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RESEARCH ARTICLE

ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF *GRANGEA MADERASPATANA* (L.) POIR. EXTRACT

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ABSTRACT

The extract of aerial parts of *Grangea maderaspatana* (L.) Poir., obtained by steam distillation was analyzed by gas chromatography and gas chromatography coupled with mass spectroscopy. Twenty one constituents were identified, constituting 91.5 % of the oil. It was characterized by the dominant presence of sesquiterpenoids (sesquiterpenoid hydrocarbons 36.1 % and oxygenated sesquiterpenoids 28.4 %). Most abundant compounds are γ-gurjunene (26.5%), terpinyl acetate (20.8%) and hinesol (11.7%). The *in vitro* antioxidant potential of the oil was evaluated using, DPPH radical scavenging, metal chelating and reducing power assays. The oil showed antioxidant potential with significant reducing power (ASE/mL 2.01 ± 0.00), chelating activity (IC_{50} 1.80 ± 0.15) and DPPH radical scavenging activity (IC_{50} 2.90 ± 0.96). Antimicrobial activity of the oil was tested against one gram positive, four gram negative bacteria and two fungi using agar well diffusion method. The ZOI values of the oil were in the range of 2.67 ± 0.58 to 11.00 ± 0.00 mm and MIC of the oil was ranged from 5 to 30 μL/ mL for tested microorganisms. The activity was more pronounced against *Candida albicans* (ZOI = 11.00 ± 0.00 mm, MIC = 5 μL/mL) followed by *Streptomyces candidus* (ZOI = 9.33 ± 0.58 mm, MIC = 5 μL/mL), while the oil was least effective against *Aeromonas hydrophila* and *Klebsiella pneumoniae*.

KEY WORDS: *Grangea maderaspatana,* essential oil, antioxidant activity, antimicrobial activity, terpenoid composition.

INTRODUCTION:

Grangea is a genus of suberect or prostate annual herbs. Fourteen species of *Grangea* are found in tropical and sub-tropical Asia and Africa. *Grangea maderaspatana* (synonym: *Artemisia maderaspatana*) is a common weed usually occurring in sandy lands and waste places[1]. Vernacular name of this plant is Mutkhari and Machi pathree. The leaves of *G. maderaspatana* are used as sedative, analgesic, carminative, stomachic and in cases of obstructed menses[2].

Steroidal constituents[3], hardwickiic acid, the corresponding 1,2-dehydro-derivative, acetylenic compounds[4], eight new clerodane diterpenes including *nor*-clerodane, a *seco*-clerodane and a *nor-seco*-clerodane derivatives along with auranamide[5], grangolide and eudesmano-lides[6] have been reported from various extracts of this species. Preliminary chemical screening of essential oil of *G. maderaspatana* has been done earlier[7,8]. Objective of the present study was to investigate chemical composition, *in vitro* antioxidant and antimicrobial activity of the essential oil of *G. maderaspatana* (L.) Pior.

4. MATERIAL AND METHODS:

4.1. *PLANT MATERIALS:*

The fresh aerial parts of *G. maderaspatana*, were collected from Sitarganj, district: Udham Singh Nagar of Uttarakhand (elevation: 298 m). Plants herbarium was identified from Botanical Survey of India, Dehradun and voucher specimen was deposited in the Phytochemistry Laboratory, Chemistry Department, Kumaun University, Nainital, India and Botanical Survey of India, Dehradun, India (Acc. No. 113563).

4.2. *EXTRACTION:*

The fresh plant materials (2 kg) were subjected to steam distillation. The distillates were saturated with NaCl and extracted with *n*-hexane and dichloromethane. The organic phase was dried over anhydrous Na_2SO_4 and the solvent was distilled off in rotary vacuum evaporator at 30°C to obtain residual oil.

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4.3. ***IDENTIFICATION OF CONSTITUENTS:***

Characterization of constituents was done on the basis of Linear Retention Index (LRI, determined with reference to homologous series of *n*-alkanes C9-C24) under identical experimental condition, co-injection with pure compounds, MS Library search (NIST and WILLEY) and by comparing with the MS literature data[9,10]. The relative contents of individual components were calculated by GC response on FID without using correction factor.

