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Original Article

Essential Oil and Bioactivity of the *Ziziphora canescens* Benth. Growing Wild in Lebanon

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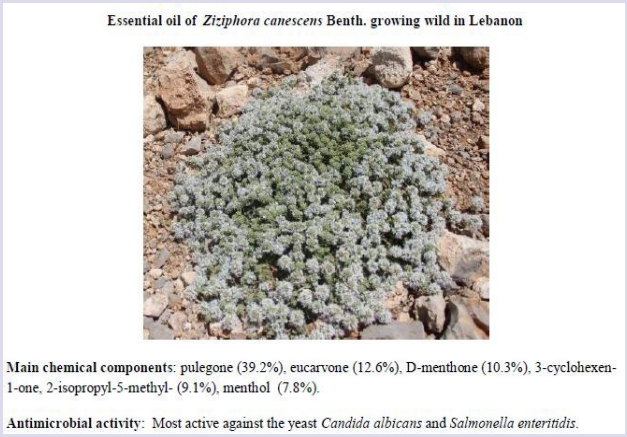
ABSTRACT

Introduction: *Ziziphora canescens* Benth. (*Lamiaceae*) is a medicinal plant widespread species in Mediterranean countries. This study was designed to evaluate the chemical composition and *in vitro* antimicrobial activities of this species growing wild in Lebanon. **Material and Methods:** The essential oil of the dry aerial flowering parts of *Z. canescens* from the Lebanese side of Mount Hermon (1843 m altitude above sea level) was obtained by hydrodistillation. The GC-MS analysis of the obtained essential oil was performed. The antimicrobial activity of the oil was tested against six certified bacteria and two certified fungi using disc-diffusion method and determination of the minimal inhibitory concentration (MIC) by agar dilution method. **Results and Discussion:** The yield of essential oil was 0.6ml/100g dry weight. GC -MS analysis revealed twenty eight components representing 93.0% of the total essential oil. Oxygenated monoterpenes (80.6%) were the predominant fraction of the essential oil with pulegone (39.2%), eucarvone (12.6%), D-menthone (10.3%), 3-cyclohexen-1-one, 2-isopropyl-5-methyl- (9.1%). The oil exhibited a broad-spectrum of antimicrobial activity against the tested pathogenic microorganisms. The yeast Candida albicans and Salmonella enteritidis were the most susceptible with growth inhibition zones of 40.5 ± 0.7 mm and 29.0 ± 0.7 mm, respectively, and MIC values of 5µg/ml. These values were similar to those of pulegone and each the antibiotics Nystatine and Amikacine, respectively whilst, the fungus *Trichophyton mentagrophytes* was the most resistant. **Conclusion:** The results of this study support the notion that the essential oil of *Z. canescens* Benth. may be important as a pharmaceutical and food preservative.

Key words: Antimicrobial activities, Chemical composition of essential oil, Eurcarvone, Pulegone, *Ziziphora canescens* Benth.

SUMMARY

The chemical composition and *in vitro* antimicrobial activities of *Ziziphora canescens* Benth. growing wild in Mount Hermon, Lebanon were investigated. The essential oil from the dry aerial flowering parts of the plant yielded 0.6ml/100g dry weight. GC and GC-MS analyses revealed twenty eight components representing 93.0% of the total essential oil. Oxygenat ed monoterpenes forming the major compounds were pulegone (39.2%), eucarvone (12.6%), D-menthone (10.3%), 3-cyclohexen-1-one, 2-isopropyl-5-methyl (9.1%), menthol (7.8%) and 1,8-cineole (3.2%). Penthylthiophene (3.6%), L- limonene (2.9%) and 1-β-pinene (2.3%) were the main of the monoterpene hydrocarbons, while germacrene D (0.5%), cedrene (0.2%) and calarene (0.2%) were the major identified sesquiterpenes. The oil exhibited a broad-spectrum of antimicrobial activity against the tested pathogenic microorganisms. The yeast *Candida albicans* and *Salmonella enteritidis* were the most susceptible with growth inhibition zones of 40.5 ± 0.7 mm and 29.0 ± 0.7 mm, respectively, and MIC values of 5 µg/ml. These values were similar to those of pulegone and both the antibiotics Nystatine and Amikacine. whilst, the fungus *Trichophyton mentagrophytes* was the most resistant.



