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Original Article

Ecological Requirenments, Essential oil Composition, Total Phenol and Flavonoid Content, Antioxidant Activity and Ethnobotanical Survey of *Ziziphora clinopodioides* Lam. in North Khorasan Razavi Province

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Abstract

In several field observation of this research ,the main ecological requirenments and ethnopharmacological data about the traditional uses of *Ziziphora clinopodioides* Lam. were recorded. The aerial parts of plant were collected in blooming from Bovanloo mountain in September 2013 (1728 m) in North Khorasan Razavi. Essential oil was obtained by hydro-distillation and analyzed by GC-MS. Methanolic extracts were obtained by maceration. Phytochemical assay: TP (total phenol) and TF (total flavonoid) were determined by spectrophotometrically, antioxidant capacity were obtained by TAC, RP and DPPH methods in compare of BHT and BHA antioxidant standard. According to results, *Ziziphora clinopodioides* Lam. (Kakuti) is the most edible aromatic montainous herb, which wild growing in cold-dry climate (1500-1800 m) in North Khorasan Razavi province, with annual raining 288 mm, annual temperature 13.7 °C in sandy loam soil, with pH= 7.8 and Ec=0.8 dc. The pulegone (46.2%), menthol (10.7%), carvacrol (9.5%), 1,8-cineole (8.37%) , p-menthan-3-one (7.5) and piperitenone (5.8%) were the main constituents of plant essential oil. The amount of total phenolic (98.13±5.9 mg GAE 100 g-1 DW) and flavonoid contents (220.9±18.65 mg QE 100g-1 DW), respectively. The highest levels of IC50 (26.5 ± 1.4 µg/ml) were detected in DPPH method against free radical scavenging in density of 5 µg/ml ($P< 0.05$) to compare of standard antioxidant (BHT and BHA). The essential oil and methanol extract of *Ziziphora clinopodioides* with high quality content and antioxidant activity can be confirmed the traditional uses of *Z.clinopodioides* in this provice as antiinflammation, antispasm, expectorant and antiinfection to treat of common cold, flu, fever, diarrhoae, gasterointestinal disorder and stomatchache in tea, yaghurt and doogh.

Key words: Antioxidant, Autecology, Essential oil composition, Ethnopharmacology, North Khorasan Razavi province TP and TF content, *Ziziphora clinopodioides* Lam.

TAC**:** (total antioxidant capacity); RP: (reducing power); DPPH: (2,2-Diphenyl-1-picrylhydrazyl)

Introduction

In recent centuries,essential oils and the extracts of many aromatic herbs have been considered to be "natural preservatives" and has been used as antioxidant and anti inflammation in controlling of many pathogens and radical scavenging. So these objects has led World Health Organization (WHO) to finding the antioxidant natural extract, especially from wild plants, which has been used along several centuries to treat of many cuurrent of human ailments [1-3].

The aromatic herbs, especially many species of *Ziziphora* genus, with more than 15 species, mainly distributed in mediterranean mountainous region in Europe, Africa and Asia and, in which 4 species of them are endemic in mountainous regions of Iran [4,5]. Theyhave been used in Iranian traditional

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medicine as a strong source of vegetable, tea and natural food additives, antispasm and anti infectionto treat of common cold, flu, stomatchache, infection disorders and inflammations [6-9].

According in many simillar reaserches the phytochemical, antioxidant and anti fungal activities of some species of *Ziziphora* were reported and they showed that they have rich monoterpenoides and phenolic compounds such as :thymol,pulegone,menthol, l,8-cineole, piperitenoneand p-menth-3-en-8-ol, previously have been studied as antioxidant [7,10,11], antibacterial [7,12], anti-inflammatory [13] and antifungal [14].

Due to poly phenols, terpenoides and flavonoides were demonstrated to posses strong antioxidant/free radical scavenging effectiveness and which have too much considerable attention to their pharmacological functions as antioxidant, antimutagenic and anti-tumor activities, that why in present study at the first time was carried on natural secondary metabolites sources (essential oil composition, TF and TP contents in extract) and the evaluate of their antioxidant activity which were collected from Bovanloo region village (1780m) in North east of Iran

Material and Methods

Study Area

The present study was carried out in Bovanloo region (1780 m) is 41 km far from Bojnoord city in north Khorasan Razavi province. this region is located in southeast of the province, in of latitude of 37 ° 32' 45" N and longitude of 58 ° 11' 04" E, Its average height is 700 to 1800 m from sea levels, semi-dry climate, the mean annual rainfall is 249.7 mm/year and the mean annual temperature is 12/9 °C .

Plant Material

The flowering aerial parts of *Z.clinopodioides* Lam. were collected in September, 2011 from Bovanloo mountain at height of 1780 m above sea level,a voucher specimen of plant was identified and preserved on (No. HRCMP:6240), is deposited at the Herbarium of RCMP (Research Center of Medicinal plants), Islamic Azad University of Gorgan branch, Golestan Province, Iran. The aerial parts of plant in blooming was dried in the shade , powdered and stored at 4 ºC until invitro tested.

