Antibacterial Activity and Composition of the Essential Oil of *Ziziphora clinopodioides* subsp. *bungeana* (Juz.) Rech. f. from Iran

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The chemical composition of the essential oil obtained from the aerial flowering parts of *Ziziphora clinopodioides* subsp. *bungeana* (Juz.) Rech. f. was analyzed by GC and GC-MS. Thirty-two components representing 97.1% of the total oil were identified. Oxygenated monoterpenes (94.3%) were the predominant fraction of the oil with pulegone (65.2%), *iso*-menthone (11.9%), 1,8-cineole (7.8%) and piperitenone (6.5%) as the main constituents. Antibacterial activity of the oil and also its two main components (pulegone and 1,8-cineole) were tested against seven bacteria. It was found that the oil exhibited interesting antibacterial activity against *Staphylococcus epidermidis, S. aureus, Escherichia coli* and *Bacillus subtilis* with MIC values of 3.75 mg/ml.

Key words: Essential Oil Composition, Antibacterial Activity, *Ziziphora*

Introduction

The genus *Ziziphora* L. (Lamiaceae) consists of four species (*Z. clinopodioides* Lam., *Z. capitata* L., *Z. persica* Bunge. and *Z. tenuior* L.) widespread all over Iran (Mozaffarian, 1996). *Z. clinopodioides* Lam. comprises nine subspecies native to Iran. *Z. clinopodioides* subsp. *bungeana* (Juz.) Rech. f. grows wild in the eastern parts of Iran (Rechinger, 1982). In Iranian folk medicine, *Ziziphora* species have been used as infusion, decoction and maceration for various purposes such as sedative, stomach tonic, heart disorders, common cold, inflammation, depression, diarrhea, expectorant, coughing, antiseptic, migraine, fever and carminative (Zargari, 1995; Naghibi *et al.,* 2005). In Iranian folklore, the dried aerial parts of aforementioned species have been frequently used as culinary and spice in food.

The antibacterial activity of the oil of *Z. taurica* subsp. *clenioides* and *Z. taurica* has already been studied (Tumen and Ayhan, 1992). A literature survey showed that the oil of *Ziziphora* species has been found to be rich in pulegone. The main constituents found in the oil of *Z. vychodceviana* and *Z. persica* collected from Kazakhestan were pulegone (57.5%–66%) and isomenthone (5.1%–15.7%) (Dembistikii *et al.,* 1995). The major constituent found in the oil of *Z. tenuior* L. has been reported to be pulegone (87.1%) (Sezik *et al.,* 1991). The essential oil of Turkish endemic *Z. taurica* subsp. *clenioides* was found to contain pulegone (81.9%), limonene (4.5%) and piperitenone (2.3%) (Meral *et al.,* 2002). Here, we report the composition and antibacterial activity of the essential oil of *Ziziphora clinopodioides* subsp. *bungeana* from Iran which has not been investigated previously.

Material and Methods

Plant material

The aerial parts of *Z. clinopodioides* subsp. *bungeana* were collected during its flowering stage in

June 2005 from Khorasan Province: Bejestan-Ferdows road, Serideh village, at an altitude of 1300 m. A voucher specimen (MP-921) was deposited in the Medicinal Plants and Drugs Research Institute Herbarium (Tehran, Iran).

Essential oil isolation

Air-dried plant material (100 g) was hydrodistilled for 3 h using a Clevenger type apparatus. The oil was dried over anhydrous sodium sulfate and was kept in a sealed vial at 4 °C until analysis and tests.

Essential oil analysis procedure

GC analysis of the oil was conducted using a Thermoquest-Finnigan instrument equipped with a DB-1 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 μm). Nitrogen was used as the carrier gas at the constant flow of 1.1 ml/min. The oven temperature was held at 60 °C for 1 min, then programmed to 250 °C at a rate of 4 °C/min, and then held for 10 min. The injector and detector (FID) temperatures were kept at 250 °C and 280 °C, respectively. GC-MS analysis was carried out on a Thermoquest-Finnigan Trace GC-MS instrument equipped with a DB-1 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temperature was raised from 60 °C to 250 °C at a rate of 5 °C/min, and then held at 250 °C for 10 min; transfer line temperature was 250 °C. The quadrupole mass spectrum was scanned over the 45–465 amu with an ionizing voltage of 70 eV and an ionization current of 150 μA.

