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Original Article

LC-MS/MS analyses of *Ziziphora clinopodioides* Lam. from Turkey: Antioxidant, anticholinesterase, antimicrobial and, anticancer activities

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ABSTRACT

Background and Aims: *Ziziphora clinopodioides* is one of the wild edible species used in Turkish folk medicine. The study was aimed to investigate the phenolic compounds of the extracts as well as the antioxidant, enzyme inhibitory, antimicrobial activities, and anticancer potential.
Methods: LC-MS/MS was used to determine phenolic compounds; antioxidant potential was evaluated by using radical scavenging assays; enzyme inhibition assays were used for anticholinesterase, tyrosinase, and urease activities; antimicrobial activities were performed by microdilution method; and XTT bioassay was used for testing anticancer potential.
Results: The LC-MS/MS results indicate that quinic acid (9020.51±73.97 µg/g; 14721.04±120.71 µg/g, respectively) is the major compound, malic acid (1972.95±22.29 µg/g; 2179.04±24.62 µg/g, respectively) and rhoifolin (1044.74±98.31 µg/g; 3593.31±338.13µg/g, respectively) are the abundant compounds in aerial and root extracts. The antioxidant activity results showed that the aerial parts extract has stronger ABTS cation radical and DPPH free radical scavenging activity than the root extract with 40.90±0.19 µg/mL, and 94.27±0.64 µg/mL IC_{50} values, respectively. The extracts showed moderate cupric reducing activity with 1.74 absorbance value at 100 µg/mL. Only the aerial parts extract exhibited weak tyrosinase inhibition (8.60±0.87%) compared with kojic acid (95.26±0.23%) at 200 µg/mL. No activity was observed in urease and anticholinesterase enzymes. Both extracts exhibited moderate antifungal activity against *Candida tropicalis* with 39.06 µg/mL MIC values. The extracts didn't show cytotoxic activity against Renal (A498, UO-31) and Colo (COLO205, KM12) cell lines, also there is no metastatic potential of the extracts against osteosarcoma cell lines (MG63.3, MG63).
Conclusion: *Z. clinopodioides* has rich phytochemical constituents with powerful health benefits, and doesn't have any harmful effect on the body.

Keywords: *Ziziphora clinopodioides*, antioxidant, enzyme inhibition, antimicrobial, anticancer, LC-MS/MS

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INTRODUCTION

Traditionally used wild edible plants have always been a main research interest in the search for new natural products as therapeutic agents. The ethno-directed research is very important and useful to demonstrate that many wild edible plants are nutritionally rich and have potential for medical treatment (Ali-Shtayeh et al., 2008; Heinrich & Gibbons, 2001).

The genus *Ziziphora* (Lamiaceae) is represented by nearly 17 species in the world, and 5 of them (*Z. clinopodioides, Z. capitata* L.*, Z. persica* Bunge*, Z. tenuior* L.*, Z. taurica* M.Bieb.) are growing in the Mediterranean region, as well as, central, western, and eastern Anatolian regions in Turkey. *Ziziphora* species are strongly aromatic plants, and are called "dağ reyhanı", "nane ruhu", and "filiskin otu" in Turkish (Kaya & Dirmenci, 2012; Selvi, Satil, Martin, Celenk, & Dirmenci, 2015).

Z. clinopodioides is one of the important wild edible plants which has ethnopharmacological uses in traditional Turkish folk medicine. The aerial parts of *Z. clinopodioides* have traditionally been used as stomachic, carminative, and antimicrobial in Anatolia (Gursoy, Sihoglu-Tepe, & Tepe, 2009; Selvi et al., 2015). Incidentally, in the Eastern part of Turkey, it is an ingredient of a very famous special cheese called "herby cheese" with some other aromatic herbs as well (Ozturk & Ercisli, 2007).

A number of investigations have already been conducted on the chemical compositions and biological activities of the essential oils obtained from *Z. clinopodioides* collected from different parts of the World. Accordingly, the major component is pulegone and other main components are thymol, limonene, menthol, isomenthone, 1,8 cineole, piperitenone, carvacrol and *β*–pinene in the essential oil. The differences in the chemical compositions of the essential oils are due to some factors such as geographical locations and climate conditions (Alp et al., 2016; Ghanbarian, Jafari, & Bahmanzadegan, 2017; Khodaverdi-Samani, Pirbalouti, Shirmardi, & Malekpoor, 2015; Maral, Taghikhani, Kaya, & Kırıcı, 2015; Nickavar & Tavakoli, 2016; Okut, Selcuk, Yagmur, & Yildirim, 2018).

