Essential Oil Composition, Antibacterial and Antioxidant Activity of the Oil and Various Extracts of *Ziziphora clinopodioides* subsp. *rigida* (Boiss.) Rech. f. from Iran

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The chemical composition of the essential oil obtained from the aerial parts of *Z. clinopodioides* subsp. *rigida* (Boiss.) Rech. f. was analysed by GC and GC-MS. Thirty-one constituents accounting to 99.5% of the total oil were identified. Oxygenated monoterpenes (93.3%) were the predominant portion of the oil with pulegone (45.8%), piperitenone (17.4%), *p*-menth-3-en-8-ol (12.5%) and thymol (8.0%) as the main constituents. Antibacterial activity of the oil and its two main compounds and various extracts of plant were tested against seven Gram-(+) or Gram-(−) bacteria. It was found that the oil and MeOH extract (M) exhibited interesting antibacterial activity. The samples were also subjected to screening for their possible antioxidant activity by using 2,2-diphenyl-1-picrazylhydrazyl (DPPH) assay. The free radical scavenging activity of MeOH extract (M) was superior to all other extracts (IC_{50}=30.7 μg/ml), while the oil was less effective.

Key words essential oil composition; pulegone; antibacterial activity; antioxidant activity; *Ziziphora clinopodioides* subsp. *rigida*; Labiatae

There is a popularity and scientific interest to screen essential oils and extracts of plants used medicinally in all over the world.[1)] Monoterpenoids, the main volatile constituents of the essential oils, have been used historically in the pharmaceutical, food and perfume industries because of their antibacterial properties, culinary and fragrance, respectively. Antioxidants have been widely used as additives to avoid the degradation of foods. Also, antioxidants have an important role in preventing a variety of lifestyle-related diseases and aging because these are closely related to the active oxygen and lipid peroxidation.[2)] However, there have been concerns about synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) because of their possible activity as promoters of carcinogenesis.[3)] Consequently, there has been much interest in the antioxidant activity of naturally occurring substances.[4,5)]

The genus *Ziziphora* L. belongs to the family Labiatae consists of four species (*Z. clinopodioides* Lam., *Z. capitata* L., *Z. persica* Bunge. and *Z. tenuior* L.) that widespread all over Iran. *Z. clinopodioides* Lam. with the common persian name "*kakuti-e kuhi*" comprised nine subspecies native to Iran. *Ziziphora clinopodioides* subsp. *rigida* (Boiss.) Rech. f., as an endemic subspecies, grows wild in Iran and also Afghanistan, Iraq, and Talish.[6)] In Iranian and Turkish folk medicine, *Ziziphora* species have been used as infusion for various purposes such as sedative, stomachache and carminative.[7)] In Iranian folklore, the dried aerial parts of this plant have been frequently used as culinary and also in cold and cough treatments.[8)] The antibacterial activity of the oil of *Z. taurica* subsp. *clenioides* and *Z. taurica* has been studied.[9)]

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%).[10)] The major constituent found in the oil of *Z. tenuior* L. has been reported to be pulegone (87.1%).[11)] The essential oil of Turkish endemic *Z. taurica* subsp. *clenioides* was found to contain pulegone (81.9%), limonene (4.5%) and piperitenone (2.3%).[12)] The chemical composition of the essential oil of *Z. clinopodioides* from Turkey has been studied by GLC.[13)] Here, we report the composition and antibacterial and antioxidant activity of the essential oil and various extracts of *Ziziphora clinopodioides* subsp. *rigida* from Iran.

MATERIALS AND METHODS

Plant Material The aerial parts of *Z. clinopodioides* subsp. *rigida* were collected during its flowering stage in July 2003 from Tabriz (East Azerbaijan province, Iran) and identified. A voucher specimen was deposited in the Medicinal Plants and Drugs Research Institute Herbarium (Mp-300).

Isolation of the Essential Oil Air-dried plant material (100 g) was hydrodistilled for 3 h using a Clevenger type apparatus. The oil was dried over anhydrous Na_2SO_4 and then was kept in a sealed vial at 4 °C until analysis.

Preparation of Extracts The hot water extract (W) was obtained by maceration of the air-dried and powdered plant material with hot water for 30 min and then held for 24 h at room temperature. The extract was filtered and concentrated on a water bath with the temperature held below 50 °C.

After completion of hydrodistillation, the liquid was separated. From this procedure deodorized hot water extract (DW) was obtained.

A portion of dried plant material (10 g) was extracted by maceration with acetone, ethyl acetate, and methanol for 24 h at room temperature. The MeOH extract (M) was suspended in water and partitioned with ethyl acetate to obtain water soluble (M-W) and water insoluble (M-E) subfractions.