The constituents of the oil were identified by calculation of their retention indices under temperature-programmed conditions on a DB-1 column for *n*-alkanes (C_6–C_{24}) and the oil under the same conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library (Wiley 7.0) or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those reported in the literature (Adams, 2001; Shibamoto, 1987). Quantitative data were obtained from FID area percentages without the use of correction factors.

Bioassay procedure

The bacterial species used in this study were: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 9372, *Enterococcus faecalis* ATCC 15753, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27852, and *Klebsiella pneumoniae* ATCC 3583. The inhibition effect on bacterial growth was determined by the disc diffusion method (Baron and Finegold, 1990). The essential oil (10 μl) and the pure constituents (10 μl of 10% solution of thymol in MeOH, and 10 μl of pure

Table I. Essential oil composition of *Z. clinopodioides* subsp. *bungeana* from Iran.

Compound	RI	%	Identification method[a]
α-Thujene	0924	t[b]	RI, MS
3-Methyl-cyclohexane	0926	t	RI, MS
α-Pinene	0934	0.5	RI, MS, Co-I
Camphene	0948	0.2	RI, MS, Co-I
1-Octen-3-ol	0961	t	RI, MS
Sabinene	0969	0.3	RI, MS
β-Pinene	0975	0.7	RI, MS, Co-I
Myrcene	0981	0.3	RI, MS, Co-I
α-Terpinene	1012	0.1	RI, MS
p-Cymene	1015	t	RI, MS, Co-I
1,8-Cineole	1027	7.8	RI, MS, Co-I
(*Z*)-β-Ocimene	1036	t	RI, MS, Co-I
γ-Terpinene	1051	0.1	RI, MS, Co-I
cis-Sabinene hydrate	1057	t	RI, MS
p-Mentha-3,8-diene	1061	t	RI, MS
Terpinolene	1081	0.1	RI, MS, Co-I
1-Octen-3-yl acetate	1090	t	MS
Menthone	1142	0.5	RI, MS
iso-Menthone	1154	11.9	RI, MS
iso-Isopulegol	1159	0.7	RI, MS
Terpinen-4-ol	1168	0.6	RI, MS
neo-Menthol	1177	t	RI, MS
neoiso-Menthol	1198	0.3	RI, MS
Pulegone	1238	65.2	RI, MS, Co-I
Piperitone	1249	0.6	RI, MS
Carvone	1255	t	RI, MS, Co-I
Bornyl acetate	1277	0.1	RI, MS
Carvacrol	1282	0.1	RI, MS, Co-I
Piperitenone	1328	6.5	RI,MS
β-Bourbonene	1390	0.1	RI, MS, Co-I
Germacrene-D	1484	0.3	RI, MS
Bicyclogermacrene	1498	0.1	RI, MS
Monoterpene hydrocarbons		2.3	
Oxygenated monoterpenes		94.3	
Sesquiterpene hydrocarbons		0.5	
Total identified		97.1	

[a] RI, retention indices relative to C_6–C_{24} *n*-alkanes on a DB-1 column; MS, mass spectroscopy; Co-I, co-injection with authentic compound.
[b] t, trace (< 0.1%).

1,8-cineole and pulegone) were applied on the paper discs (the disc diameter was 6 mm). Then disc papers were placed in the inoculated plates. After 24 h of incubation at 37 °C the diameters of inhibition zones were measured.

For the determination of MIC (minimum inhibitory concentration) values, a microdilution broth susceptibility assay was used as recommended by NCCLS (1999). A standard reference antibiotic (ampicillin) was used as positive control. All experiments were performed in triplicate.

