.87	2.14	136	$C_{10}H_{16}$	(41, 51, 77, 91, 93, 105, 121, 136)	α-Phellandrene
4	22.39	2.38	136	$C_{10}H_{16}$	(41, 51 65 77, 93, 105, 121, 136)	α-Terpinene
5	23.93	6.90	152	$C_{10}H_{16}O$	(41, 43, 55, 69 79, 81, 95, 108, 121, 152)	2-Pentyl-2-cyclopenten-1-one
6	25.24	19.01	154	$C_{10}H_{18}O$	(43, 55, 69, 71, 86, 93, 111, 125, 136, 154)	α-Terpineol
7	25.74	8.62	196	$C_{12}H_{20}O_2$	(43, 53, 55, 59, 71, 79, 93, 111, 121 136, 154, 196)	α-Terpinyl acetate
8	27.0	9.95	------	-------	(41, 43, 55, 67, 69, 81, 95, 109, 123, 138, 156)	Unknown
9	34.61	3.38	204	$C_{15}H_{24}$	(41, 55, 67, 79, 93, 95, 105, 121, 136, 147, 161, 189, 204)	(−)-δ-Elemene
10	35.24	8.75	198	$C_{13}H_{26}O$	(58, 59, 71, 85, 96, 198)	2-Tridecanone
11	35.53	8.92	222	$C_{15}H_{26}O$	(58, 59, 121, 136, 189, 204)	Elemol
12	35.81	3.71	204	$C_{15}H_{24}$	(58, 94, 105, 135, 161, 204)	E-Santaline
13	37.22	3.54	218	$C_{15}H_{22}O$	(41, 53, 69, 81, 93, 121, 175, 218)	Dendrolasin
14	40.85	5.83	284	$C_{15}H_{26}O$	(41, 43, 55, 67, 81, 109, 119, 161, 204, 222)	β-Bisabolol
15	41.85	9.78	138	$C_9H_{14}O$	(41, 67, 69, 81, 93, 107, 136, 138)	Camphenilone
16	47.80	0.74	272	$C_{20}H_{32}$	(41, 55, 69, 81, 91, 105, 120, 133, 161, 257, 272)	Isophyllocladene
17	59.25	3.64	------	-------	(57, 71, 83, 113, 121, 149, 167, 279)	Unknown
Oxygenated monoterpenes		25.91%				
Oxygenated sesquiterpenes		18.29%				
Monoterpenes		7.25%				
Sesquiterpenes		7.09%				
Diterpenes		0.74%				
Others		(27.15%)				
Unknown		13.59%				
Total identified		**86.43 %**				

R_t: Retention time; M.W.: Molecular weight; M.F.: Molecular formula.

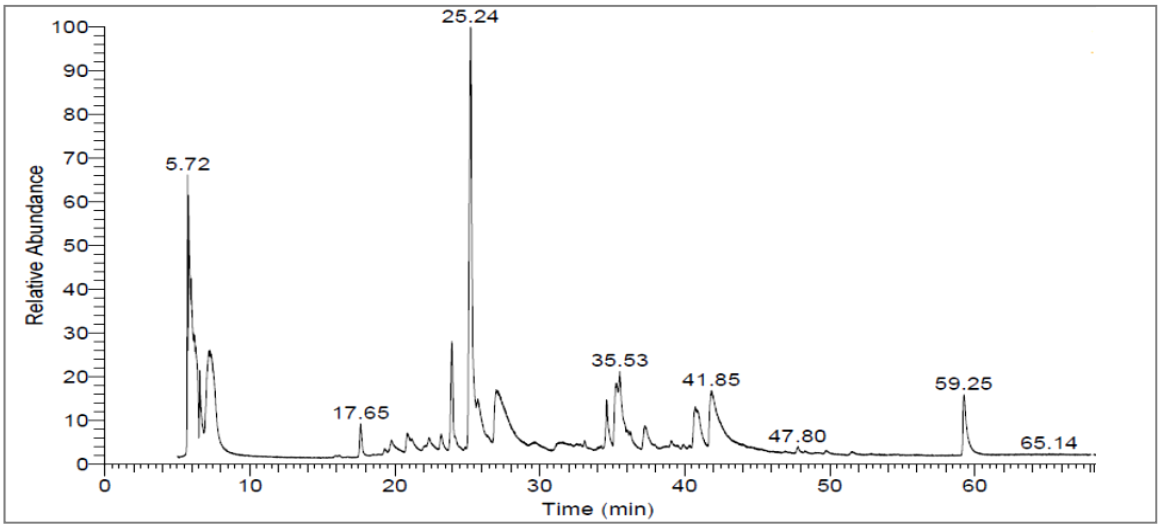

Fig. 1: GC-MS chromatogram of *C. macrocarpa* essential oil.

