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Elemental Determination and Essential Oil Composition of *Ziziphora clinopodioides* and Consideration of its Antibacterial Effects

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Ziziphora clinopodioides belongs to the Lamiaceae family, that are widespread all over Iran. The leaves, flowers and stems of the plant are frequently used as wild vegetables or additives in food to offer aroma and flavour. The chemical composition of the essential oils obtained from the aerial parts of *Z. clinopodioides* Lam. were analyzed by GC and GC-MS and 29 components were identified. The major components were pulegone (41.5 %), piperitenone (18.54 %), carvacrol (5.21 %) menth-2-en-1-ol (4.29 %) and neomenthol (4.16). The essential oils of *Ziziphora clinopodioides* antibacterial activity were tested against two strains of gram positive bacteria (*Bacillus cereus*, *Staphylococcus aureus*) and two strains of gram negative bacteria (*Escherichia coli*, *Klebsiella pneumonia*). The average MICs and MBCs of essential oils were tested against the organisms by agar dilution. Eight metal elements in *Ziziphora clinopodioides* Lam were determined by FAAS. The work conditions and precision of the method were studied. The results showed that there were comparatively rich metal elements, among which comparatively high are calcium, potassium, sodium and magnesium in *Ziziphora clinopodioides* Lam. It was noted that level of essential elements was high as compared to the level of toxic elements. It provided useful data for discussing the relationship between the content of the metal elements in *Ziziphora clinopodioides* Lam and clinical application of the traditional medicine.

Key Words: *Ziziphora clinopodioides* Lam., Essential oil, GC/MS, Biological activity, Metal elements.

INTRODUCTION

The genus *Ziziphora* L. belongs to the family Lamiaceae, which consists of four species (*Ziziphora clinopodioides*, *Z. capitata*, *Z. tenuir* and *Z. persica*) are widespread all over Iran[1]. *Ziziphora clinopodioides* is an edible medicinal plant, which is widely distributed in the Anatolia. The leaves, flowers and stem of the plant are frequently used as wild vegetables or additives in food to offer aroma and flavour[2]. The plant of *Ziziphora clinopodioides* Lam, which is habitant of Iran, has been used in Iranian Traditional Medicine for treatment of some infectious conditions. In Iranian folklore, the dried aerial parts of this pant have been frequently used as culinary additives and also as a cold and cough treatment[3]. The plant, known locally as 'kakuti-e kuhi', comprises nine subspecies native to Iran[1] and it is used in the preparation of an aromatic tea for gastrointestinal disorders and as carminative, antiseptic.

Volatile oils are a complex mixture of compounds, mainly monoterpenes, sesquiterpenes and their oxygenated derivatives (alcohols, aldehyde, esters, ethers, ketones, phenols and oxides)[4]. Other volatile compounds include phenylpropenes and specific sulphur- or nitrogen-containing substances. Generally, the oil composition is a balance of various compounds, although in many species one constituent may prevail over all others[5].

The aims of this work are to identify of the chemical compositions and elemental determination some element and a brief study of antibacterial activity. Essential oil of *Ziziphora clinopodioides* was obtained by using a Clevenger distillation apparatus. The chemical compositions of the essential oil were evaluated by using gas chromatography flame ionization detector (GC-FID) and gas chromatographymass spectrometry (GC-MS). Some elements were determined by atomic absorption spectrometry (AAS).

EXPERIMENTAL

The aerial parts of *Ziziphora clinopodioides* were collected in the pre-flowering stage in April-May 2011 from the East North of Iran (Bojnord) in North Khorasan Province of Iran and identified. A Voucher specimen (No.2863) has been deposited at the Herbarium of the Islamic Azad University of Mashhad, Iran.

A Hitachi Z-2000 atomic spectrometer (Hitachi, Japan) with a graphite furnace atomizer (a pyro tube) was used for atomic absorption measurements. Zeeman Effect was employed during all experiments. The hollow cathode lamps for determination of elements (Hitachi, Japan) were used as radiation source at their maximum absorption wavelength.

Preparation of the crude extraction: Air-dried, powdered plant material (100 g) was successively extracted (three times) for 24 h at room temperature with methanol (70 % solution in distilled water). The solvent (methanol) was rotary evaporated after filtration of combined extracts and the extract was freeze-dried.

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 sodium sulphate and then was kept in a sealed vial at 4 ºC until analysis.

Preparation plant for elemental analysis: Wet digestion was applied. 2 g of dried (80 ºC) plant material that has been ground (0.5-1.0 mm) and thoroughly homogenized and place in a tall-form beaker or digestion tube 5 mL concentrated HNO_3 and covered beaker on hot plate or digestion tube into block digester and heat at 125 ºC for 1 h. Then cooled and added 2 mL 30 % H_2O_2 and digest at the same temperature until digest is clear. The filtrated solution diluted to100 with distilled water.