Gas Chromatography Analysis GC analysis of the oil

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was conducted using a Varian CP-3800 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. A polar DB-Wax column (30 m×0.25 mm i.d., film thickness 0.25 μm) was also used for analysis. In this case, the oven temperature was raised from 40 to 250 °C at a rate of 4 °C/min, then held at 250 °C for 10 min with the transfer line temperature adjusted at 250 °C. Helium was used as the carrier gas at a flow rate of 1.1 ml/min; split ratio was, 1/50. The quadrupole mass spectrometer 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 for *n*-alkanes (C_6—C_{24}) and the oil on DB-1 and DB-Wax columns 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 of reported in the literature.[14,15] Quantitative data was obtained from FID area percentages without the use of correction factors.

Test for Antibacterial Activity 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.[16] The essential oil (15 μl) and the pure constituents (10 μl of 10% solution of thymol in MeOH, and 7.5 μl of pure pulegone) and their mixtures (7.5 μl+ 7.5 μl) were applied on the paper discs (the disc diameter was 6 mm). Each concentrated extract was dissolved in its own solvent (100 mg/ml) and 25 μl of each solution was delivered on the disc and left for complete evaporation of the solvent. Then disc papers were placed in the inoculated plates. After 24 h of incubation at 37 °C the diameter of growth inhibition zones were measured.

For the determination of MIC (Minimum Inhibitory Concentration), a microdilution broth susceptibility assay was used, as recommended by NCCLS.[17] Technical data have been previously described.[18] Methanol and DMSO were used as negative controls. Standard reference antibiotics (ampicillin and penicillin) were used as positive control. All experiments were performed in triplicate.

Antioxidant Activity: Free Radical Scavenging Capacity (RSC) RSC was evaluated by measuring the scavenging activity of examined extracts and essential oil on the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical. The DPPH assay was performed as described before.[19] Samples in various concentrations were mixed with 1ml of 90 μM DPPH solution and filled up with 95% methanol to a final volume of 4 ml. The absorbance of the resulting solutions and the blank (with same chemicals, except for the sample) were recorded after 1 h at room temperature against *tert*-butylated hydroxytoluene (BHT) as a positive control. For each sample, three replicates were recorded. The disappearance of DPPH was measured spectrophotometrically at 515 nm on a Shimadzu 2501UV spectrophotometer. The percentage of RSC was calculated using the following equation:

$$\mathrm{RSC}\,(\%)=100\times\left(\frac{A_{\mathrm{blank}}-A_{\mathrm{sample}}}{A_{\mathrm{blank}}}\right)$$

The IC_{50} value, which represented the concentrations of the essential oil and extracts that caused 50% inhibition, was determined by linear regression analysis from the obtained RSC values.

Assay for Total Phenolics Total phenolic constituents of the aforementioned extracts were determined by the literature methods involving the Folin–Ciocalteu reagent and gallic acid as standard.[20] Twenty microliters of extract solution was taken in a cuvette, then 1.58 ml of distilled water and 100 μl of Folin–Ciocalteu reagent were added, and cuvette was shaken thoroughly. After 3 min, 300 μl of the sodium carbonate solution (7% w/v) was added, and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm.

RESULTS AND DISCUSSION

Essential Oil Analysis The hydrodistillition of aerial parts of *Z. clinopodioides* subsp. *rigida* gave an oil in 1.0% (w/w) yield, based on the dry weight of the plant. Thirty-one components were identified representing 99.5% of total oil. The qualitative and quantitative essential oil compositions are presented in Table 1, where compounds are listed in order of their elution on the DB-1 column. The major constituents of the oil were pulegone (45.8%), pipertenone (17.4%), *p*-menth-3-en-8-ol (12.5%), thymol (8.0%), 1,8-cineole (2.7%), neomenthol (2.1%), menthone (1.8%) and isomenthol (1.6%). The result of this research is in accordance with other earlier studies on *Ziziphora* species that all found to be rich in pulegone.[10—12] Compared to the other *Ziziphora* species, pulegone content of the essential oil of *Z. clinopodioides* subsp. *rigida* (45.8%) was lower than those of *Z. tenuior* (87.1%),[11] *Z. taurica* subsp. *cleonioides* (81.9%),[12] *Z. persica* (66%) and *Z. vychodceviana* (57.5%).[10] Piperitenone (17.4%) as the second major oil component of *Z. clinopodioides* subsp. *rigida* was only found in the oil of *Z. taurica* subsp. *cleonioides* (2.3%), while the other major compound, *p*-menth-3-en-8-ol (12.5%), was only reported from *Z. tenuior* essential oil. Thymol (8.0%), as one the main constituents of the oil of *Z. clinopodioides* subsp. *rigida* has not been identified as the oil component of other species.

