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Chemical Composition and Antioxidant Activity of Essential Oil and Methanol Extract of Aerial Parts of *Ziziphora clinopodioides* Var. *rigida*

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Abstract

Introduction: Plants are a rich source of phenolic compounds that as natural antioxidants prevent oxidative stress and are very good for health. *Ziziphora clinopodioides* belongs to Lamiaceae family and its aerial parts are used in pharmaceutical and food industries. It is effective in the treatment of heart disorders, cold, depression, diarrhea, coughing, migraine and fever. This study aimed to identify the composition and antioxidant activity of the essential oil and extract of *Ziziphora clinopodioides* as an alternative to synthetic antioxidants.

Method: *Ziziphora clinopodioides* was collected from Bardsir Mountains (Kerman province) and dried in shade. Essential oil was obtained by hydro distillation method using Clevenger apparatus. Essential oil was analyzed using GC/MS apparatus. Methanol extract was concentrated by rotary evaporator. Possible antioxidant activities of the essential oil and extract were studied using beta carotene linoleic acid and DPPH methods.

Results: The major constituents of essential oil were (+) –pulegone (52.41%), dihydrocarvyl acetate (14.13 %), 1.8-cineole (12.98%) and D-neoisomenthol (4.19 %). The extract of flower had the highest antioxidant activity (the least IC_{50}) in DPPH assay while in beta carotene linoleic acid, the essential oil of flower had the highest antioxidant activity.

Conclusion: The main component of essential oil of *Ziziphora clinopodioides* collected from Bardsir (Kerman province) was pulegone. The essential oil and methanol extract of this plant showed remarkable antioxidant activities; therefore, it can be used as an antioxidant in food and pharmaceutical industries.

developed in the left eye. Two patients had no family history suspicious for keratoconus.

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Introduction

Free radicals are powerful reactants that intend to receive electrons and pair them. Thus, they cause other molecules lose their function. (1, 2). Oxidative stress is the outcome of an imbalance between the production of free radicals in body and antioxidant defense mechanisms. In living organisms, free radicals cause peroxidation of lipids in the cell membrane. In this condition, not only the wall structure, but also some products resulting from oxidation like Malondialhyde can react with biomolecules and show cytotoxic and genotoxic effects (3).

Oxidative damage of DNA, proteins, and macromolecules is one of the internal causes of degenerative diseases such as obesity, cancer, cardiovascular disease, immune deficiency and abnormal brain function. Singlet oxygen, a high-energy and mutagenic form of oxygen, can be produced by transfer of energy from light, the respiratory burst from neutrophils, or lipid peroxidation (4). Antioxidants are compounds that prevent lipid oxidation (5). Today, antioxidants are used in industry to delay lipid oxidation which is interesting for researchers because of its undesirable effects and the growing interest for consuming natural compounds (6). The plant phenolic compounds are among the best natural antioxidant sources (7). Today, biological activities of essential oils are more important than the past.

Ziziphora, a genus from lamiaceae family, is annual or perennial herb with wooden stem like *Thymus kotschyanus* (8). Among important genus of lamiaceae family, mint, *Ziziphora*, *Thymus kotschyanus* can be mentioned (9). *Ziziphora* has 4 species of herbaceous and perennial plant which grow in Torkamanestan, Afghanistan, Armenia, Anatolia, Pakistan, central Asia and West ciboria, in addition to Iran (8).

The leaves, flowers and stems of *Ziziphora clinopodidoes* which obtained from natural places are used as natural drug and food additive to make it tasty (9). In most parts of Iran, this plant is consumed with yoghurt and diaries (10).

In Iranian ethnomedicine, *Z. clinopodioides* is used as a stomach boosting and anti-inflammatory agent. Also, it is used in the treatment of heart problems, cold, depression, diarrhea, cough, migraine, and fever. Because of these features, several studies have already been done on its essential oil (11,12). In some parts of Kerman, this plant is used as tea to reduce fatigue and cold symptoms. Different studies have been conducted in different regions to determine the composition of *Z. clinopodioide*. In a study conducted in the north east of Iran (north Khorasan), the main compounds of *Ziziphora*oil were thymol, menthol, borboene, piperitene, pulegone, isomenthol and menthene. The results of the mentioned study indicated that this plant has the maximum growth in low PH soils (11). In another study, the quality and quantity of the *Ziziphora* oil obtained from four regions of Hamedan and Kordestan were studied and in whole 26 compounds were identified in the *Ziziphora clinopodiodes* oil that 15 ones were common in all regions. In three regions, pulegone and in one region, 1,8-cineol were more abundant. In four regions, chemical compositions of essential oil were different. The difference of components was attributed to the effect of different growth conditions (13). In another research on *Ziziphora* by Ozturk and Ercisli (2006), the main components were pulegone, 1,8-cineole, limonene, menthol, β-pinene, menthone, piperitenone and piperitone (14).