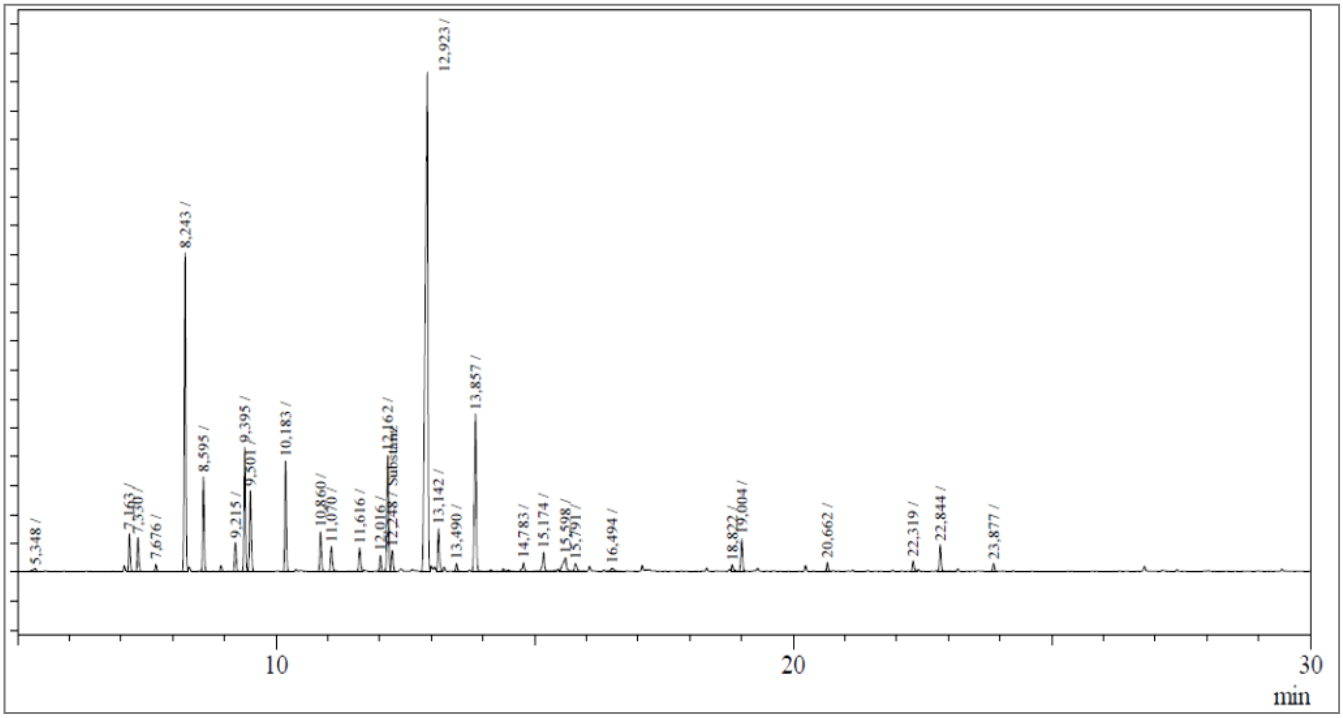

Fig. 2: GC/FID chromatogram of *C. macrocarpa* essential oil.

Antimicrobial activity

The antimicrobial activity of the essential oil was tested against G +ve and G -ve bacteria and fungi. Generally, it has been found that the activity was decreased with increasing the dilution titer of the tested microbes. EO at dilution (1:5, v/v) showed higher antifungal activity against *F. oxysporum* followed by *A. niger* and *F. solani* (17, 16 and 15 mm), respectively, and it recorded activity against *C. albicans* with (19 mm). The potent antibacterial activity resulted at dilution of 1:5, v/v against *P. aeruginosa* followed by *E. coli* with 21 and 15 mm for G-ve, and *B. subtilis* followed by *S. aureus* with 20 and 14 mm for G +ve, respectively (**Table 2**). The MIC values proved that the oil inhibited the growth of the tested fungi and bacteria at dilution 1:12.5-50 (v/v), with *Escherichia coli* being the most sensitive (**Table 3**).

EOs exhibited promising antimicrobial activity against a wide range of bacteria, and are very useful in food industry and clinical practice (Nakatani, 1994). Usually, Gram +ve organisms are highly susceptible than Gram -ve (Burst, 2004) The activity of the EOs (*i.e.*, antimicrobial) may be attributed to its major components (Lahlou, 2004), which may act synergistically (Peschel *et al*., 2006) to provide the activity.

