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Research Article

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Antioxidant Activity and Total Phenols Content of Different Solvent Extracts of *Ziziphora clinopodioides* from Three Geographical Locations in Iran

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ABSTRACT

Antioxidant capability, total phenolic contents and total flavonoids of various solvent extracts of Ziziphora clinopodioides aerial parts from three geographical locations of Iran were evaluated in this work. Antioxidant activities of the samples were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The aqueous methanol extract from Lar region exhibited the greatest antioxidant capacity (IC_{50}=91.27±1.89 µg/mL) than that of other extracts (ethanol and methanol), which was probably due to its high content of polyphenols (78.74±1.17 mg gallic acid equivalent/g dry extract). The ethanol extracts of three population of the plant has been found to be rich in flavonoids. There was a correlation between the antioxidant activity potential and amount of phenolic compounds in all extracts. The observed differences in antioxidant activity of Ziziphora clinopodioides extracts could be attributed to the variations of composition in the polyphenol contents of the plant grown in different geographical locations due to diverse environmental or climatic factors.

Keywords: Ziziphora clinopodioides; Antioxidant activity; Total phenolics; Flavonoids contents

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INTRODUCTION

The processes of oxidation are intrinsic in the management of energy of all living organisms and are, therefore, kept under strict control by several cellular mechanisms [1]. However, the production of excessive free radicals and the antioxidant protection due to unbalanced mechanisms result in the onset of numerous diseases and accelerate ageing. The antioxidants of low molecular weight are considered as possible protection agents reducing oxidative damage of the human body, when the internal enzymatic mechanisms fail or are inadequately efficient [2]. These compounds play an important role in preventing chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases [3, 4]. In recent years, natural phenolic compounds are used as functional and nutraceutical ingredients and natural alternatives to synthetic antioxidants in food industry [5]. These compounds are secondary metabolites which are biosynthesized by shikimate and phenylpropanoid metabolic pathways in plants [6]. Most of the herbal infusions, commonly used as home medicines have antioxidative and pharmacological properties related to the presence of phenolic compounds, especially phenolic acids derivatives and flavonoids. Polyphenols, such as phenolic derivatives and flavonoids are also known for their ability to prevent fatty acids from oxidative decay and provide an additional value to plants used as food ingredients [7].

The Lamiaceae (Labiatae) is a large family which its aromatic species are used for a variety of purposes, such as culinary and as traditional medicines [8]. *Ziziphora clinopodioides* Lam., a perennial plant belonging to the Lamiaceae, consists of four species (*Z. clinopodioides* Lam., *Z. capitata* L., *Z. persica* Bunge and *Z. tenuior* L.) that are widespread all over Iran [9]. *Ziziphora clinopodioides* Lam. With the common Persian name "*kakuti-e kuhi*" comprised nine subspecies native to Iran. In Iranian and Turkish folk medicine, *Ziziphora* species have been used as stomachic, carminative and wound healing material [10]. This plant is also used to treat hypertension, fever, edema,

heart disease, neurasthenia, insomnia, tracheitis, lung abscess, and hemorrhoids [11, 12]. Previous pharmacological investigations revealed that this species exhibits antimicrobial [13], antifungal [14], antioxidative [15], and anti-hypertensive [16] properties. Phytochemical research has shown that this genus is a source of flavonoids, polyphenols, polysaccharides, fatty acids and sterols [17].

This study was planned to evaluate the antioxidant properties of *Ziziphora clinopodioides* extracts from three different geographical regions of Iran for the first time. The choice of solvent was showed to have a significant influence on the concentration of antioxidants [18, 19]. Thus, we investigated the ethanol, methanol and aqueous methanol extracts. Further experiments were conducted to determine phenol and flavonoid contents.

MATERIALS AND METHODS

Chemicals and analytical instruments

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Butylated hydroxyanisole (BHA), gallic acid, quercetin, aluminium chloride, potassium acetate and standard Folin–Ciocalteu's phenol reagent were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Analytical grade methanol, ethanol, sodium carbonate were obtained from Merck (Darmstadt, Germany). Double distilled water was used for the experiments. The spectrophotometric measurements were carried out using a S2100SUV spectrophotometer (UNICO, USA).

Plant materials

The aerial parts of *Ziziphora clinopodioides* were collected during the flowering period in June 2014 from Vardavard-Varij region; Alborz Province; at 2187 m altitude (sample Z1), Chalus-Polekhab region; Alborz Province; at 1914 m altitude (sample Z2) and Lar region; Tehran Province; at 2298 m altitude (sample Z3). Voucher specimens have been deposited at the Herbarium of Islamic Azad University, North Tehran Branch (Voucher no. 3006 (sample Z1), 2967 (sample Z2) and 16152 (sample Z3)). The samples were air-dried in shadow and ground into fine powder by a laboratory mill.