PICTORIAL ABSTRACT

Abbreviations used:
MIC: Minimal Inhibitory Concentration, GC: Gas Chromatography, GC-MS: Gas Chromatography-Mass Spectrometry

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INTRODUCTION

The genus *Ziziphora* L. belonging to the Lamiaceae family consists of 10 species (*Z. brantii, Z.clinopodioides, Z. capitata, Z.tenuior, Z. hispanica, Z. persica, Z. puschkinii, Z.raddei, Z.taurica, Z.woronowii)* is widespread in East Mediterranean countries and south Europe.[1] Three of these species are cited in the flora of Lebanon.[2]

The species *Z. canescens* Benth., a synonym of *Z. clinopodioides* Lam. and *Z. clinopodioides* subsp. *Clinopodioides*,[3] locally known as Na'ana'a Jurdi is an edible medicinal species and is commonly used by rural people in Lebanon alone or with *Origanum syriacum, O. Ehrenbergii, Thymbra spicata* and *Satureja thymbra* as a herbal tea and a food additive to offer aroma and flavor.[4,5] The aromatic subfrutescent, subshrub *Ziziphora clinopodioides* has been widely used in Arabic and folk medicine in the region to treat various ailments and as a sedative, expectorant to treat cough, bronchitis, common cold, influenza, inflammation, stomach trouble, aerocoly, diarrhea, wounds and inflammation.[4-6]

Several studies on *Ziziphora clinopodioides* and its subsp. and ecotypes have shown that the main constituents in the essential oil of this species are pulegone, isomenthone, menthol, menthone, 1,8-cineol, thymol, p-cymene, carvacrol, terpinen-4-ol, linalool, piperitone, menth-3- en- 8-ol, limonene and β- pinene.[7-14]

To our knowledge *Z. canescens* Benth. which grows wild in Lebanon, has not yet been investigated. The objective of the present study was to identify the essential oil constituents *in vitro* and assess its antibacterial and antifungal activities.

MATERIAL AND METHODS

Plant material

The aerial parts of *Z. canescens* were collected during the flowering stage from the Lebanese side of Mount Hermon (33º 26' 24"N, 35º 50' 48"E) at 1843m altitude above sea level, in July 2014. The species identification was performed using the determination keys of Mouterde.[2] Nomenclature and plant family delimitation was based on Euro+Med.[1] A voucher specimen (RCED92) was deposited at the herbarium of the Research Center for Environment and Development, Beirut, Arab University, Lebanon).

Bacterial and fungal strains

Certified bacterial and fungal stains (Medi Mark, Europe) were used in the study. They were three Gram positive: *Bacillus cereus (*ATCC 10876*); Staphylococcus aureus* (ATCC-1026); *Enterococcus faecalis* (ATCC 29212), three Gram negative bacteria: *Salmonella enteritidis* (ATCC 13076); *Escherichia coli* (ATCC 8739); *Pseudomonas aeruginosa* (ATCC 9207), one yeast: *Candida albicans* (ATCC 10231) and one fungus: *Trichophyton mentagrophytes* (ATCC 9533).

Essential oil isolation

The essential oil of dried aerial parts (leaves and flowering tops) was hydrodistillled using a Clevenger-type apparatus for three hours. The oil was then dried over anhydrous sodium sulphate overnight and stored in sterile sealed vials at 4ºC.