Extract Preparation for Phytochemical and Antioxidant Tests:

One gram of plant parts with 100 ml (methanol 80%) were extracted by maceration. Extracts were filtered with Whatman No. 1 filter paper. The filtrates obtained from extracts were evaporated into dry rotary evaporator at 40 °C and were stored at 4 °C [15].

Chemicals**:** 2,2'-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St., Louis, USA), Gallic acid, Folin-Ciocalteu reagent and methanol were purchased from Merck Co. (Germany). BHT (butylated hydroxytoloane), BHA (butylated hydroxyanisole) and methanol were purchased from Merck Co. (Germany).

Antioxidant Activity Tests

Reducing Power Assay

This assay is based on Arabshahi-Delouee method. First, The dried extract (12.5–1000 µg) in 1 ml of the corresponding solvent was combined with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (K3Fe(CN)6; 10 g l-1), after the mixture was incubated at 50 °C for 30 min. Then, 2.5 ml of trichloroacetic acid (100 g l-1) were added and the mixture centrifuged at 1650g for 10 min. Then, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml of FeCl3 (1 g l-1), and the samples absorbance was measured at 700 nm [16].

1,1-diphenyl-2-picryl hydrazyl radical scavenging capacity Assay

The ability of the extracts for free radical scavenging was assessed by the method suggested by [16]. Briefly, 1 ml of a 1 mM methanolic solution of DPPH was mixed with 3 ml of extract solution in methanol (containing 12.5-1000 µg of dried extract). The mixture was then vortexes vigorously and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm and activity was expressed as percentage DPPH scavenging relative to control using the following equation:

DPPH scavenging activity (%) =[(Acontrol-Asample) /Acontrol] ×100

Total Antioxidant Capacity

This experimental procedure was adapted from Arabshahi-Delouee method, which is based on the reduction of Mo (VI) to Mo (V) by the sample and observation of a green phosphate/Mo (V) complex

at acidic pH. An aliquot of 0.1 ml of sample solution, containing 12.5-1000 µg of dried extract in corresponding solvent, was combined in a tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). They were incubated in a thermal block at 95 °C for 90 min. Then we got cold the samples and measured their absorbance at 695 nm. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent was used for the sample, and was incubated under the same conditions as the rest of the samples [16].

Essential oil

The plant samples in blooming stage were dried at room temperature for 4 days. They were hydro-distillated in a Clevenger-type apparatus for 3 h according to the method recommended in the British Pharmacopoeia. The oil was dried over anhydrous sodium sulphate and deoxygenated under nitrogen gasand the oil obtained was kept refrigerated and protected from directlight until the analysis time.

Gas Chromatography-mass Spectrometry (GC-MS) Analysis

The essential oil was analyzed by GC-MS. The GC-MS analysis was carried out on a Shimadzu GC-MS (QP5050). The capillary conditions were as follows; carrier gas, helium with a flow rate of 1.7 ml/min; injected 0.1 µL of the essential oil and ionization potential 70 ev. The initial temperature of column was 60 °C (held for 1 min) then heated to 280 °C with a rate of 3 °C/min, then heated to 250 °C and kept constant for 4 min. The same condition of temperature programming was used for n-alkenes mixture to calculate the retention indexes (RI). The identification of each component was studied by mass spectral data, literature and National Institute of Standards and Technology (NIST) computer library. The relative percentage of the oil constituent was calculated.

Determination of Total Phenolic Content

Total phenolic contentwas determined using the Folin-Ciocalteu Reagent based on the procedure suggested by [15]. Then 0.5 ml of plant extracts or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu Reagent (5 ml) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined bycolorimetry at 765 nm. Total phenol valueswere expressed in terms of mg equal gallic acid in 1 gr powder dry plant [15].

Determination of Total Flavonoid Content

Total flavonoids content were determined by aluminum chloride method. Extract plants (0.5 ml) were separately mixed with 1.5 ml of solvent, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. They were kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer. Total flavonoid values were expressed in terms of mg equal
quercetin in one gram powder dry plant [15].