4.4. ***GC AND GC-MS ANALYSIS:***

The residual extract (oil) was analyzed by using Nucon 5765 gas chromatograph equipped with Rtx-5 non-polar fused silica capillary column (30 m × 0.32 mm, 0.25 µm film coating). The oven temperature (60-210˚C) was programmed at 3˚C min^{-1} using N_2 as carrier gas at 4 Kg cm^{-2}. The injector temperature was 210˚C, detector temperature 210˚C and the injection volume 0.5 µL, using a 10% solution of the oil in *n*-hexane. GC-MS was conducted on a ThermoQuest Trace GC 2000 interfaced with a Finnigan MAT PolarisQ ion trap mass spectrometer equipped with Rtx-5 non-polar fused silica capillary column (30 m × 0.25 mm, 0.25 µm film coating). The oven temperature (60-210˚C) was programmed at 3˚C min^{-1} using helium as carrier gas at 1.0 mL/ min. The injection, ion source and MS transfer line temperatures were 210˚C, 220˚C and 275˚C, respectively, the injection volume was 0.1µL, and the split ratio was 1:40. MS were taken at 70 eV with mass range of 40-450 amu.

4.5. ***ANTIOXIDANT ACTIVITY:***

Antioxidant activity of the *G. maderaspatana* extract was evaluated as 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability and its effect on the chelation of Fe^{2+} as well as its reducing power in comparison with standard antioxidants butylated hydroxytoluene (BHT) and citric acid as positive control. Antioxidant activity was evaluated at concentrations ranging from 1-20 µL/ mL.

4.5.1. ***DPPH RADICAL SCAVENGING ASSAY*****:**

The DPPH radical scavenging activity was determined by using the method of Yen & Duh 1994[11]. Different aliquots were added to 2.9 mL of freshly prepared solution of DPPH (6 × 10^{-5} M in MeOH). The absorbance was recorded at 517 nm after 1 h of incubation. Percent absorbance of DPPH (I%) was calculated according to formula:

$$I\,\% = [1-(A_t/A_o)]\times 100$$

where A_o = absorbance of the control reaction (containing all reagents except the test sample), A_t = absorbance of the test sample. The IC_{50} was estimated and calculated as described by Kroyer (2004)[12]. IC_{50} value is the concentration of sample required to scavenge 50% DPPH free radical and was calculated from a calibration curve by a linear regression.

4.5.2. ***REDUCING POWER ASSAY*****:**

Reducing power was determined using ferric reducing-antioxidant power assay taking butylated hudroxytoluene (BHT) as standard as described by Apati et al. (2003)[13]. Different aliquots of sample maintained to 1 mL, followed by the addition of 2.5 mL of phosphate buffer (pH 6.6) and 2.5 mL of 1% w/v potassium ferricyanide in each reaction mixture, obtained mixtures were incubated at 50 ˚C for 20 min. After incubation, reaction was terminated by addition of 2.5 mL of 10% w/v trichloroacetic acid solution; 2.5 mL of above solution from each reaction was diluted with equal amount of distilled water. Aliquot of 0.5 mL $FeCl_3$ (0.1%) was added in each and absorbance was recorded after 10 min at 700 nm. Reducing power was expressed as ascorbic acid equivalent (1 mM = 1 ASE).

4.5.3. ***METAL CHELATING ASSAY:***

The ability of the extract to chelate ferrous ions (Fe^{2+}) was determined according to Decker & Welch (1990)[14]. Aliquots of 1 mL of different concentrations (1, 2, 5, 10, 15 and 20 µL/ mL) of the oil were mixed with 3.7 mL of deionized water. The mixture was left for reaction with $FeCl_2$ (2 mM, 0.1 mL) and ferrozine (5mM, 0.2 mL) for 10 min at room temperature, and then the absorbance was measured at 562 nm. The chelation of Fe^{2+} by the oil was compared to that of BHT at 0.01 mM and gallic acid at 0.025 M. Percent chelating activity was calculated according to the following equation.

$$\%\ \text{Chelating activity} = [1-(A_t/A_o)] \times 100$$

(Where A_t is the absorbance of the sample at 700 nm and A_o is the absorbance of the control at 700 nm). The IC_{50} was estimated and calculated as described by Kroyer (2004)[12].