GC and GC-MS analyses

GC and GC-MS analyses of the oil were performed by Agilent Technologies 7890 gas chromatography equipped with a Flame Ionization Detector (FID) and a HP- 5 MS 5% capillary column (30 m x 0.25 mm x 0.25 µm film thickness). Mass spectra were recorded at 70 eV of electron energy and a mass range of 50-550 m/z. The carrier gas was Helium at a flow of 0.8 ml/min. The initial column temperature was 60ºC programmed to increase to 280ºC at a rate of 4ºC/min. The split ration was 1:40. The injector temperature was set at 300ºC. The purity of helium gas was 99.99%. A sample of 1 ml was injected manually in the split mode.

Components identification was based on retention indices and comparison with mass spectral data of authentic standards and computer matching with Wiley 229, NIST 107, NIST 21 libraries as well as by comparing the fragmentation patterns of the mass spectra with those reported in the literature.[15] Quantitative data were obtained by FID relative area percentages.

Antibacterial and antifungal activity tests

Two different methods were applied for the evaluation of the antimicrobial activity:

Disc diffusion method

The antibacterial and antifungal activity of essential oil was carried out by disc diffusion method using 100µl of suspension containing 10^8 CFU/ml of bacteria spread on Muller-Hinton agar medium (Merck). Sterile 6 mm diameter filter paper discs (Whatman No. 3) were impregnated with 10 µl of essential oil and were placed on the agar. Standard reference discs of the antibiotics erythromycin 30 µg/disc, amikacin 30 µg/disc and nystatin 10µg/disc, were used as positive controls. Each test was run in triplicate and the mean values were considered. A blank disc was used as a negative control. The bacterial cultures were incubated at 37ºC for 24 hrs. Whereas *Candida albicans* and *Trichophyton mentagrophytes* were incubated at 27ºC for 48 hrs and 5 days, respectively. The diameters of growth inhibition zones around discs were measured using a caliper.

Table 1: Chemical composition by GC and GC-MS of the essential oil of Ziziphora canescens Benth

No.	Retention Index	Compounds	Composition(%)
		Oxygenated Monoterpenes	
1	9.2981	3-Octanol	0.1
2	10.2308	1,8-Cineole	3.2
3	16.1588	D-Menthone	10.3
4	16.874	Isopulegone	1.1
5	18.0756	Menthol	7.8
6	20.2958	cis-Carvotanacetol	0.1
7	22.802	Pulegone	39.2
8	24.0952	3-Cyclohexen-1-one, 2-isopropyl-5-methyl-	9.1
9	25.6916	Seudenone	0.2
10	27.3624	Bornyl acetate	0.1
11	33.153	Eucarvone	12.6
	Total		**80.6**
	Monoterpene Hydrocarbons		
12	7.4442	α-Pinene	0.8
13	7.8448	Camphene	0.2
14	9.4126	l-β-Pinene	2.3
15	11.5411	l-Limonene	2.9
16	12.8801	α-Terpinolene	0.8
17	15.3806	Pentylthiophene	3.6
18	20.9767	β-Cymene	0.3
19	26.2237	1-Menthene	0.1
	Total		**11.0**
	Sesquiterpenes Hydrocarbons		
20	32.5694	Bicycloelemene	0.1
21	34.595	β-Bourbonene	0.1
22	35.6535	Calarene	0.2
23	35.9339	β-Cubebene	0.1
24	37.1641	Germacrene D	0.5
25	37.4845	Cedrene	0.2
26	39.8821	(+)-Epi-bicyclosesquiphellandrene	0.2
	Total		**1.4**
	Grand Total		**93.0**

Minimum inhibitory concentration (MIC) by agar dilution method (NCCLS, 1997)

MICs were determined by agar dilution method approved by NCCLS.[16] A series of four concentrations of oil (5, 10, 25 and 50 µl/ml) were prepared into 10 ml of Muller Hinton broth with 0·5% (v/v) Tween-20 to enhance oil solubility. The essential oil and Tween-20 (Sigma) were added to the broth on cooling after autoclaving. The mixtures were poured into plates aseptically in a laminar flow cabinet were allowed to dry at 37°C for 30 min prior to inoculation with 100 µl of microbial suspension containing approximately 10^4 CFU of each microorganism. Mueller Hinton agar, with 0·5% (v/v) Tween-20 with no oil, was used as a positive growth