Antimicrobial (Celik, Tutar, Karaman, Hepokur, & Atas, 2016; Hamedi, Kargozari, Shotorbani, Mogadam, & Fahimdanesh, 2017; Yasser Shahbazi, 2015), antifungal (Ma et al., 2016), antibacterial (Pakdel et al., 2017; Y Shahbazi, Shavisi, & Mohebi, 2017), antibiofilm (Celik et al., 2016), antioxidant (Alp et al., 2016; Hamedi et al., 2017), anti-inflammatory (Abu-Darwish et al., 2016), and insecticidal (Kheirkhah, Ghasemi, Yazdi, & Rahban, 2015) activities of essential oils obtained from *Ziziphora* species have been reported in recent years.

The flavonoid components of *Z. clinopodioides* extract were identified by using UPLC-Q-TOF-MS. The researchers reported that ten flavonoid compounds were present in the extract: "baicalein, quercetin, hyperoside, quercetin-3-O-*β-D*-glucopyranoside, apigenin, kaempferol, chrysin, diosimin, linarin and rutin" (Zhang, An, Guo, Yang, & Zhang, 2018).

Many studies exist on the essential oil composition of *Z. clinopodioides* growing in different parts of Turkey. This study focused on the chemical composition and biological activities of the ethanol extracts of *Z. clinopodioides* aerial and root parts collected from Ardahan, Turkey. According to the literature survey, there has never been a study conducted about the LC-MS/MS profile and the biological activities of the ethanol extracts of *Z. clinopodioides* collected from Ardahan.

The aim of this study was to evaluate phenolic compounds and antioxidant potential by using DPPH free radical scavenging, ABTS cation radical scavenging, cupric reducing, and *β*-carotene bleaching activities methods, anticholinesterase, tyrosinase, and urease enzyme inhibition activities, antimicrobial activities, and anticancer potential of *Z. clinopodioides*.

MATERIALS AND METHODS

Chemicals and instruments

Chemical compositions of *Z. clinopodioides* extracts were determined by using LC–MS/MS (Shimadzu, Kyoto, Japan). A Shimadzu UV spectrophotometer and BioTek PowerWave XS microplate reader (USA) were used for the activity assays. All chemical compounds which were used in the LC-MS/MS analysis and the biological assays were purchased from Merck (Germany), Sigma (Germany); and Fluka (Germany). All solvents were of analytical grade.

Plant material

Ziziphora clinopodioides was collected from Ardahan, Turkey in July 2014 and identified by Dr. Y. Yesil. A voucher specimen was deposited in the Herbarium of Faculty of Pharmacy of Istanbul University (ISTE 116052).

Preparation of the extracts

10 g samples (aerial /root parts) were macerated in 100 mL of ethanol for 24 hours at room temperature. The extract was filtered through Whatman No 1 filter paper and the residue was re-macerated under the same condition with 100 mL of ethanol two more times. The combined filtrate was concentrated in a vacuum at 35ºC to remove the organic solvent. The extract was stored at -20ºC until ready to be used for LC–MS/MS analysis and biological activities. Dry filtrates were diluted to 250 mg/L and filtrated with 0.2 µm microfiber filter prior to LC-MS/MS analysis.

LC–MS/MS instrument and chromatographic conditions

Quantitative analyses of 37 compounds were carried out by using Shimadzu UHPLC (Nexera) coupled to a tandem MS detector. The equipment, all conditions of the method, and method validation parameters were used in the study of Yilmaz et al. (Yilmaz et al., 2018).

Total phenolic and flavonoid contents of the extracts

The colorimetric assay was carried out to determine the total phenolic content expressed as micrograms of pyrocatechol equivalents (PEs) of the extracts by using the method which has been explained in the literature in detail (Boga et al., 2016). The flavonoid contents of the extracts were measured by using the method expressed by Moreno et al., and the results were given as quercetin equivalents (QEs) (Boga et al., 2016; Moreno, Isla, Sampietro, & Vattuone, 2000).

DPPH free radical scavenging activity

The DPPH free radical scavenging potential was evaluated by using a method described by Blois (Blois, 1958; Boga et al., 2016). Absorbance at 517 nm was determined after 30 min against a blank. Inhibition % was calculated using the following equation:

DPPH scavenging effect (Inhibition, %) =

$$\frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

"$A_{Control}$ is the absorbance of the control, A_{Sample} is the absorbance of the extracts or positive controls."