Table 1 Ecological requirements of *Z.clinopodioides* Lam. in Bovanloo region (1728 m)

Characterastic Regions	Hight (m)	Rainfall (mm per year)	Temperture ($^{\circ}$C per year)	Soil characters					
				Ec	PH%	TEXT.S	Clay%	Silt%	Sand%
Bovanloo	1728	288	13.7	0.8	7.8	silty Loam	14	50	36

Table 2 Antioxidant activity of *Z.clinopodioides* Lam. in three different methods from North Khorasan province 1728 m (Bovanloo village)

Antioxidant Activity IC50 (µg/ml)	Aerial part extracts	BHA	BHT
TAC	42.5±1.3		
RP	37.08±1	41.05±0.3	35.3±0.5
DPPH	26.5±1.4		

Table 3 Essential oil composition of *Ziziphora clinopodioides* Lam. in Bovanloo region (1780m)

1	2- hexenal	4.287	0.115	849
2	α-thujene	6.410	0.040	929
3	α- pinene	6.647	0.427	936
4	Camphene	7.162	0.128	951
5	Sabinene	8.093	0.294	975
6	β- pinene	8.216	0.601	978
7	Carbinol or 1- octen- 3- ol	8.421	0.037	983
8	β- myrcene	8.788	0.225	992
9	Ethyl amyl carbinol or 3- octanol	9.065	0.234	998
10	1,8- cineole	10.570	8.326	1037
11	γ-terpinene	11.668	0.108	1062
12	3,8- menthadiene or p-mentha- 3,8- diene	12.243	2.028	1074
13	Carvacrol	16.303	9.571	1163
14	p- menthan- 3- one	16.767	7.588	1173
15	Cis- isopulegone	17.114	0.777	1180
16	Menthol	18.758	10.721	1213
17	Pulegone	21.170	46.060	1262
18	2- cyclohen- 1- one or 3- carvomenthenone	21.511	2.446	1268
19	Azolidine- 4- carboxylic acid	22.052	0.059	1278
20	Borneol acetate	22.632	0.271	1289
21	Menthol acetate	23.136	1.846	1298
22	Geraniol formate	23.402	0.041	1303
23	Thymol	23.686	0.238	1311
24	Piperitenone	25.288	5.801	1351
25	β-bournonene	26.767	0.235	1386
26	Germacrene-D	30.278	0.495	1483
	Total		98.712	

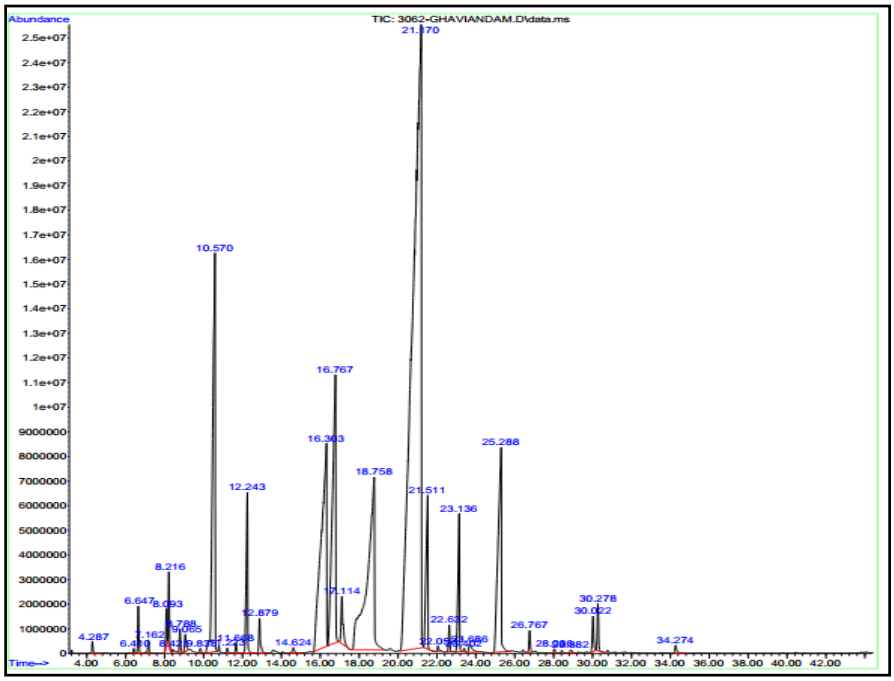

Fig. 1 Chromatogram of essential oil analysis from *Ziziphora clinopodioides* (1780m) in Bovanloo region

Results

Ziziphora clinopodioides Lam. (Lamiaceae) with localy name Kakuti (Annekh in turkish language in this rural region) is widely distributed as wild edible, aromatic annual herb, often grow in north slob and suuny position around the hills of Bovanloo mountain(1728 m), with annual raining 288 mm, annual temperature 13.7 °C , dry cold climate and in sandy loam soil ,with Ec=0.8 desizimence and pH= 7.8 (Table 1).