Table 2: Comparison between yield and composition of essential oil of *Ziziphora clinopodioides* cited from other geographical origins

Species or subspecies	Essential oil yield %	Chemical composition %	Country	Citation
Z. clinopodioides subsp. canescens (syn. Z. canescens)	0.6	Pulegone (39.2), eucarvone (12.6), D-menthone (10.3), 3-cyclohexen-1-one, 2isopropyl-5-methyl (9.1), menthol (7.8), 1,8-cineol (3.2), germacrene D	Lebanon	Current
Z. clinopodioides subsp. rigida	1.0	Pulegone (45.8), piperitenone (17.4), *p*-menth-3-en-8-ol (12.5)	Iran	7
Z. clinopodioides subsp. bungeana	1.0	Pulegone (65.2), isomenthone (11.9), 1,8-cineole (7.8), piperitenone (6.5)	Iran	8
Z. clinopodioides	0.4	Pulegone (31.86), 1,8-cineole (12.2), limonene (10.5)	Turkey	9
Z. clinopodioides	N.R.	Pulegone (36.45), piperitenone (19.12), menth-2-en-8-ol (5,31), carvacrol (5.10), neomenthol (4.78), menthone (4.46)	Iran	10
Z. clinopodioides	0.44-0.90%	Pulegone (7.258-16.038), L- menthone (2.148-2.248), α-fenchene (2.714), L. (-)-menthol (1.526-189.0),1,8-cineole (0.211-0.220)	Iran	11
Z. clinopodioides	0.96	Pulegone (34.4), piperitinone (15.1), 1,8-cineol (6.5), neo-menthol (5.8), menth-2.en-1-ol (5.3), menthol (5.2), menthone (4.5)	Iran	12
Z. clinopodioides	0.30	Pulegone (33.27), piperitone (14.28), limonene (10.66)	Turkey	13
Z. clinopodioides	N.R.	Pulegone (44.5), terpineol (14.5), methyl acetate (10.9), iso-neomenthol (7.1), 1,8-cineole (4.1)	Iran	14
Z. clinopodioides	1.861	Pulegone (61.67), cis-ciaran trans-2-ol (12.66), 1,8-cineole (10.23), 2-B-pinene (2.16)	Iran	17
Z. clinopodioides subsp. rigida	N.R	Pulegone (0.7-44.5), 1,8-cineole (2.1-26.0), neomenthol (2.5-22.5)	Iran	18
Z. clinopodioides (four ecotypes)	0.4-1.0	Pulegone (22.3-60.1), 1,8-cineole (6.3-29.9), *p*-menth-3-en-8-ol (tr-14)	Iran	20
Z. clinopodioides (2 Locations)	N.R.	Thymol (53.6%), p-cymen (10.5), carvacrol (8.7), γ-terpinene (6.7), 1,8-cineole (5.4) 1,8-cineole (21.6), terpinen-4-ol (18.2), linalool (7.9), pulegone (7.7), α- terpineol (5.3)	Iran	19,21

N.R. Not reported

Table 3: The zone of inhibition of antimicrobial activities (mm) of the essential oils of *Ziziphora canescens* Benth