4.6. ***ANTIBACTERIAL ACTIVITY:***

4.6.1. ***GROWTH AND MAINTENANCE OF TEST MICROORGANISM:***

The *in vitro* antibacterial activity was evaluated against a total of five bacteria including one gram positive *Bacillus subtilis* (MTCC-441) and four gram negative bacteria *Klebsiella pneumoniae* (MTCC-3384), *Pseudomonas aeruginosa* (MTCC-424), *Aeromonas hydrophila* subspecies *hydrophila* (MTCC-646) & *Escherichia coli* (MTCC-443). The antifungal activity of the oil was performed against *Streptomyces candidus* subspecies *azaticus* (MTCC-703) and *Candida albicans*

(MTCC-227). The test strains were procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India. MTCC (Microbial Technology Culture Collection) numbers represent the standard strain numbers assigned to these microorganisms.

4.6.2. ***Determination of zone of inhibition (ZOI):***

The antimicrobial activity was investigated by the well diffusion method using 24–48 h grown strains reseeded on nutrient broth for bacterial strains and Malt yeast agar for fungal strains[15]. The cultures were adjusted to 1×10^6 CFU/mL with sterile water. Then 100 µL of the suspensions were spread over nutrient agar and malt yeast plates to obtain uniform microbial growth. In petri plates well prepared with the help of borer (bore 2 mm diameter). The wells were filled with 20 µL of essential oil solutions in the inoculated nutrient/malt yeast extract agar plates. The petri dishes were kept at 4 °C for 2 h. The plates were incubated at 37 (24 h) and at 30 °C (48 h) for bacterial and fungal strains, respectively. The diameter of the inhibition zones were measured and considered as the zone of inhibition (ZOI). All experiments were performed in triplicate.

4.6.3. ***DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC):***

The performed agar dilution susceptibility test was based on modified methods of NCCLS and CLSI[16,17]. Serial dilutions of the essential oil were prepared by diluting oil with DMSO to achieve a decreasing concentration range from 50 to 10 µL/ mL using 100 µL of a suspension containing 1×10^6 CFU/mL of bacteria spread on nutrient agar plates, whereas the fungal strains were reseeded on Potato dextrose agar (PDA) plates. The wells were filled with 20 µL of essential oil solutions in the inoculated nutrient/malt yeast extract agar plates. The bacterial plates were incubated at 37 ± 2 °C for 24–72 h., while fungal cultures were incubated at 30 ± 2°C for 48 h. The MIC was defined as the lowest concentration of the oil inhibiting the visible growth of each bacterium on the agar plate so the least concentration of each essential oil showing a clear zone of inhibition was taken as the MIC. DMSO was used as the negative control. Gentamicin and kanamycin were used as positive control for bacteria and nystatin for fungi.

4.7. ***STATISTICAL ANALYSIS:***

All the measurements were done in triplicate and statistical analysis was performed by Duncan test as available in SPSS 20. Results are presented as mean ± S.D.

RESULTS AND DISCUSSION:

2.1. ***CHEMICAL COMPOSITION OF ESSENTIAL OIL:***

The quantitative and qualitative analysis of the oil by GC and GC-MS, led to the identification of 21 constituents, constituting 91.5 % of the oil. The identified compounds are listed in Table 1 in order of their elution in Rtx-5 non-polar fused silica capillary column. The oil was dominated by sesquiterpene hydrocarbons (36.1 %) with the major presence of γ-gurjunene (26.5 %). The other constituents were terpinyl acetate (20.8 %), hinesol (11.7 %), 10-*epi*-γ-eudesmol (5.8 %), α-guaiene (4.8 %) and *epi*-cubebol (3.5 %). The chemical class distributions of the volatile constituents are summarized as the major constituents of the oil were sesquiterpene hydrocarbons (36.1 %) followed by oxygenated sesquiterpenes (28.4 %) and oxygenated monoterpenes (22.9 %). Monoterpene hydrocarbons and diterpene hydrocarbons were 2.8 % and 1.3 %, respectively.