Ethno pharmacological survey showed that dry leaves *of Z.clinopodioides* Lam. especially in bloomong, is one of the most edible aromatic tea, which have been used in traditional medicines of mountainous village of this province as a strong tonic, anti-inflammation, antispasm, anti-infection, carminative and sedative to treat of stomatchache, diarhoea, gastetointestinal disorder, cold, flu, fever, toothache, backache and digestive infection as a single or in combination with other herbs such as below:

- As strong anti-inflammation, warmer, smoother and anti infection to treat of cold, bronchitis, flu, Ulcer and aphtus: The tea or gargle of *Z. clinopodioides* with combination of *Thymus carmanicus, Satureja mutica*, the flower of *Sambucus nigra*, *Adiantum cappilus veneris* and *Malva neglecta.*
- Backache and rheumatic pain : the massage of high density extract of *Ziziphora clinopodioides* with mixed to *Thymus carmanicus, Mentha longifolia* L., *Capsicum annuum, Artemisia sieberi* and *Perovskia abrotanoides* Karel. As a strong sedative and anti-spasm to treat of rheumatic pain, backache,sciatic and gout.
- Anticarminative : the leaves powder of *Ziziphora clinopodioides* with *Mentha aquatica* and *Artemisia annua* in Yaghurt and Doogh to treat of Diarrhoae and stomatchache.

According to table 2. The amount of total phenolic (98.13±5.9) mg GAE 100 g-1 DW) and flavonoid contents (220.9±18.65 mg QE 100 g-1 DW) were evaluated by spectrophotometric method, respectively. The highest levels of IC50 (26.5 ± 1.4 µg/ml) were detected in DPPH method against free radical scavenging in density of 5 µg/ml (p-value less than 0.05) to compare of standard antioxidant (BHT and BHA).

According to results (Table 3), 26 constituents accounting to 98.7% of the total oil were identified. pulegone (46.2%), menthol (10.7%), carvacrol (9.5%), 1,8-cineole (8.37%), p-menthan-3-one (7.5) and piperitenone (5.8%) were the main constituents of plant essential oil.

Disscusion

It has been recognized that secondary metabolites (terpenoides, phenols and flavonoid contents) especially in wild medicinal herbs have a good antioxidant agents through scavenging on human and animal health. So Increasing damages from oxidant agents in free radicals and its necessary to finding new natural antioxidant compounds from natural plants, oriented researchers to evaluate the effect of plants and their active compounds [17].

According to above, the methanol extract of plant in Bovanloo region has better potential antioxidant capacity against free radical, especially in DPPH test, due to their traditional uses as anti-inflammation and antidiabetes against hypertension, diabetes, cold and related infections.

The species of *Ziziphora*, which are contain many different secondary metabolites including terpenoids, flavonoid and phenolics [2,7,18,19]. On the other hand, these classes of secondary compounds are responsiblefor the most pharmacological effects such as antioxidant, anti-inflammation and antibacterial activity [2,20]. Among them :menthol, thymol, carvacrol, pulegone,piperitenone, p-menth-3-en-8-ol and 1,8-cineole were themost important as antioxidant, anti-inflammatory, antinociceptive, antispasmodic and antibacterial[9,13]and also with the strongly radical scavenging in providing health-beneficial effects and especially in prevent molecular damage, damage by pathogens and hypoglycemic properties effect [2,21-24].

inconfirming to ourresultsand another works were reported that these phenolic and flavonoid compounds, especially terpenoides such as pulegone, menthol, carvacrol and 1,8-cineole were the main constituent of *Z. clinopoides* essential oil are responsible for the antimicrobial, antispasm, anti-infection and antioxidant effects which can be exactly confirmed the traditional uses of this plant as anti-inflammation, sedative and anti infection.

In relation to our data, Khodaparest et al., 2007 were showed that in traditional medicine of Iran the *Z. clinopodioides* with another species of *Ziziphora* (*Z. multiflorais* , *Z. tenuior*), *Zataria* and *Thymus* which has been used in decoction, yoghurt and doogh to relieve spasm, stomachache, gastric complications ,common cold and flue among the people, it had a significant as

antimicrobial, antioxidative and antibacterial potential on *Listeria* and its may be useful for the treatment of infectious disease which caused by *Listeria* , *Shigella* and *Escherichia coli* in many bacterial dysentery [13,25,26]. Any way the medicinal effects of many species of Lamiaceae family such as *Ziziphora clinopoidioides*, which can be influenced by many ecological factors such as geographic location, environmental and climate conditions, season, soil type, the method of drying and extraction of the plants extract [9,27].

Conclusion

In conclusion, These findings and another researches, indicated that terpenoides and polyphenols are the most secondary antioxidant compounds in medicinal plants, which have important role in blocked activity of free radicals and so there was a positive correlation between total phenolic content and antioxidant activity. Mean while, our result can be confirmed the traditional uses of this plant as an antispasm, anti inflammation, anti-infection, feverfew and expectorant to treat of cold, flu, diarrhae, gasterointestinal disorder and stomatch ache. So it represented that the methanol extract of *Z. clinopoidioides* like other mentioned *ziziphora* species can has a great potent antimicrobial and antioxidant properties and additives in the preservation of processed food, drug industrial and anticancer agent.

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