Results and Discussion

Essential oil analysis

The essential oil obtained from hydrodistillition of the aerial flowering parts of *Z. clinopodioides* subsp. *bungeana* yielded 1.0% [(w/w) based on the dry weight of the plant] of a yellow oil. Thirty-two components were characterized representing 97.1% of total oil. The percentage composition of the essential oil is presented at Table I, where all compounds are listed in order of their elution on a DB-1 column. As can be seen at the end of Table I, the classification of the identified compounds based on their functional groups is summarized and it was found that oxygenated monoterpenes (94.3%) were the main group. The major constituents were pulegone (65.2%), *iso*-menthone (11.9%), 1,8-cineole (7.8%) and piperitenone (6.5%). The result of this research is in accordance with earlier studies on *Ziziphora* species all found to be rich in pulegone as the major component, and it may be considered as a chemical characteristic of the genus *Ziziphora* (Sezik *et al.,* 1991; Dembistikii *et al.,* 1995; Meral *et al.,* 2002). Compared to other *Ziziphora* species, the pulegone content of the essential oil of *Z. clinopodioides* subsp. *bungeana* (65.2%) was lower than that of *Z. tenuior* (87.1%) (Sezik *et al.,* 1991), *Z. taurica* subsp. *clenioides* (81.9%) (Meral *et al.,* 2002), *Z. persica* (66.0%) and higher than that of *Z. vychodceviana* (57.5%) (Dembistikii *et al.,* 1995). Piperitenone (6.5%) as another major oil component of *Z. clinopodioides* subsp. *bungeana* was also found in the oil of *Z. taurica* subsp. *clenioides* (2.3%).

Antibacterial activity

As shown in Table II the results obtained from the disc diffusion method followed by the measurement of MIC values indicated that *S. epidermidis*, *B. subtilis*, *E. coli* and *S. aureus* were the most sensitive microorganisms with the lowest MIC value of 3.75 mg/ml in the presence of the essential oil isolated from *Z. clinopodioides* subsp. *bungeana*. Moderate inhibitory activity of the oil was determined against *E. faecalis* and *K. pneumoniae* with MIC values higher than 15 mg/ml. No activity was observed against *P. aeruginosa* which was resistant to the oil and also its two main components. Compared to the positive control, ampicillin, in many cases except for *P. aeruginosa*, the oil showed higher antibacterial activity.

For investigation the responsible activity of the major compounds, two monoterpenes 1,8-cineole and pulegone, was tested against the test bacteria. From our results, it may be concluded that the antibacterial activity of the oil may in part be associated with the presence of these two monoterpenes,

Table II. Antibacterial activity of the essential oil of *Z. clinopodioides* subsp. *bungeana* and its two main components.

Microorganism	Essential oil (10 μl/disc)		Main compounds (10 μl/disc)				Ampicillin (10 μg/disc)
			1,8-Cineole		Pulegone		
	IZ [mm]	MIC [mg/ml]	IZ [mm]	MIC [mg/ml (mM)]	IZ [mm]	MIC [mg/ml (mM)]	IZ [mm]
Bacillus subtilis	20 ± 0.2	3.75 ± 0.2	25 ± 0.6	0.9 (5.84) ± 0.2	16 ± 0.3	3.6 (23.64) ± 0.3	14 ± 0.4
Staphylococcus epidermidis	22 ± 0.3	3.75 ± 0.1	18 ± 0.2	1.8 (11.68) ± 0.4	19 ± 0.1	1.8 (11.82) ± 0.3	19 ± 0.5
Staphylococcus aureus	19 ± 0.1	3.75 ± 0.3	15 ± 0.1	3.6 (23.36) ± 0.3	18 ± 0.2	1.8 (11.82) ± 0.3	13 ± 0.3
Enterococcus faecalis	14 ± 0.3	> 15 ± 0.2	10 ± 0.4	7.2 (46.72) ± 0.2	11 ± 0.4	7.2 (47.28) ± 0.3	11 ± 0.3
Klebsiella pneumoniae	11 ± 0.6	> 15 ± 0.3	8 ± 0.1	7.2 (46.72) ± 0.1	13 ± 0.5	7.2 (47.28) ± 0.3	na
Pseudomonas aeruginosa	na	nt	na	nt	na	nt	9.7 ± 0.2
Escherichia coli	20 ± 0.5	3.75 ± 0.1	20 ± 0.1	1.8 (11.68) ± 0.5	12 ± 0.4	7.2 (47.28) ± 0.3	12 ± 0.2

IZ, inhibition zone including diameter of disc (6 mm); MIC, minimum inhibitory concentration; na, not active; nt, not tested; (8–14), moderately active; (> 14), highly active.

which showed the same type of inhibitory properties against test bacteria. Pulegone has a similar structure to carvone which has been shown to affect the cell membrane by dissipation of pH gradient and membrane potential of cells (Burt, 2004).

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