Table 1: Terpenoid composition of *G. maderaspatana*

Compound	RI	Percentage composition	Mode of identification*
Myrcene	994	0.4	a,b
α-Terpinene	1019	1.0	a,b
γ-Terpinene	1054	1.4	a,b
cis-*β* –Terpeneol	1092	*t*	a,b
trans-Thujone	1110	1.4	a,b
Geraniol	1259	0.7	a,b
Terpinyl acetate	1389	**20.8**	a,b,c
Longifolene	1408	*t*	a,b
α-Guaiene	1419	4.8	a,b
***γ*-Gurjunene**	1457	**26.5**	a,b,c
Epizonarene	1481	3.0	a,b
epi-Cubebol	1483	3.5	a,b
Cubebol	1494	3.2	a,b

Cadina-1,4-diene	1497	1.8	a,b
1,10-di-*epi*-Cubebol	1517	1.8	a,b
10-*epi*-γ-Eudesmol	1622	**5.8**	a,b,c
Cubenol	1635	*t*	a,b
α-Muurolol	1640	0.2	a,b
Hinesol	1644	**11.7**	a,b,c
α-Bisabolol	1685	2.2	a,b
(3-Z) Cembrene A	1963	1.3	a,b
Monoterpene hydrocarbons		2.8	
Oxygenated monoterpenes		22.9	
Sesquiterpene hydrocarbons		36.1	
Oxygenated sesquiterpenes		28.4	
Diterpene hydrocarbons		1.3	
Total identified		91.5	
Oil yield (% v/w)		0.3	

*Mode of identification: Retention Index (LRI, Based on homologous series of n-alkanes; C_8-C_{24}), coinjection with Standards/Peak enrichment with known oil constituents, MS (GC-MS), *t*= trace (<0.1%); (-) = not detected, a = Retention Index (RI) on Rtx-5 capillary column; b = MS (GC/MS); c= co-injection with authentic sample; Compounds > 5.0% are represented in bold face.

2.2. *ANTIOXIDANT ACTIVITY*

Numerous antioxidant methods and modifications have been proposed to evaluate antioxidant activity and to explain how antioxidants function. Of various methods the total antioxidant activity, reducing power, DPPH assay, metal chelating, active oxygen species such as H_2O_2, $O_2^{\bullet -}$ and $OH^{\bullet}$ quenching assays were used for the evaluation of antioxidant activities of extracts[18].

Figure 1 shows the reductive capabilities of the oil compared to BHT and linoleic acid. For the measurements of the reductive ability, we observed the transformation of Fe^{3+} - Fe^{2+} in the presence of oil samples using the method of Apati et al. (2003)[13]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[19]. Like the antioxidant activity, the reducing power of essential oil increased significantly with concentration. By Figure 1, it may be concluded that reducing power activity of the oil is higher than linoleic acid but less than butylated hydroxytoluene. Mean absorbance values were significantly different at *P<0.05.* Reducing power of the essential oil was 2.01 ± 0.00 ASE/ mL (Table 2).

The effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The maximum absorption of a stable DPPH radical in ethanol is at 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radical, which results in the scavenging of the radical by hydrogen donation. This is visualized as a discoloration from purple to yellow color with the result of which percent absorbance value increases. Figure 2 illustrates a significant increase in percentage absorbance value (as the concentration of DPPH radical decreased due to the scavenging ability of the oil and standards). The scavenging effect (% inhibition) of the oil and standards on the DPPH radical decreased in the order of BHT (97.7 %) > Essential oil (91.01 %) > Gallic acid (85.95 %) at 20 µL/ mL (Figure 2). Mean values were significantly different at *P<0.05.* IC_{50} value of the essential oil for DPPH radical scavenging activity was 2.90 ± 0.96 µL/ mL (Table 2).