Microorganisms	Zone of Inhibition (mm)						
	Essential oil 10µl/disc	Main compounds			Antibiotics		
		Pulegone 10 µg/disc	Menthone 10 µg/disc	Eucarvone 10 µg/disc	Erythromycine 30 µg/disc	Amikacine 30 µg/disc	Nystatine 100 µg/disc
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
S. enteritidis	29.0 ± 0.7	30.5 ± 0.7	4.5 ± 0.70	15.5 ± 0.7	NA	24.2 ± 1.2	NT
E .coli	11.0 ± 1.4	34.5 ± 0.7	2.5 ± 0.70	16.0 ± 0.0	NA	24.5 ± 0.7	NT
P. aeruginosa	9.4 ± 0.9	26.5 ± 2.1	1.0	3.5 ± 2.1	29 ± 1.41	23.5 ± 2.1	NT
B. cereus	10.5 ± 0.7	39.5 ± 0.7	3.5 ± 2.12	17.0 ± 0.0	13 ± 1.41	22.0 ± 1.4	NT
S. aureus	18.8 ± 1.1	29.3 ± 1.0	2.5 ± 0.70	15.8 ± 0.4	NA	11.7 ± 0.4	NT
E. faecalis	17.8 ± 0.4	34.8 ± 0.4	2.0	9.7 ± 0.4	NA	20.5 ± 0.7	NT
C. albicans	40.5 ± 0.7	38.0 ± 0.0	5.0	20.5 ± 0.7	NT	NT	38.5 ± 2.12
T. mentagrophytes	NA	21.5 ± 2.1	NA	3.7 ± 1.8	NT	NT	NA

NA: Not active, NT: Not tested

control. Plates inoculated with bacteria were incubated at 37°C for 24 hrs and plates inoculated with *Candida albicans* and *Trichophyton mentagrophytes* were incubated at 27°C for 48 hrs and 5 days respectively. MICs were determined as the lowest concentration of oil inhibiting the visible growth of each microorganism on the agar plate.

RESULTS AND DISCUSSION

Chemical composition of the essential oil

The hydrodistillation of the flowering aerial parts of *Z. canescens* Benth. in this study yielded 0.6% of essential oil. As shown in Table 1, the GC-

Table 4: The MIC Antimicrobial activities of the essential oils of *Ziziphora canescens* Benth

Microorganism	MIC			
	Essential oil concentration µg/ml	Main compounds		
		Pulegone µg/ml	Menthone µg/ml	Eucarvone µg/ml
S. enteritidis	5	5	50	10
E. coli	25	5	50	10
P. aeruginosa	25	10	NA	50
B. cereus	25	5	50	10
S. aureus	10	5	10	5
E. faecalis	10	5	NA	25
C. albicans	5	5	25	10
T. mentagrophytes	NA	50	NA	25

MS analysis of the essential oil led to the identification of 28 different components representing a total of 93.0% of the essential oil. The oil was typically a complex mixture of mainly oxygenated monoterpenes (80.6%), monoterpenes hydrocarbons (11.0%) and oxygenated sesquiterpenes (1.4%). The other compounds were identified in minor concentrations of (+)-epi-bicyclosesquiphellandrene 0.2%, cis-carvotanacetol, 0.1% and bornyl acetate 0.1%.

The oxygenated monoterpenes forming the major compounds were pulegone (39.2%), eucarvone (12.6%), D-menthone (10.3%), 3-cyclohexen-1-one, 2-isopropyl-5-methyl (9.1%), menthol (7.8%) and 1,8-cineole (3.2%). Penthylthiophene (3.6%), L-limonene (2.9%) and 1-β-pinene eole (2.3%) were the main compounds of the monoterpene hydrocarbons, while germacrene D (0.5%), cedrene (0.2%), bicycloelemene (0.1%) were the identified sesquiterpenes.

In accordance with published literatures on *Z. clinopodioides* and its subspecies from other countries, the essential oil of *Z. canescens (Z. clinopodioides* subsp. *clinopodioides*) in this study was found rich in pulegone (39.2%) which is recognized as a chemical characteristic of the genus *ziziphor*a. This high content of pulegone was, however, relatively higher than *Z. clinopodioides* from Turkey (31.86% and 20.18%).[9,13] and Iran (36.45%).[10] Nevertheless, a review of studies on *Z. clinopodioides* subsp. *bungeana* (65.2%)[8] and Z. *clinopodioides* subsp. *rigida* (45.8%) both from Iran revealed higher pulegone levels.[7]