In the study of Verdian-Rizi (2008), 26 compounds were identified in *Ziziphora clinopodioides;* pulegone (36.45%), piperitone (9.12%), mentha-2-en-1-ol (5.3%) and carvacrol (5.1%) were the main ones (15). In Shahbazi (2015) study, carvacrol (64.2%), thymol (19.2%), para-cymene (4.8%) and γ-

terpinene (4.6%) were the major components of *Ziziphora clinopodioides* (16).

According to the importance and broad use of this plant, especially in Kerman, we decided to evaluate chemical composition and antioxidant activity of essential oil and methanol extract of aerial parts of *Ziziphora clinopodioides Var. rigida*

Method

To conduct this study, *Ziziphora clinopodiodies* var.*rigida* was gathered in spring and summer in flowering stage from the west of Bardsir in Kerman and dried in shadow. This plant was identified in the Pharmacy faculty of Kerman University of Medical Sciences and the result was confirmed by experts in Kerman University (code: 3511).

Essential oil and extract preparation

To prepare the essential oil, 100 g of dried aerial parts was powdered and subjected to for 2h using Clevenger apparatus. The obtained hydrodistillation essential oil was kept in refrigerator and away from sun.

To provide methanol extract, 100g of the leaves, flowers and stems underwent three stages; first, they were kept for 72 hours in 1 liter methanol 70%. Then, the solution was purified by using watman paper no.1 and concentrated by using rotatory evaporator in+ 40 ˚ C.

Essential oils analysis

To analyze the essential oil, GC andGC/MS methods were used. GC analysis was done by using chromatograph Shimadzu 15A. N2 as the carrier gas (1 mililitre /min) and DB5 column were used. Column temperature was kept at 60 ^{o}C for three minutes and then increased with the speed of 5-220^{o} C and then it got fixed for 5 minutes at 220^{o} C. Relative percent was estimated using chromatopack C-R4A without using correction factor .GC/MS analysis was done by Hewlett-pakard 5973 equipped with HP-5M5 columns. The temperature of column was kept at 60^{o} C for 3 minutes and increased until 220^{o}C, then it got fixed at 220 ^{o}C for 5 minutes. The speed of Helium gas as the carrier was at 70 eV. The identification of constituents was done using mass spectrum (17).

Investigation of antioxidant activity

To investigate the antioxidant activity of *Ziziphora clinopodioides,* two methods were used: 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and β-caratone linoleic acid. These experiments were conducted randomly with 4 concentrations in three repeats.

In DPPH, the activities of hydrogen, electron of extracts were measured by colorless extracts. In this spectrophotometery test, free radicals were used as reagent. 50µL of different extracts were mixed by 5 ml methanol 0.0004 % DPPH. After 30 minutes absorption of samples was measured at 517 nm wavelength. Free radical inhibition was calculated by using the following equation:

$I\% = (A_{blank} - A_{sample}/A_{blank})$

Where $A_{blank}$is negative control absorb (including all reagents, except the defined concentration of the given extract). IC_{50} levels indicate the extracts and essential oils concentration that causes 50 percent inhibition for oxidative products (18). It is obvious that the less value of IC_{50}, the more the power of

free radicals. BHT was used as the control positive and all experiments were performed in triplicate

In β-caraton method, the antioxidant activities of extracts and essential oils are measured by colorless β-caraton/linoleic acid assay. (19).