Antibacterial Activity As can be seen in Table 2, all extracts were found to have moderate to high activity against *B. subtilis*, *S. epidermidis*, and *E. coli*. Water extract (W) and water soluble fraction of methanol extract (M-W) inhibited slightly the growth of *K. pneumoniae* and *E. faecalis*. *P. aeruginosa* was resistant in all experiments. Results obtained from disc diffusion method, followed by measurements of

Table 1. Chemical Composition of the Essential Oil of *Z. clinopodioides* subsp. *rigida*

Compound	RI[a] (DB-1)	RI (DB-wax)	%	Method of identification[b]
α-Thujene	926	1018	0.1	MS, RI
α-Pinene	935	1014	0.3	MS, RI, Co-I
Sabinene	970	1098	0.2	MS, RI
β-Pinene	976	1111	0.6	MS, RI, Co-I
Myrcene	981	1154	0.2	MS, RI
α-Terpinene	1013	1681	tr[c]	MS, RI
p-Cymene	1015	1257	0.3	MS, RI, Co-I
1,8-Cineole	1026	1198	2.7	MS, RI
γ-Terpinene	1053	1208	0.2	MS, RI, Co-I
Terpinolene	1064	1233	0.5	MS, RI
trans-Sabinene hydrate	1082	1707	0.1	MS, RI
Linalool	1085	1562	tr	MS, RI, Co-I
cis-Verbenol	1111	—	0.1	MS, RI
trans-Verbenol	1124	—	0.1	MS, RI
p-Menth-3-en-8-ol	1140	1600	12.5	MS, RI
Menthone	1152	1445	1.8	MS, RI
Neomenthol	1160	1586	2.1	MS, RI
Menthol	1166	1589	0.1	MS, RI, Co-I
Terpinen-4-ol	1171	1580	0.4	MS, RI
Isomenthol	1182	1653	1.6	MS, RI
Verbenone	1186	—	0.2	MS, RI
Cuminyl aldehyde	1202	1984	0.8	MS, RI
Pulegone	1225	1631	45.8	MS, RI, Co-I
Piperitone	1234	1715	1.4	MS, RI
Carvone	1246	—	0.1	MS, RI
Thymol	1268	2178	8	MS, RI, Co-I
Isomenthyl acetate	1280	1547	0.5	MS, RI
Eucarvone	1287	1756	0.2	MS, RI
Piperitenone	1320	1891	17.4	MS, RI
β-Bourbonene	1391	1499	0.1	MS, RI
Germacrene D	1484	1720	1.1	MS, RI
Monoterpene hydrocarbons			5.1	
Oxygenated monoterpenes			93.3	
Sesquiterpene hydrocarbons			1.2	
Total identified			99.5	

a) RI, retention indices relative to C_6—C_{24} *n*-alkanes. *b*) MS, mass spectrum; Co-I, coinjection with an authentic sample. *c*) tr, trace (<0.1%).

Table 2. Antibacterial Activity of the Various Extracts of *Z. clinopodioides* subsp. *rigida*

Microorganism	Inhibition zone (mm)						
	M[a]	M-E[b]	M-W[c]	DHW[d]	W[e]	A[f]	E[g]
Bacillus subtilis	14	14	18	16	13	na	na
Staphylococcus epidermidis	17	15	13	12	15	na	na
Staphylococcus aureus	13	14	na	na	na	na	na
Enterococcus faecalis	na	na	na	na	na	na	na
Klebsiella pneumoniae	na	na	8	na	11	na	na
Pseudomonas aeruginosa	8	na	na	na	na	na	na
Escherichia coli	15	15	na	13	13	na	na

a) Methanol extract (M). *b*) Water insoluble of methanol extract (M-E). *c*) Water soluble of methanol extract (M-W). *d*) Deodorized hot water extract (DHW). *e*) Water extract (W). *f*) Acetone extract (A). *g*) Ethyl acetate extract (E). na, not active; (7—14) moderately active; (>14) highly active.