Extraction

A quantity (50 g) of each powdered plant was extracted in a Soxhlet apparatus with different solvents namely ethanol, methanol and aqueous methanol (70% methanol-30% water). All the extracts were filtered and evaporated to dryness under reduced pressure in a rotary evaporator. Then the dry extracts were weighed and percentage of different extractive values was calculated with respect to the air dried powdered plant material. The extracts were transferred to vials and kept at +4 ˚C until use.

Antioxidant assay using DPPH method

The free radical-scavenging activities of the plant extracts were measured using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method [20] with some modifications. Aliquots (1 mL) of different concentrations of the plant extract in methanol (25-1000 µg/mL) were mixed with 2 mL of a 0.004% (w/v) methanol solution of DPPH. The mixture was shaken vigorously and left standing at room temperature for 30 min. Then the absorbance of the resulting solution was measured at 517 nm against pure methanol. The DPPH solution was freshly prepared daily, stored in a flask covered with aluminium foil, and kept in the dark at +4 ˚C between measurements. All experiments were carried out in triplicate. The radical-scavenging activities of the tested samples, expressed as percentage inhibition of DPPH, were calculated according to the formula: $I\% = (A_{blank}\text{-}A_{sample}/A_{blank}) \times 100$. Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the test sample. The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage against sample concentration. Butylated hydroxyanisole (BHA) was used as the positive control.

Determination of total phenols content

The level of total phenols in the crude extracts was determined as described [21], with Folin–Ciocalteu reagent and galic acid used as a standard. Briefly; 0.5 mL of extract solution and 2.5 mL of Folin–Ciocalteu reagent (diluted 10 times with water) were added and the contents mixed thoroughly. After 5 min, 2 mL of 20% Na_2CO_3 was added, and then the mixture was allowed to stand for 2 h at normal temperature. The absorbance was measured at 760 nm. The same procedure was also applied to the standard solutions of gallic acid, and a standard curve was obtained. The total phenol contents were expressed as mg gallic acid equivalents per g of the extract.

Determination of total flavonoids

The flavonoid contents of the aforementioned extracts were assessed using the aluminium chloride colorimetric method [22], with some modifications. The extract solutions (0.5 mL), 10% aluminium chloride (0.1 mL), 1 M potassium acetate (0.1 mL) and distilled water (4.3 mL) were mixed. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm. Total flavonoid content was calculated from a calibration curve using quercetin as standard, and expressed as mg quercetin equivalents (QE) per g of dry extract.

Statistical analysis

The measurements of DPPH radical-scavenging activity, total phenolic compounds and total flavonoids were carried out for three replicates. The results are expressed as mean±SD.

RESULTS AND DISCUSSION

Extraction yield

The waxy extracts from the Soxhlet extractions were weighed and percentage of different extractive values was calculated with respect to the air dried powdered plant material. These results are presented in Table 1. As shown, the most extractive solvent was aqueous methanol. The highest extraction yield of *z. clinopodioides* aerial part was the extract from Lar with 23.2% (percentage of dry matter w/w), followed by the extract from Vardavard-Varij (21.4%) then Chalus-Polekhab (19.5%).

Table 1: Residues yields (% of dry matter) of *ziziphora clinopodioides* in the organic solvents

Regions	Samples	Yields (%)
Z1	Ethanol	5.5
	Methanol	14.3
	Aqueous methanol (70%)	21.4
Z2	Ethanol	8.5
	Methanol	12.5
	Aqueous methanol (70%)	19.5
Z3	Ethanol	9.3
	Methanol	18.1
	Aqueous methanol (70%)	23.2

Z1: Vardavard-Varij; Z2: Chalus-Polekhab; Z3:Lar

DPPH scavenging activity

The effects of antioxidants in the DPPH-radical-scavenging test reflect the hydrogen-donating capacity of a compound. When the radical form of DPPH is scavenged by an antioxidant through the donation of a hydrogen atom to form a stable DPPH molecule, this leads to a colour change from purple to yellow, and a decrease in absorbance.

In this study, ethanol, methanol and aqueous methanol extracts of *z. clinopodioides* from three regions were investigated for their antioxidant activity with DPPH assay. Antioxidant effect of *z. clinopodioides* extracts in model system of DPPH is presented in Figure 1. As is expected, an increscent in activity was observed when the concentration of extracts increased.