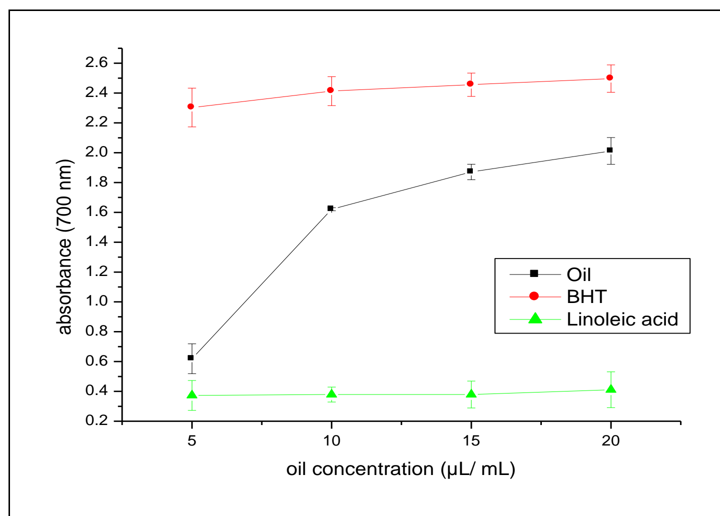

Figure 1: ***Reducing power activity***

Table 2: Antioxidant activity of *G. maderaspatana* extract.

Sample/standard	DPPH (IC_{50} µL/mL)	RP (ASE/mL)	Chelating (IC_{50} µL/mL)
GMO	2.90 ± 0.96	2.01 ± 0.00	1.80 ± 0.15
Gallic acid	1.60 ± 0.86	ND	0.71 ± 0.34
BHT	0.38 ± 0.47	1.48 ± 0.01	0.78 ± 0.46
Linoleic acid	ND	2.05 ± 0.24	ND

*GMO= *Grangea maderaspatana* essential oil; BHT= butylated hydroxy toluene; na = not active; ND = not determined.

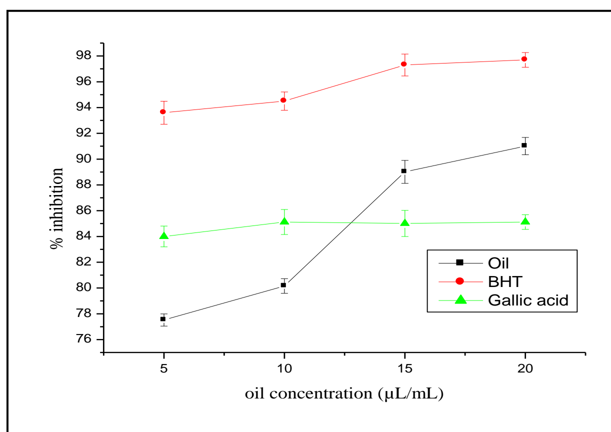

Figure 2*: DPPH Radical scavenging activity*.

The chelating of ferrous ions by the extract oil was estimated. In this assay, the extract oil and standard antioxidant compound interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and capture ferrous ion before ferrozine. As shown in Figure 3, the formation of the Fe^{2+}-ferrozine complex was not completed in the presence of the oil, indicating that the oil chelates the iron. The absorbance of Fe^{2+}-ferrozine complex was linearly decreased dose-dependently (from 5 to 20 µL/ mL) as a result of which percentages of metal chelating capacity increased simultaneously. The percent chelating activity of essential oil, BHT and gallic acid were found as 89.07, 88.11, 84.12%, respectively at 20 µL/ mL. Mean values of percent chelating activity were significantly different at $P<0.05$. Metal chelating activity of the essential oil appeared significant having IC_{50} 1.80 ± 0.15 µL/ mL (Table 2).