ABTS cation radical decolorization assay

ABTS cation radical decolorization activity of the extracts was assessed using a method informed by Re, et al (Boga et al., 2016; Re et al., 1999). The percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to a blank absorbance (methanol). Potential of the scavenging capability of $ABTS^{\cdot+}$ was calculated using the following equation:

$ABTS^{\cdot+}$ scavenging effect (Inhibition, %) =

$$\frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity (CUPRAC) of the extracts was determined based on the method summarised by Apak, et al. The absorbance was measured at 450 nm, and the results were given as absorbance value (Apak, Güçlü, Özyürek, & Karademir, 2004; Boga et al., 2016).

ß-Carotene linoleic acid test system

The *β*-carotene linoleic acid test system was used to evaluate the antioxidant capacity of the extracts (Miller, 1971). The absorbance values of the samples and standard compounds (*α*-Tocopherol and BHT) were measured by using a 96-well microplate reader at 470 nm (BioTek Power Wave XS, USA).

"The bleaching rate (R) of *β*-carotene was calculated according to the following equation:

$$R = \frac{\ln \frac{a}{b}}{t}$$

ln is natural log, a is the absorbance at time zero, b is the absorbance at time t (120 min).

The inhibition % was calculated by using the following equation:

$$\text{Inhibition, \%} = \frac{R_{Control} - R_{Sample}}{R_{Control}} \times 100$$

Anticholinesterase activity

The acetyl- and butyryl-cholinesterase inhibitory activities were detected using a method developed by Ellman et al. (Ellman, Courtney, Andres Jr, & Featherstone, 1961).

BioTek Power Wave XS at 412 nm was used to monitor the hydrolysis of these substrates.(Boga et al., 2016). Inhibition % was calculated using the following equation:

$$\text{Inhibition, \%} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Anti-tyrosinase activity

In vitro anti-tyrosinase activity of the extracts were performed according to the method designed by Hearing and Jimenez. (Hearing & Jiménez, 1987).

Firstly, the inhibition of diphenolase function of the compounds was evaluated and *L*-DOPA was used as substrate. Tyrosinase from mushroom (E.C. 1.14.18.1) (30 U, 28 nM) was dissolved in Na-phosphate buffer (pH=6.8, 50 nM) and the compounds were added to the solution for pre-incubation at room temperature for ten minutes. To start the enzymatic reaction, 0.5 mM *L*-DOPA was added to the mixture and the change in absorbance was measured at 475 nm at 37ºC. For the positive control, kojic acid was used.

The following formula was used to calculate the percentage of all enzyme inhibitions:

Inhibition, % = ($A_{control}$ – A_{sample}) / $A_{control}$ x 100

Antiurease activity

Urease inhibition activity of the studied extracts was investigated using the protocol reported by Zahid et al. (2015). (Zahid et al., 2015).

5 µL of sample solutions (4000 ppm, in methanol) were mixed with 25 µL of urease (from *Canavalia ensiformis* type III) solution and this mixture was incubated at 30°C for 15 minutes. The substrate solution was prepared by mixing urea (100 mM) and 40 µL of phosphate buffer. The enzyme and substrate solutions then stirred and reincubated for 30 minutes. After that, 50 µL each of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 µL of alkali reagent (0.5% w/v sodium hydroxide NaOH and 0.1% sodium hypochlorite NaOCl) were added to the mixtures. After incubation for 50 minutes, the change in absorbance was read at 630 nm.

The following equation was used to calculate the antiurease activity:

Antiurease activity (Inhibition %) = ($A_{control}$ – A_{sample}) / $A_{control}$ x 100

Antimicrobial activity

In vitro antibacterial activities of the *Z. clinopodioides* extracts and standard compounds were investigated by microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI, 1997, 2006, 2010). Minimum inhibitory concentrations (MICs) of the samples were determined against *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, and *Candida tropicalis* ATCC 750. Minimum inhibitory concentrations (MICs) of compounds were determined by microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI, 1997, 2006). Cefuroxime-sodium, cefuroxime, ceftazidime, amikacin, amphotericin B, and clotrimazole were used as standard compounds for bacteria and yeast. The antimicrobial effects of the solvents were tested against the microorganisms as a control.