The remaining major compounds of *Z. canescens* oil in this study also qualitatively and quantitatively varied from the other studies (Table 2). Eucarvone (12.6%), D-menthone (10.3%), 3-cyclohexen-1-one, 2-isopropyl-5-methyl- (9.1%) and menthol (7.8%) have not been reported among the major components of *Z. clinopodioides* from other countries [8,14,17] neither in *Z. clinopodioides* subsp. *bungeana,*[8] nor in Z. *clinopodioides* subsp. *rigida*[7,18] from Iran. The moderate level of 1,8-cineole (3.2%) in this study was, apparently, considerably less than Z. *clinopodioides* from Turkey (12.2%)[9] and Iran (10.23% and 21.6%)[17,19] and the subspecies *Z. clinopodioides* subsp. *bungeana* (7.8%)[8] and Z. *clinopodioides* subsp. *rigida*[18] from Iran. These differences may be due to genetic differences as well as edaphic and environmental factors like geographic origin, altitude, temperature, among others which play key roles in determining the chemical composition of Z. *clinopodioides* growing wild.

Antibacterial and antifungal activities

The antibacterial and antifungal activities of *Z. canescens* essential oil were in vitro tested by agar diffusion method against six bacteria, one-yeast and one fungus (Table 3). According to the results, the essential oil was found most active against *Candida albicans* (40.5 mm) followed by *Salmonella enteritidis* (29 mm), *Staphylococcus aureus* (18.8 mm), *Enterococcus faecalis* (17.8 mm) , *Escherichia coli* (11 mm) and *Pseudomonas aeruginosa* (9.4 mm). No activity was observed against *Trichophyton mentagrophytes*.

Among the three tested main compounds of the oil, pulegone exhibited the highest antimicrobial activity against all tested microorganisms causing inhibition zones in the range 26.5-39.5 mm. With the exception to *Candida albicans* and *Salmonella enteritidis*, the activity of pulegone was much higher than that of the essential oil. In addition, moderate inhibitory activity was recorded against eucarvone whereas that of menthone was the weakest.

Compared to the positive control, the antimicrobial activity of the essential oil against *S. enteritidis* and *S. aureus* on one hand and *C. albicans* on another was higher than amikacine (30 µg/disc) and nystatine (100 µg/disc), respectively.

As indicated in Table 4, the results obtained by MIC values of the essential oil and its three main compounds (pulegone, menthone, eucarvone) show that the MIC values of the essential oil were in the range 5-25 µg/ml and that those of the three tested monoterpenes were in the range of 5-50 µg/ml (pulegone and eucarvone) and in the range of 25-50 µg/ml for menthone. Table 4 also shows that *C. albicans* and *S. enteritidis* were the most sensitive to the oil having the lowest MIC values of 5 µg/ml, followed by *S. aureus* and *E. faecalis* (10 µg/ml) and *E. coli and P. aeruginosa* (25 µg/ml). No activity was observed against *T. mentagrophytes* which was also resistant to menthone.

Based on these results, it can be concluded that *Z. canescens* essential oil seems to be a valuable source for antibacterial drugs especially against *S. enteritidis and C. albicans* which is in accordance with the traditional uses of *Z. canescens* by rural communities of Mount Hermon. The antimicrobial activity of the oil is likely due to pulegone and its possible association with the other tested main monoterpenes. The varying degree of sensitivity of tested microorganisms to the oil may be due to their intrinsic tolerance and the nature of oil compounds as well as their combinations.

CONCLUSION

This is the first study to provide details on the chemical composition of essential oil of *Z. canescens* from Lebanon and its *in vitro* antibacterial and antifungal activities against pathogenic microorganisms. The findings demonstrate the occurrence of pulegone, eucarvone and menthone as the major compounds which is indicate that the studied *Z. canescens* is different from the other chemotypes described in earlier studies of *Z. canescens* species or subspecies as pulegone, piperitone, limonene in *Z. clinopodioides*[13] or pulegone, piperitenone[12] (Table 2).

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CONFLICT OF INTEREST

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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