Table 2: Antimicrobial activities of the essential oil from *C. macrocarpa* leaves.

Dilution v/v	*Fusarium solani*	*Fusarium oxysporum*	*Aspergillus niger* NRRL	*Candida albicans*	*Pseudomonas aeruginosa*	*Staphylococcus aureus* NRRL B-	*Escherichia coli* NRRL B-210	*Bacillus subtilis* NRRL 543
	Fungi	Fungi	Fungi	Yeast	(G -ve)	(G +ve)	(G -ve)	(G +ve)
1:5	15	17	16	19	21	14	15	20
1:25	13	14	13	17	18	13	14	16
1:50	12	12.5	12	15	15	12	12	12
1:100	11	12	11	11	11	11	10.5	11.5
Streptomycin	--	--	--	--	21	12	14	15
Nystatin	17	18	15	15	--	12	--	11

Inhibition zone diameter (mm).

Table 3: MICs of the essential oil from *C. macrocarpa* leaves.

Dilution v/v	*Fusarium solani*	*Fusarium oxysporum*	*Aspergillus niger* NRRL	*Candida albicans*	*Pseudomonas aeruginosa*	*Staphylococcus aureus* NRRL B-	*Escherichia coli* NRRL B-210	*Bacillus subtilis* NRRL 543
	Fungi	Fungi	Fungi	Yeast	(G -ve)	(G +ve)	(G -ve)	(G +ve)
MIC (ml % v/v)	--	--	--	12.5	--	12.5	50	12.5

Minimum Inhibition Concentration (MIC).

El-Ghorab *et al*., (2007) reported that the EO of Egyptian *C. macrocarpa* strongly inhibited the growth of *S. aureus*, *P. aeruginosa*, *E. coli*, *A. niger* and *C. albicans*, and concluded that, this may be attributed to the presence of neral, geraniol, eugenol dihydro, carvacrol acetate and phenol (2,6-dimethoxy). Moreover, Manimaran *et al*. (2007) reported the potent antimicrobial activity of the EO of the Indian *C. macrocarpa* and concluded that, such activity mainly due to the EO components *i.e.*, caryophyllene, *α*-terpineol etc. In addition, a comparative antimicrobial study was done on the EOs content of *Cupressus glauca, C. funebris, C. lawsonia, C. macrocarpa* and *C. sempervirens*. The results showed that *C. macrocarpa* possessed the potent antibacterial and antifungal activities at concentration of 100 mcg/mL while against *C. albicans* was 50 mcg/mL (Manivannan *et al*., 2005).

In the current study, *α*-terpineol was reported to be the major component of the EO of *C. macrocarpa*, which was suspected to be responsible for the antimicrobial activity. Previous studies are in full agreement with our findings which correlated the antimicrobial activity to one or several major constituents. Yang *et al*. (2014) concluded that *α*-terpineol and *p*-cymene are the reason for the antimicrobial activity of the EO of *Glossogyne tenuifolia*. Moreover, Krist *et al*. (2008) investigated the antimicrobial effect of five aroma compounds, among them *α*-terpineol showed the highest activity. Kubo *et al*. (1991) proved that *α*-terpineol has high antimicrobial activity against *S. aureus*, and *P. aeruginosa*.

In fact, the oxygenated monoterpenes (e.g. monoterpene alcohols) are promising antimicrobial agents due to the presence of the alcoholic part, which increase their water solubility **(**Hammer *et al*., 2003**)**. Their mode of action may be attributed to protein denaturation or dehydration on the vegetative cells (Dorman and Deans, 2000). In addition, *α*-terpineol as a member of the oxygenated monoterpenes can act as antimicrobial agent via the cell barrier destruction, and initiate seepage of proteins and lipids (Oyedemi *et al*., 2009). On the other hand, some studies have concluded that the antimicrobial activities of the whole essential oil were greater than the activities ascribed to certain individual constituents, and the synergistic effect should be taken in our minds due to a complex interaction between such individual constituents (Gill *et al*., 2002; Savelev *et al*., 2003).

CONCLUSION

The essential oil separated from the leaves of *C. macrocarpa* exhibited strong antimicrobial activity against Gram +ve & -ve pathogenic strains, which may be attributed to the presence of *α*-terpineol (19.01%) as major component, or due to the synergistic effect between the overall constituents *α*-terpineol (19.01%), camphenilone (9.78%), elemol (8.92%), 2-tridecanone (8.75%), *α*-terpinyl acetate (8.62%), 2-pentyl-2-cyclopenten-1-one (6.90%) and *β*-bisabolol (5.83%), therefore, it could be used in the treatment of microbial infections.

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