MIC, indicated that *B. subtilis* was the most sensitive microorganism tested, with the lowest MIC value (3.8 mg/ml) in the presence of the oil isolated from *Z. clinopodioides* (Table 3). Strong inhibition against *S. epidermidis* and *S. aureus* were observed with a similar MIC value (7.5 mg/ml). Moderate inhibitory activity of the oil against *E. faecalis*, *K. pneumoniae*, and *E. coli* were also determined with MIC values equal or bigger than 15 mg/ml. No activity was observed against *P. aeruginosa*. In some cases, the oil showed the same type of antibacterial activity compared to ampicillin, while, penicillin showed higher activity in some other cases than the essential oil (Table 3).

For investigation the responsible activity of the major compounds, two monoterpenes, thymol and pulegone and also their mixture, were tested against the mentioned microorganisms at a concentration corresponding to their percentages in the total oil. From our results, it may be concluded that, the antibacterial activity of the oil, may in part be associated with the presence of thymol and pulegone, which showed the same type of inhibition zones against tested bacteria.

The molecular mechanism of the antibacterial activity of

Table 3. Antibacterial Activity of the Essential Oil of *Z. clinopodioides* subsp. *rigida*

Microorganism	Essential oil		Antibiotics[c]		Main compounds		
	DD[a]	MIC[b]	Pen	Amp	Thymol[d]	Pulegone[e]	Thy+Pul[f]
B. subtilis	18	3.8	22	14	46	14	22
S. epidermidis	16	7.5	13	12	38	16	32
S. aureus	15	7.5	32	13	34	15	20
E. faecalis	12	>15	16	11	25	9	18
K. pneumoniae	11	15	na	12	24	10	12
P. aeruginosa	na	nt	na	9.7	12	na	10
E. coli	15	15.0	8	12	32	9	17

a) Diameter of inhibition zone including diameter of disc 6 mm (tested at a volume of 15 μl/disc). *b*) Minimum Inhibitory Concentration (values in mg/ml). *c*) Tested at a concentration of 10 μg/disc; Pen, Penicillin; Amp, Ampicillin. *d*) Tested at a volume of 10 μl/disc of 10% solution in MeOH. *e*) Tested at a volume of 7.5 μl/disc. *f*) Tested at a concentration of (7.5+7.5) μl/disc. na, not active; nt, not tested; (8—14), moderately active; (>14), highly active.

Table 4. Total Phenolic Compounds and Radical Scavenging Capacity of Various Extracts of *Z. clinopodioides* subsp. *rigida* against DPPH (IC_{50})

Extracts	Gallic acid equivalents (mg/l)[a]	IC_{50} (μg/ml)
Methanol extract (M)	174.8±1.2	30.7±0.6
Water extract (W)	166.2±3.1	34.7±0.3
Water soluble of methanol extract (M-W)	129.2±1.1	42.3±0.6
Water insoluble of methanol extract (M-E)	24.5±2.5	150.0±1.6
Acetone extract (A)	100.1±0.0	46.0±2.0
Ethyl acetate extract (E)	95.1±0.4	47.9±1.3
Deodorized hot water extract (DHW)	26.0±1.1	118.2±2.1
Control (BHT)	—	26.5±1.0

a) Results are given as mean±S.D. of three different experiments.

thymol has already been investigated and shown to cause disintegration of cell membranes.[21] Pulegone has a similar structure to carvone which has been shown that affect the cell membrane by dissipation of pH gradient and membrane potential of cells.[22]

Antioxidant Activity The antioxidant activity of the *Z. clinopodioides* extracts and the essential oil were evaluated in a series of *in vitro* tests. Free radical scavenging capacities of the extracts, measured by DPPH assay, are shown in Table 4 and Fig. 1. The free radical scavenging activity of methanol extract (M) (IC_{50}=30.7 μg/ml) was superior to all other extracts. Polar extracts exhibited stronger antioxidant activity than non-polar extracts. The stronger activity of M should be related to its higher phenolic content (174.8±1.2 mg/l) as measured by gallic acid test (Table 4).

Amount of Total Phenolics Based on the absorbance values of the various extract solutions, reacting with Folin–Ciocalteu reagent and compared with the standard solution of gallic acid equivalents, as described above, the total phenolics content was highest in M (17.5%), followed by water soluble of methanol extract (M-W) (12.9%), water extract (W, 16.6%), acetone extract (A, 3.8%), and ethyl acetate extract (E, 7.6%). The lowest amount of total phenolics was recorded in water insoluble of methanol extract (M-E) (0.5%).

The molecular mechanism of radical scavenging activity of polar extracts from *Z. clinopodioides* could be attributed to the presence of polyphenolic compounds. It has already been shown that polyphenolic compounds were responsible for radical scavenging activity in Lamiaceae family due to ease of their hydrogen atom donation to active free radical.[23]

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