Cytotoxicity assay on renal and colon cell lines

A two-day assay was used to determine the cytotoxic potential of the extracts. XTT bioassay is an in vitro antitumor colorimetric assay developed by the MTP (Molecular Targets Program), Assay Development and Screening Section, NCI (National Cancer Institute). The renal cancer cell lines (UO-31 and A498) and colon cancer cell lines (COLO205 and KM12) were used in the assay. All details of the assay was performed according to Cho et al. (Cho et al., 2017).

Metastatic potential assay

The metastatic potential of the extracts was identified by using XTT assay, developed by the MTP (Molecular Targets Program), Assay Development and Screening Section, NCI (National Cancer Institute), via comparing the effects of the extracts on high (MG63.3) and low (MG63) metastatic potential osteosarcoma cell lines. The cells were plated in tissue culture plates and allowed to attach overnight followed by 2 day treatment with the extracts. Relative cell numbers we re assessed using the XTT assay. The results were evaluated to determine which extract has activity ≤50% for MG63.3 and ≥50% for MG63, and the difference between the two values must be ≥50%.

Statistical analysis

All measurements were repeated three times. The results were evaluated using t test with Microsoft Excel and expressed as mean ± standard deviation. Differences were considered significant at $p<0.05$

RESULTS AND DISCUSSION

LC-MS/MS results

The chemical composition of the extracts is presented in Table 1. In the ethanol extracts obtained from aerial parts and roots of *Z. clinopodioides*, the major compounds were detected as quinic acid, malic acid, and rhoifolin by LC-MS/MS. The quantities of the compounds in the aerial parts extract were calculated as 9020.51±73.97 µg/g, 1972.95±22.29 µg/g, and 1972.95±22.29 µg/g, respectively. In the roots extract, the amounts of the compounds were determined as 14721.04±120.71 µg/g, 2179.04±24.62 µg/g, and 3593.31±338.13 µg/g, respectively. The current study results also showed that the extracts contain some flavonoids: hesperidin, naringenin, rutin, apigenin, and isoquercetin, as well as some phenolic acids.

Despite the fact that there are an abundance of studies focused on the chemical composition of the essential oil of *Z. clinopodioides*, to our knowledge, there is only one study about the identification of flavonoid content of the extracts obtained from *Z. clinopodioides*. According to that previous study, ten flavonoid compounds present in the extract, namely, baicalein, quercetin, hyperoside, quercetin-3-O-*β*-*D*-glucopyranoside, apigenin, kaempferol, chrysin, diosmin, linarin and rutin (Zhang et al., 2018). It must be noted that, the current study is an original study to contribute to the chemical profile of the species in detail. The quinic acid, malic acid, rhoifolin, hesperidin, p-coumaric acid, caffeic acid, salicylic acid, ferulic acid, chlorogenic acid, rosmarinic acid, protocatechuic acid, fumaric acid, vanillin, hesperetin, chrysin, apigetrin, nicotiflorin, naringenin, and isoquercetin were observed in *Z. clinopodioides* extracts for the first time.

Total phenolic and flavonoid content

The results of the total phenolic and flavonoid content of ethanol extracts *Z. clinopodioides* aerial and root parts are represented in Table 2. In the study of Unal et al. (2008), total phenolic compounds of the acetone, ethanol and water extracts of *Z. clinopodioides* were given as gallic acid equivalents. The highest total phenolic content was found in the acetone extract (Unal et al., 2008). The total polyphenolic and flavonoid content of petroleum ether, chloroform, ethyl acetate, n-butanol and ethanol extracts of *Z. clinopodioides* were studied in Tian et al. (Tian, Shi, Zhou, Ge, & Upur, 2011). The total polyphenolic content of the extracts were determined as gallic acid equivalents and ethyl acetate (19.27%), chloroform (4.99%), n-butanol (3.94%), ethanol (1.64%) and petroleum ether (0.23%), respectively (Tian et al., 2011). The total flavonoid content of the extracts were determined as rutin equivalents and concentrated in parts of ethyl acetate (65.61%), chloroform (14.36%) and n-butanol (10.76%) extracts (Tian et al., 2011). In the study Alp et al. (2016), the total phenolic content of essential oils of eight *Z. clinopodioides* ecotypes were investigated and determined as gallic acid equivalents. These ranged from 43.41 to 55.71 mg GAE/100 g fresh weight (Alp et al., 2016). In the study of Mahboubi et al. (2014), five plants belong to Labiatae family including *Thymus vulgaris, Thymus caramanicus, Zataria multiflora, Ziziphora clinopodioides* and *Ziziphora tenuior* hydroethanolic extracts were evaluated for antimicrobial properties and total phenolic content. The total phenolic content were determined as gallic acid equivalents and amount of total phenolics ranged from 3.785 to 10.247% of the dry extract. The total phenolic content of *Ziziphora clinopodioides* and *Ziziphora tenuior* were found to be 3.785 and 4.007%, respectively (Mahboubi, Kamalinejad, Ayatollahi, & Babaeian, 2014). Gursoy et al. studied the total phenolic content of methanol extracts of three different plants including *Z. clinopodioides* and the amount of the total phenolics was highest in *Z. clinopodioides* extract (129.55±2.26 µg/mg) in the studied plants (Gursoy et al., 2009). In the study