To prepare the β-carotene/linoleic acid solution, 0.5 mg β-caratone was solved in 1 ml chloroform and 25 µL linoleic acid and 200 mg Tween-40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100mL oxygen-saturated distilled water was added and the container was vigorously shaken. Then, 2500 ml reaction mixture and 350µL of the different concentrations of the extract and essential oils were added to the test tube. The absorbance of the specimens was measured at 490nm immediately and 24 hours after the preparation of the test tubes, using the spectrophotometer. The same method was used for BHT as positive control without antioxidant. The antioxidant capacityof essential oils and extracts were compared with BHT and negative control. The activity was expressed as inhibition percentage. Then, the obtained data were analyzed using statistical software packages such as SPSS and Minitab. Duncan test was used to compare data.

Results

The major constituents of essential oil were (+) - pulegone (52.41 %), Dihydrocaryl acetate (14.13 %), 1,8 - cineole (12.98%) and D-neoisomenthol (4.19 %). In DPPH assay, the extract of flower had the highest antioxidant activity (the least IC_{50}) while in β- carotene linoleic acid, the essential oil of flower had the highest antioxidant activity.

Table 1. The essential oil composition of *Ziziphora clinopodioides*

No	component	%Total	RI
1	α-Pinene	0.80	935
2	Camphene	0.47	952
3	Sabinene	0.88	980
4	ß-Pinene	1.35	989
5	ß-Myrcene	0.52	1031
6	Cyclohexene	1.69	1036
7	1,8-Cineole	12.98	1072
8	R- (+)-Limonene	0.76	1067
9	Dihydrocarvyl acetate	14.13	1074
10	Menthone	2.37	1090
11	D- Neoisomenthol	4.19	1095
12	Borneol	2.91	1099
13	(+)-Isomenthol	0.44	1187
14	α-Terpineol	0.38	1217
15	Isolimonen	0.78	1248
16	(+)-Pulegone	52.41	1259
17	Piperitone	0.47	1288
18	Bornyl acetate	0.22	1245
19	Cis-Verbenol	2.24	
	Total	99.99	-

The results of variance analysis of antioxidant activity using DPPH method is shown in table 2. Based on the results, IC_{50} values of extract and essential oil comparing with IC_{50} values of BHT are significantly different ($p < 0.01$). To compare the obtained results, Duncan test was used.

Table 2. The Variance analysis of antioxidant activity using DPPH method in different parts of *Ziziphora clinopoioides*

Sources of Variation (S.O.V)	Degree of freedom (Df)	Mean square IC50 (MS)
test	4	2047***
error	10	574
total	14	

*ns

The results of Duncan test for different parts of the plant have been presented in table 3. According to the results, it is obvious that the least inhibition is for flower essential oil in alcohol and the highest activity (the least IC_{50}) is for *Ziziphora* flower extract that has better activity than control samples. In addition, antioxidant activity of stem methanol extract is more than that of control samples. It means that they need less antioxidant compounds to suppress free radicals. Comparison of means shows no statistically significant difference between flower extract, stem extract and control, but flower essential oil in hexane has statistically significant difference with extracts.

The changes of IC_{50} are shown in figure 1. The results show that the values of IC_{50} of flower extract (0.39 μg/ml), stem extract (1.87μg/ml), essential oil of flower in hexane (23.94μg/ml) and essential oil of flower in alcohol (61.48μg/ml) in comparison to BHT do not have significant difference. In this experiment, antioxidant capacities of flower essential oil in alcohol and hexane are very weaker than of BHT. The order of the antioxidant activity of all samples is as follows: methanolic extract of flower> methanolic extract of stem>BHT>hexane oil of flower> methanolic oil of flower.

Table 3. Comparison of antioxidant activity ofdifferent parts of *Z. clinopoioides* in DPPH assay

Different parts of plant	Mean IC_{50}
Methanolic extract of flower	0.39 ± 0.03^{ab}
Methanolic oil of flower	61.48 ± 53.35^{c}
Hexaneoil of flower	33.94 ± 4.61^{abc}
Methanolic extract of stem	1.87 ± 0.06^{ab}
Control BHT	2.73 ± 0.08^{abc}