DPPH scavenging activity is presented as an IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. Lower IC_{50} value indicates higher antioxidant activity. The IC_{50} value ranged from 91.27 µg/mL to 400.44 µg/mL as shown in Table 2. Results showed an important antioxidant power of *z. clinopodioides* extracts compared to the standard product and the aqueous methanol was the most effective as organic solvent (Table 2). The antioxidant activity of the aqueous methanol extract from Lar region was superior to all samples tested with an IC_{50} value of 91.27 µg/mL which was near to the inhibition capacity of the positives control BHA (IC_{50}=61.07±1.19 µg/mL), followed by aqueous methanol extract from Vardavard-Varij (IC_{50}=106.18 µg/mL) then Chalus-Polekhab (IC_{50}=120.97 µg/mL). The DPPH-radical-scavenging activity of the extracts decreased in the order of aqueous methanol > methanol > ethanol.

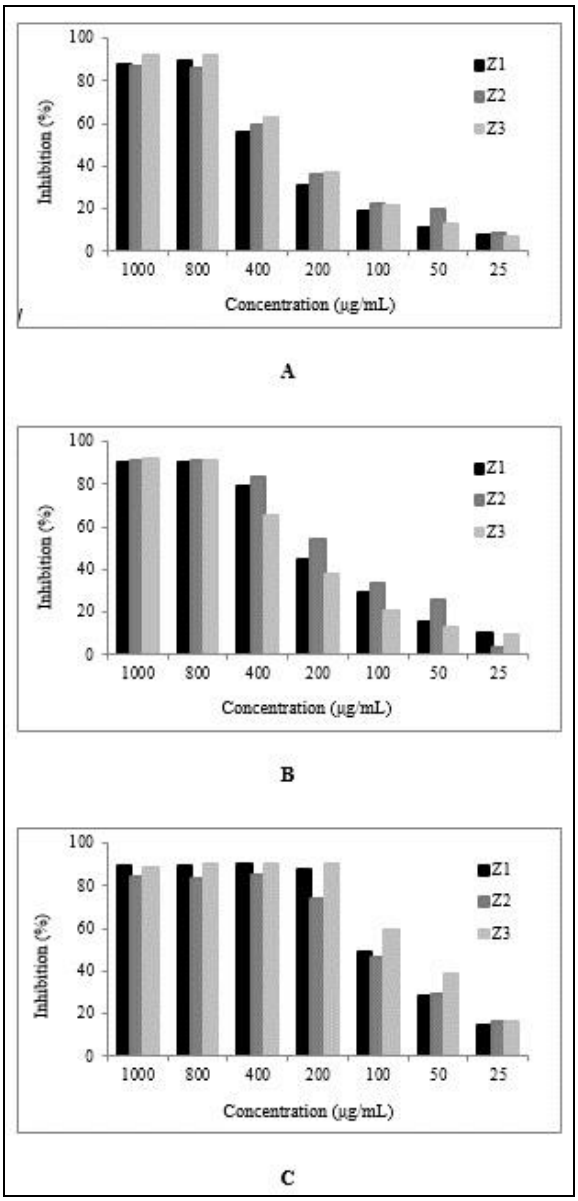

Figure 1: Scavenging activities of different concentrations of *Ziziphora clinopodioides* ethanol, methanol and aqueous methanol extracts on the DPPH radical. A: ethanol extract; B: methanol extract; C: aqueous methanol extract. Z1: Vardavard-Varij region; Z2: Chalus-Polekhab region; Z3: Lar region

Table 2: IC_{50} (µg/mL) values of different solvents extracts of *Ziziphora clinopodioides* according to DPPH assay

Regions	Samples	IC_{50} (µg/mL)
Z1	Ethanol	400.44 ± 3.42
	Methanol	234.94 ± 2.91
	Aqueous methanol (70%)	106.18 ± 2.50
Z2	Ethanol	382.05 ± 2.87
	Methanol	207.44 ± 4.73
	Aqueous methanol (70%)	120.97 ± 3.11
Z3	Ethanol	367.88 ± 5.36
	Methanol	293.59 ± 3.12
	Aqueous methanol (70%)	91.27 ± 1.89

Assay performed in three replicates and the data were reported as means±SD. Z1: Vardavard-Varij; Z2: Chalus-Polekhab; Z3:Lar