Table 1. Chemical compositions of *Ziziphora clinopodioides* extracts.

No	Analytes	RT[a]	Parent ion (m/z)	Daughter Ions	Ion. Mode	Quantification (µg analyte/g extract)[b]	
						ZCH	ZCR
1	Coumarin	17.40	147.05	91.0-103.2	Poz	N.D.	N.D.
2	Hesperidin	12.67	610.90	303.1-465.1	Poz	429.27±11.25	12.32±0.32
3	*p*-Coumaric acid	11.53	162.95	119.25-93.25	Neg	243.22±12.55	38.25±1.97
4	*o*-Coumaric acid	15.45	162.95	119.35-93.25	Neg	N.D.	N.D.
5	Gallic acid	3.00	168.85	125.2-79.2	Neg	N.D.	N.D.
6	Caffeic acid	8.80	178.95	135.2-134.3	Neg	272.71±9.65	27.61±0.98
7	Vanilic acid	8.57	166.90	152.25-108.25	Neg	N.D.	N.D.
8	Salicylic acid	11.16	136.95	93.3-65.3	Neg	132.42±4.36	28.56±0.94
9	Quinic acid	1.13	190.95	85.3-93.3	Neg	**9020.51±73.97**	**14721.04±120.71**
10	p-Hydroxybenzoic acid	7.39	136.95	93.3-65.3	Neg	N.D.	N.D.
11	Ferulic acid	12.62	192.95	178.3	Neg	46.16±2.28	97.79±4.83
12	Chlorogenic acid	7.13	353.15	191.2	Neg	22.03±0.15	N.D.
13	Rosmarinic acid	14.54	359	161.2-197.2	Neg	180.19±12.85	N.D.
14	Protocatechuic acid	4.93	152.95	108.3	Neg	118.15±4.86	199.48±8.20
15	Cinnamic acid	25.61	147.00	103.15-77.3	Neg	N.D.	N.D.
16	Sinapinic acid	12.66	222.95	208.3-149.2	Neg	N.D.	N.D.
17	Fumaric acid	1.48	115.00	71.4	Neg	687.11±8.52	810.45±10.05
18	Vanillin	10.87	151.00	136.3-92.2	Neg	47.42±1.32	437.01±12.24
19	Pyrocatechol	6.48	109.00	108.35-91.25	Neg	N.D.	N.D.
20	Malic acid	1.23	133.00	115.2-71.3	Neg	**1972.95±22.29**	**2179.04±24.62**
21	Syringic acid	9.02	196.95	182.2-167.3	Neg	N.D.	N.D.
22	Hesperetin	31.76	300.95	164.2-136.2	Neg	N.D.	2.09±0.12
23	Naringenin	30.68	270.95	151.2-119.3	Neg	21.47±1.12	N.D.
24	Rutin	12.61	609.05	300.1-271.1	Neg	90.85±1.44	N.D.
25	Quercetin	28.17	300.90	151.2-179.2	Neg	N.D.	N.D.
26	Quercitrin	16.41	447.15	301.15-255.15	Neg	N.D.	N.D.
27	Apigenin	31.43	268.95	117.3-151.2	Neg	108.74±7.07	N.D.
28	Chrysin	36.65	252.95	143.3-119.4	Neg	65.35±1.31	484.9±9.74
29	Liquitrigenin	25.62	254.95	119.25-135.15	Neg	N.D.	N.D.
30	Isoquercitrin	13.42	463.00	300.15-271.15	Neg	28.11±0.37	N.D.
31	Apigetrin	16.59	431.00	268.2-239.2	Neg	82.82±4.94	N.D.
32	Rhoifolin	16.11	577.05	269.2-211.15	Neg	**1044.74±98.31**	**3593.31±338.13**
33	Nicotiflorin	14.68	593.05	285.1-255.2	Neg	591.75±16.33	261.68±7.22
34	Fisetin	19.30	284.95	135.2-121.25	Neg	N.D.	N.D.
35	Luteolin	28.27	284.75	133.2-151.2	Neg	N.D.	N.D.
36	Myricetin	18.72	317.00	179.15-151.25	Neg	N.D.	N.D.
37	Kaempferol	31.88	284.75	255.1-117.3	Neg	N.D.	N.D.