Gas chromatography analysis: Gas chromatographic analysis was carried out on Shimadzu GC-14A chromatograph equipped with flame ionization detector fitted with 25 m × 0.22 mm (id.) SE-30 capillary column at carrier gas flow rate of 1 mL/min with split ratio was 1:30 and sample size 0.1 µL. The column temperature was programmed at 70 ºC for 4 min. with 4 ºC/min rise to 210 ºC while detector and injector temperature were maintained at 250 ºC. Percentage composition of individual components was calculated on the basis of peak area using Shimadzu C-R4A chromatopac electronic integrator.

The GC-MS analysis was carried out on a Shimadzu GC-MS model QP 5050. The capillary column was DB-5 (30 mm × 0.2 mm, film thickness 0.32 µm). The initial temperature of column was 60 ºC (held 1 min) and then heated to 200 ºC with a 3 ºC/min rate and then heated to 220 ºC and kept constant for 2 min. The flow rate of helium as carrier gas was 1.0 mL/min. The analysis was used split ratio 1:30. The injector and detector temperatures were both at 250 ºC; volume injected 0.1 µL of the essential oil and ionization potential 70 eV. The same condition of temperature programming used for *n*-alkenes mixture to calculate the retention index (RI).

Identification of components: The linear retention indices for all the compounds were determined by injection of the sample with a solution containing the homologous series of C8-C22 *n*-alkanes[6].

The individual constituents were identified by their identical retention indices, referring to known compounds from the literature[7] and also by comparing their mass spectra with either the known compounds or with the Wiley mass spectral database. The relative percentage of the oil constituent was calculated.

RESULTS AND DISCUSSION

The hydrodistillation of aerial parts of *Z. clinopodioides* Lam. gave an oil in 1.1 % (w/w) yield, based on the dry weight of the plant. Twenty-nine components were identified representing 98 % of the 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-5 column. The major constituents of the oil were pulegone (41.5 %), piperitenone (18.54 %), menth-2-en-1-ol (4.29 %), carvacrol (5.21 %), neomenthol (4.16) and menthone (3.26 %). The result of this research is in accordance with other earlier studies on *Ziziphora* species that all found to be rich in pulegone[8-10]. Compared to the other *Ziziphora* species, pulegone content of the essential oil of *Z. clinopodioides* Lam. (41.5 %) was lower than those of *Z. tenuior* (87.1 %), *Z. taurica* subsp. *cleonioides* (81.9 %),12) *Z. persica* (66 %) and *Z. vychodceviana* (57.5 %)[11]. Piperitenone (19.12 %) as the second major oil component of *Z. clinopodioides* Lam. was only found in the oil of *Z. taurica* subsp. *cleonioides* (2.3 %), while the other major compound was only reported from *Z. tenuior* essential oil.

TABLE-1
CHEMICAL COMPOSITION OF THE ESSENTIAL OIL FROM *Z. clinopodioides* LAM.

No	Compound	Retention index	%
1	α-Thujene	926	0.08
2	α-Pinene	934	0.19
3	Camphene	939	0.24
4	Sabinene	946	0.36
5	1,8-Cineole	1012	2.40
6	γ-Terpinene	1045	1.12
7	Terpinolene	1068	0.19
8	Linalool	1079	0.41
9	Menth-2-en-1-ol	1123	4.29
10	Menthone	1135	3.26
11	Menthofuran	1140	0.21
12	Neomenthol	1145	4.16
13	4-Terpineol	1155	0.35
14	Menthol	1167	3.11
15	Isomenthol	1178	0.98
16	Verbenone	1186	2.10
17	Cuminyl acetate	1197	1.08
18	Pulegone	1219	41.5
19	Isomenthone	1224	0.35
20	Piperitenone	1227	1.86
21	Carvone	1238	0.55
22	Thymol	1245	2.50
23	Carvacrol	1263	5.21
24	Isomenthyl acetate	1275	0.23
25	Piperitone	1291	18.54
26	β-Caryophyllene	1305	0.34
27	Germacrene-D	1354	0.58
28	Bisabolene	1402	0.78
29	Caryophyllene oxide	1478	0.53

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 Kazakhstan were pulegone (57.5-66 %) and isomenthone (5.1-15.7 %)[8]. Also pulegone (79.33 %) and limonene (6.78 %) were the main components in the oil of *Z. persica* collected from Turkey[12]. The main component found in the oil of *Z. tenuior* L. has been reported to be pulegone (87.1 %). The volatile oil of Turkish endemic *Z. taurica* subsp. clenioides was found to contain pulegone (81.9 %), limonene