The same letter (s) are not significantly different at $p\leq0.05$ probability

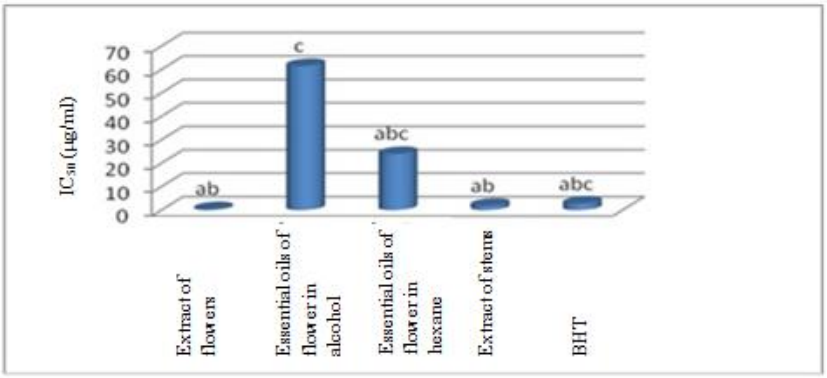

Figure1. IC50 values of different parts of Z. *clinopoioides* in DPPH assay. The same letter (s) is not significantly different at p≤0.05 probability.

The results of β-carotene/linoleic acid assay showed that the essential oil of flower had the highest antioxidant activity which it is statistically significantin comparison with other samples.The differences of antioxidant activities of flower and stem extracts and BHT are not statistically significant (Table 4). The essential oil of flowers has the highest antioxidant capacity in comparison to other samples (Fig 2).

Table 4. Antioxidant activity of essential oil and methanol extract of different parts of Z. *clinopodioides*.

Different parts of plant	Antioxidantactivity percent
flower extract	60.60±2.49a
stem extract	59.42±2.16a
Flower essential oil	108.69±81.03b
control (BHT)	60.61±8.25a

The same letter(s) are not significantly different at p≤0.05 probability

As it is seen in figure 2, flower extract, stem extract and *Ziziphora* essential oil at concentrations of 0.001 and 0.0001 have more antioxidant activity in comparison with BHT. It can be said that flower essential oil, flower and stem methanolic extract have the most antioxidant activity respectively. The order of the antioxidant activity in concentration of 0.01was as follows: BHT>flower essential oil> flower extract>stem extract

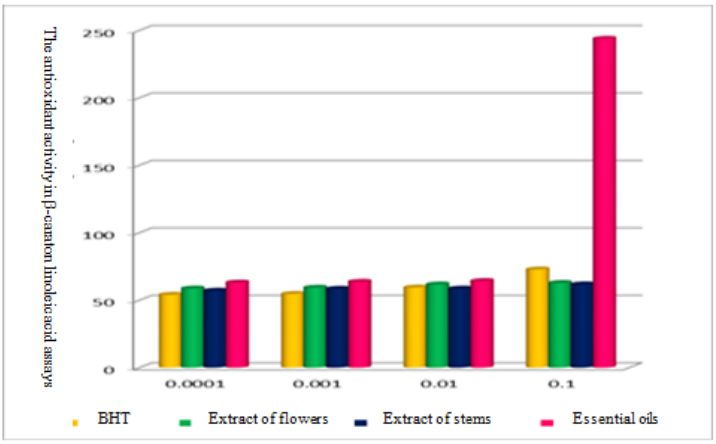

Figure 2. Antioxidant activity of methanol extract and control BHT in β-caraton linoleic acid

Discussion

Main composites of lamiaceae are thymol and carvacrol that have powerful antimicrobial effects (18,19). Several studies on antibacterial effects of *Z. clinopoioides* were conducted by Mehrabian et al (11), Salehi (20), Ozturk,Ercili (21), and SoltaniNejad (22). According to these studies, antibacterial and antifungal effects of *Z. clinopoioides* essential oil is due to the presence of pulegone

According to table 1, the main components were pulegone (52.41%), dihydrocarvylacetate (14.13%), 1,8-cineole (12.98%) and isomenthone (4.19%). Table 5 shows the results of the present study and other studies on essential oil analysis of *Ziziphora clinopodiodes* that have been done in different regions and as it is seen, in the present study, like other studies, the main compound is pulegone but with different values. In Izmir and Vancity (Turkey), pulegonehad comprised respectively 81.86% and79.33% of the sample (16, 23). In Palandocan (Erzurum, Turkey), this value has been31.86% (19). In the presentstudy,pluegone comprised 52.41% of the studied sample, while in other regions like Ferdoos in values have been 63.5% and 65.2% respectively. But, in Razan valley in Khoramabad 25), Lar and Lavasan in Tehran (26) and Khorasan (19), the amount of pulegone has been less than 50 %. These differences can be attributed to different conditions of regions in terms of weather, altitude, humidity, the slope of growth area and the amount of precipitation.