Total phenolic compounds and flavonoids contents

Total phenol content of the extracts was determined spectrometrically according to the Folin-Ciocalteu procedure, and calculated as gallic acid equivalents. The standard curve equation is y = 10.15x + 0.1748, R^2 = 0.9975. The amounts of total phenols found in the plant different extracts were shown in Table 3. The organic extracts of *z. clinopodioides* had an important charge of phenols and their values varied widely for all three organic solvent used and in the different origin area ranging from 29.67 to 78.74 mg GAE/g dry extract. The most extractible solvent of phenols was the aqueous methanol and the highest amount of total phenolics was found in *z. clinopodioides* from Lar (78.74±1.17 mg GAE/g dry extract), followed by Vardavard-Varij (74.99±0.79 mg GAE/g dry extract) and Chalus-Polekhab (72.33±0.91 mg GAE/g dry extract).

Table 3: Total phenolic compounds of the different extracts of *ziziphora clinopodioides*

Regions	Samples	mg GAE/g extract
Z1	Ethanol	29.67 ± 1.23
	Methanol	56.37 ± 0.85
	Aqueous methanol (70%)	74.99 ± 0.79
Z2	Ethanol	50.76 ± 0.94
	Methanol	54.70 ± 1.67
	Aqueous methanol (70%)	72.33 ± 0.91
Z3	Ethanol	43.96 ± 0.87
	Methanol	41.79 ± 0.96
	Aqueous methanol (70%)	78.74 ± 1.17

Z1: Vardavard-Varij; Z2: Chalus-Polekhab; Z3:Lar. Assay performed in three replicates and the data were reported as means±SD. Results are expressed in mg gallic acid per g of dry extract. Concentration of sample was 1.0 mg/mL

The standard curve equation for the determination of flavonoids with quercetin is y = 7.4358x + 0.0347, R^2 = 0.9993. The results of flavonoid contents of the three extracts from different regions were shown in Table 4. The ethanol extract has been found to be rich in flavonoids toward the other extracts. The greatest amounts of total flavonoids was found in *z. clinopodioides* ethanol extract from Chalus-Polekhab (69.30±0.87 mg QE/g dry extract), followed by Vardavard-Varij (52.76±1.03 mg QE/g dry extract) and Lar (43.61±0.79 mg QE/g dry extract).

Table 4: Total flavonoids contents of *ziziphora clinopodioides* extracts

Regions	Samples	mg QE/g extract
Z1	Ethanol	52.76 ± 1.03
	Methanol	40.25 ± 0.73
	Aqueous methanol (70%)	27.74 ± 0.53
Z2	Ethanol	69.30 ± 0.87
	Methanol	51.95 ± 1.49
	Aqueous methanol (70%)	28.82 ± 0.72
Z3	Ethanol	43.61 ± 0.79
	Methanol	33.39 ± 0.67
	Aqueous methanol (70%)	23.44 ± 0.98

Z1: Vardavard-Varij; Z2: Chalus-Polekhab; Z3: Lar. Assay performed in three replicates and the data were reported as means±SD. Results are expressed in mg quercetin per g of dry extract. Concentration of sample was 1.0 mg/mL

The results indicated there is a relationship between the antioxidant ability and total phenol contents. Phenolic antioxidants are products of secondary metabolism in plants, and the antioxidant activity is mainly due to their redox properties and chemical structure, which can play an important role in chelating transitional metals, inhibiting lipoxygenase and scavenging free radicals [23].

Environmental differences in different production locations contribute to the differences in active ingredient contents and antioxidant activity of medicinal plants [24, 25]. Therefore, the observed differences in antioxidant activity and phenolic content of *z. clinopodioides* different populations could be attributed to the environmental or climatic factors. The altitude and temperature are important factors to influence the metabolism and accumulation of secondary metabolites [26-28]. In this work, the plant sample from Lar region was collected from higher altitude (2298 m) compared to two other species (2187 and 1914 m). This might be one of the reasons that *z. clinopodioides* collected from Lar had the highest phenolic content and antioxidant activity. It was reported, ultraviolet changes in altitudes might be the reason for increasing total phenolic content. Low temperature at high altitudes can lead to increase the biosynthesis of some kind of antioxidants [29].

CONCLUSION

According to the results of this study, the aqueous methanol extracts of *Z. clinopodioides* may be suggested as a good source of natural antioxidants compounds which can be considered as a natural additive in the food and pharmaceutical industries. The variability in antioxidant activity of *Z. clinopodioides* extracts could be explained by diverse environmental or climatic factors among different population. Lower temperatures in higher altitude can result in increasing the rate of biosynthesis of some kind of antioxidants.

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