(4.5 %) and piperitenone (2.3 %)[10]. In the literature there are some reports on the chemical constitutions of *Z. clinopodioides* growing in the former USSR and west part of Turkey[13,14]. According to these studies, major components of the essential oil were pulegone (13.2-21.9 %), isomenthone (2.0-10.8 %), menthone (4.6-5.44 %), limonene (1.8-8.19 %) and 1,8-cineole (2.3-14.5 %). The changes in the essential oil compositions might have arisen from several differences (climatic, seasonal, geographical and geological), as mentioned by Daferera *et al.*[15]. Previous study on analysis of the essential oil of *Z. clinopodioides* subsp. regida collected from Azerbaijan from the northwest of Iran show that pulegone (45.8 %), piperitenone (17.4 %), *p*-mentha-3-en-8-ol (12.5 %) and thymol (8.0 %) were the main constituents[16]. Also pulegone (65.2 %), isomenthone (11.9 %), 1,8-cineol (7.8 %) and piperitenone (6.5 %) were identified as the major components in the oil of *Z. clinopodioides* subsp. *bungeana* (juz) Rech. from Iran[16,17]. The major components of the oil of *Z. clinopodioides* obtained from Turkey were pulegone (31.86 %), 1,8-cineol (12.21 %), limonene (10.48 %), menthol (9.13 %), -pinene (6.88 %) and menthone (6.73 %)[2].

Elemental analysis: After the sample was decomposed and dissolved, the trace elements were determined by atomic absorption spectrometry (AAS) in flame mode (FAAS), only As determined with a graphite furnace device (GFAAS). The standard addition method was used for determination of elements in sample. The results were showed in Table-2. The results showed that there were comparatively rich metal elements, among which are comparatively high calcium, potassium, sodium and magnesium in *Ziziphora clinopodioides* Lam. It was noted that level of essential elements was high as compared to the level of toxic elements.

TABLE-2
DETERMINATION OF ELEMENTS (mg/g) IN DRY
Ziziphora clinopodioides (MEAN ± SD), n = 3

Element	Concentration (mg/g)	Element	Concentration (mg/g)
Na	8.96 ± 0.20	Co	0.014 ± 0.12
K	18.27 ± 0.30	Zn	1.75 ± 0.14
Ca	26. 64 ± 0.24	Cu	6.72 ± 0.25
Mg	3.51 ± 0.23	As	0.065 ± 0.011

Bacterial strains: The bacterial strains that used in this study were obtained from the Biology Laboratory, Department of Biology, Faculty of Basis Sciences, Ferdowsi University of Mashhad, Iran. The essential oil and crude extract from methanol were individually tested against two strains of gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and two strain of gram negative bacteria (*Escherichia coli* and *Klebsiella pneumonia*).

Antibacterial activity: Antibacterial activity of essential oil and crude extract of *Ziziphora clinopodioides* were tested against the above mentioned gram-positive and gram-negative bacteria. Minimal inhibitory conentrations (MICs) were determined by the agar serial dilution method[18] at concentration ranging from 1-300 µg/mL. Two fold serial dilutions were made from essential oil in molten Mueller Hinton agar (Pronadisa- Madrid) cooled to 45-50 ºC in a water bath. The essential oil was dispersed in mixture using dimethyl sulfoxide (DMSO).The amount of 0.01 mL of every bacterial suspension, equivalent to McFarland tube No. 1.5×10^8 (CFU/mL), inoculated on the agar of every well. The culture plates were then incubated at 37 ºC for 24 h. The MIC was defined as the lowest concentration at which no visible growth was observed[19]. The Mueller Hinton agar were contained DMSO without essential oil was used a negative control while gentamycine and kanamycin were used as positive control. As shown in Table-3, the results indicate that essential oil of *Ziziphora clinopodioides* exhibited higher activity against *S. aureus* and *E. coli* than average other tested bacteria. The average MBC of both essential oils against *B. cereus* and *S. aureus* were higher than MIC values. So, the essential oils showed bacteriosatatic activity against tested Gram positive bacteria contrast in gram negative bacteria. The essential oils had bactericidal effects gainst tested Gram negative bacteria (the MIC and MBC values were the same). The microbes were not highly sensitive against the crude extract and sensitivity was found to decrease with increasing concentration of the crude extract.

TABLE-3
AVERAGE MICS AND MBCS OF ESSENTIAL OIL
FROM *Ziziphora clinopodioides* ON TESTED ORGANISMS
BY AGAR DILUTION METHOD

Bacteria	MIC (µg/mL)	MBC (µg/mL)
S. aureus	4.25	90
B. cereus	1.25	4.50
K. pneumonia	7.25	7.25
E. coli	5.75	5.75

Values are mean of triplet experiments.

Conclusion

The results suggest that *Ziziphora clinopodioides* has potent antibacterial effects on some gram-positive and gram-negative bacterial species especially on *S. aureus* and *E. coli*. Gas chromatographic analysis revealed 29 different compounds in essential oil which five of them comprise more than 73 % of oil and pulegon is the highest one.

As it is expected, there are a lot of differences between the types of components and the amount of them in the essential oils of *Ziziphora clinopodioides*. These differences not only depend to the type of spices but might be relate to the regional of the growing of the plant.

Analysis of the elemental composition is accordingly an important tool for relationship between the content of the metal elements in *Ziziphora clinopodioides* Lam and clinical application of the traditional medicine.

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