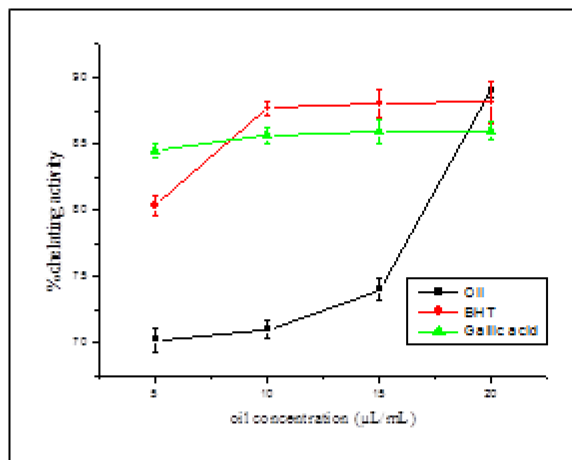

Figure 3: ***Metal chelating activity*.**

2.3 ANTIMICROBIAL ACTIVITY:

In this study, seven microbial species were used to determine the possible antimicrobial activity of the oil. Of the species used, *Candida albicans* is the microbe responsible for most clinical yeast infections, e.g. in mouth infections. Interestingly, the oil showed good activity against *C. albicans* with ZOI 11.00 ± 0.00 mm and 5 µL/ mL MIC value. As shown in Tables 3, the generation of most microbial species was inhibited by the oil. Activity of the oil has been compared with standard antimicrobial compounds in Fig 4. *Escherichia coli*, belonging to the normal flora of humans, is an enterohemmoragic bacterium causes serious cases of food poisoning and preservatives to eliminate its growth are needed. Oil was found to be active against *E. coli* with 7.33 ± 0.58 mm ZOI and 10 µL/ mL MIC value. The activity was more pronounced against *S. candidus,* and *B. subtilis* (9.33 ± 0.58 and 8.00 ± 0.00 mm, respectively) with MIC value of 5 µL/mL for each while least effective against *Aeromonas hydrophila* and *Klebsiella pneumoniae*. Gentamicin and kanamycinwere used as positive control for bacteria and nystatin for fungi.

Table 3: Antimicrobial activity (zone of inhibition and minimum inhibitory concentration) of steam volatile extract of *G. maderaspatana*

Sample	EC		KP		PA		BS		AH		SC		CA	
	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC
GMO	**7.33 ± 0.58**	**10**	5.33 ± 0.58	30	6.00 ±0.50	10	**8.00 ± 0.00**	**5**	5.00 ± 0.00	30	**9.33 ± 0.58**	5	**11.00 ± 0.00**	5
Positive control														
Gn	9	50	10	50	13	50	10	50	10	50	ND	ND	ND	ND
Kn	7	100	9	50	13	50	10	75	10	50	ND	ND	ND	ND
Ns	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	10	250	11	500

GMO = *Grangea maderaspatana* essential oil; Positive control - Gn = gentamicin, Kn = kanamycin, Ns = nystatin; Zone of inhibition (ZOI) is given in mm at 50 µL/ mL (mean ± SD) and minimum inhibitory concentration (MIC) is given in µL/mL for oil and µg/ mL for positive control; ND = not determined; EC = *Escherichia coli*, KP = *Klebsiella pneumoniae*, PA = *Pseudomonas aeruginosa*, BS= *Bacillus subtilis*, AH = *Aeromonas hydrophila*, SC = *Streptomyces candidus*, CA= *Candida albicans.*

CONCLUSION:

To the best of our knowledge, no data have been published on antioxidant, antibacterial activity of the extract oil of this plant species. The oil was mainly characterized by the presence of sesquiterpene hydrocarbons 36.1 % followed by oxygenated sesquiterpene 28.4 %. The major constituents were γ-gurjunene (26.5 %), terpinyl acetate (20.8 %), hinesol (11.7 %), 10-*epi*-γ-eudesmol (5.8 %), α-guaiene (4.8 %) and *epi*-cubebol (3.5 %). The oil showed less antioxidant potential than standard antioxidant, butylated hydroxy toluene. High level of antibacterial activity against *C. albicans* indicates good bioactive properties of the oil. Further antimicrobial study for other microbial strains can produce significant results.

Antimicrobial activity is difficult to correlate to a specific compound due to their complexity and variability. It has been mainly explained through C10 and C15 terpenes with aromatic rings and phenolic hydroxyl groups able to form hydrogen bonds with active site of target enzymes, although other active terpenes, as well as alcohols, aldehydes and esters can contribute to the overall antimicrobial effect of essential oils[20]. Therefore, the antibacterial results observed in this investigation might be

related to the presence of γ-gurjunene, terpinyl acetate, hinesol, 10-*epi*-γ-eudesmol, α-guaine and *epi*-cubebol, although the synergistic effects of the diversity of major and minor constituents present in the oil should be taken into consideration to account for their biological activity.

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