In regard to other composites, in Lar and Lavasan in Tehran, β-myrecene (19.02%), neomenthol (11.6%) and piperitenone (9.43%) have been the main composites of the essential oil following pulegone. In Razan valley in Khorram Abbad, thymol (21.3%), P-mentha-3-en-8-ol (12.9%) and in Taftan, 1,8-cineole (10.23%) as well as cis-carene-trans-2-ol (12.66%) have been the main composites after pulegone.

Table 5. Comparison of the composition of Ziziphora clinopodioides reported indifferent studies

Study region / Compounds	erzerum,turkey *Z. clinopodioides Lam*	Van city.Turkry *Z. clinopodioides* Lam	Lar-Lavasan,Tehran *Z. clinopodioides* Lam	Razanvalley.Kordestan *Z. clinopodioides Lam*	Ferdos,Khorasan *Z.clinopodioides* Lam	Khorasan *Z. clinopodioides*Lam	Ezmir-Turkey *Z. persica*Bunge	Taftan-Baloochestan *Z. clinopodioides* Lam
ß-Pinene	6.88	1.88	0.58	0.6	0.7	0.43	0.88	2.16
ß-Myrcene	0.7	0.5	19.02	-	0.3	1.02	0.1	0.74
Bornyl acetate	-	-	1.17	4.7	0.1	-	-	-
1,8-Cineole	12.21	-	4.48	4.1	7.8	2.61	0.21	10.23
Limonene	10.48	6.78	0.44	-	-	2.25	4.48	-
Menthone	6.73	-	3.62	2.4	--	6.82	-	-
Isomenthone	0.38	0.56	1.78	1.6	11.9	1.63	0.28	
(+)-Pustemone	31.86	79.33	29.3	32.1	65.2	27.15	81.86	63.5
Piperitone	4.18	4.20	2.43	9.3	0.6	-	-	-
Piperitenone	5.13	-	9.48	-	6.5	0.82	2.30	-
P-mentha-3-en-8-ol	-	-	-	12.9	-	-	-	-
Neomenthol	-	-	11.6	2.5	-	-	-	-
Thymol	-	-	-	21.3	-	9.71	-	-
Geraniol	-	-	-	-	-	8.26	-	-
α-terpinyl acetate	-	-	-	-	-	10.83	-	-
(-)cis-caran-trans-2-ol	-	-	-	-	-	-	-	12.66

In Palandoken (Erzurum, turkey), 1,8-cineole(12.21%) and limonene (10.48%) have been the main composites. In Vancity, limonene (6-78%) and piperitone (4-20%) have been reported as the main oil composites. The differences are due to different weather conditions.

The antioxidant activity of *Ziziphora clinopodioides* by using DPPH is shown in Fig.1 and as it is seen, the values are not statistically significant ($p > 0.05$). In this study, the antioxidant capacity of essential oils in alcohol and essential oil in hexane were weaker than BHT but the antioxidant activities of extract of flower and stem were more than that of BHT. Therefore, methanol extract of different parts of *Ziziphora clinopodioides* has more suppressive effect on DPPH free radicals than BHT. In comparison of mean IC_{50} values, flower and stem extracts showed no significant difference with BHT, but there is significant difference between extracts and flower essential oil in hexane.

In another study conducted by Amiri on *Ziziphora clinopodioides* from Razan valley in Khoerram Abad, mean $IC_{50}$of the extract was 55.3 ± 0.85 and for methanol extracts, it was 21.4±50 and for BHT, it was18.0± 40µg/ml. Moreover, the antioxidant activity of methanol extract was more than that of BHT (22). In another study by Salehi et al on *Ziziphora clinopodiodes*, the value of IC_{50} for this plant was 30.7µg/ml (20).

Conclusion

According to the obtained results in the present study and other studies, due to the presence of pulegone, as the main natural antibacterial, antifungal and antioxidant in the essential oil of *Ziziphora clinopodiodes*, we can use the product obtained from this plant in food and pharmaceutical industries. This essential oil can be used instead of the synthetic antioxidants in order to prevent nutrient oxidation. This species belonged to lamiaceae family can also beused as additive to make the taste of foods and also for cosmetic and sanitary purposes.

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