[a]RT: Retention time, [b]Values in µg/g (w/w) of plant extracts. N.D: not detected; ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR**:** The ethanol extract of *Z. clinopodioides* root

of Salehi et al. (2005), the total phenolic content of various extracts (methanol, water, water soluble methanol extract, water insoluble methanol extract, acetone, ethyl acetate and deodorized hot water extract) of *Ziziphora clinopodioides* subsp. *rigida* were investigated and methanol extract showed the biggest total phenolic content (174.8±1.2 gallic acid equivalents) (Salehi, Sonboli, Eftekhar, Nejad-Ebrahimi, & Yousefzadi, 2005). In this study, the total phenolic and flavonoid content

Table 2. Total phenolic and flavonoid contents of *Ziziphora clinopodioides* extracts[a].

Extracts	Phenolic content (μg PEs/mg extract)[b]	Flavonoid content (μg QEs/mg extract)[c]
ZCH	18.73±0.90	27.97±1.31
ZCR	13.67±0.73	4.74±0.22

a: Values are means ± SD of 3 parallel measurements; b: PEs: pyrocatechol equivalents (y=0,0395x + 0,0607 R^2=0,9980); c: QEs: quercetin equivalents (y=0,0325x + 0,0601 R^2=0,9984); ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

of ethanol extracts of aerial and root parts of *Z. clinopodioides* were investigated. Total phenolic content was given as pyrocatechol equivalents and total flavonoid content was given as quercetin equivalents. Results showed that the total flavonoid content (27.97±1.31 μg QE/mg extract) of the aerial parts of *Z. clinopodioides* extract was higher than the total phenolic content (18.73±0.90 μg QE/mg extract), and the total phenolic and flavonoid contents of aerial parts of *Z. clinopodioides* were higher than the root extract.

Antioxidant capacity

All literature related to DPPH free radical scavenging activity of *Ziziphora* species were given in this section. In the study of Salehi et al. (2005), the DPPH scavenging activities of essential oil and various extracts (methanol, water, water soluble methanol extract, water insoluble methanol extract, acetone, ethyl acetate and deodorized hot water extract) of *Z. clinopodioides* subsp. *rigida* were investigated and the methanol extract showed the highest free radical scavenging activity with IC_{50}:30.7±0.6 μg/mL (Salehi et al., 2005). In the study of Unal et al. (2008), chloroform, acetone, ethanol and water extracts of 25 plants including *Z. clinopodioides,* mostly used as remedies against various diseases in Turkish traditional medicine, were investigated for antimicrobial and antioxidant activities. Inhibition percentages of acetone, ethanol and water extracts of *Z. clinopodioides* were determined at 30-45% at 100 μg/mL concentration (Unal et al., 2008). Petroleum ether, chloroform, ethyl acetate, n-butanol and ethanol extracts of *Z. clinopodioides* were investigated for DPPH free radical scavenging activity in the study of Tian et al. (2011) The ethyl acetate extract showed the best activity with 34.11±0.54% at 1mg/mL concentration in all extracts and standard compounds of Vitamin C (Tian et al., 2011). *Z. capitata* methanol extract was investigated for DPPH radical scavenging activity and found IC_{50}:206.6±1.3 μg/mL value in the study of Mohammadhosseini et al. (2016) (Mohammadhosseini et al., 2016). Alp et al. studied eight ecotypes of *Z. clinopodioides* essential oils and they found DPPH free radical scavenging activity ranged from IC_{50}:3.60 to 4.20 mg/mL (Alp et al., 2016). In the other study related to the DPPH free radical scavenging activity of *Z. clinopodioides*, methanol extracts of three plants *Ziziphora clinopodioides, Cyclotrichium niveum*, and *Mentha longifolia* ssp. *typhoides* var. *typhoides* were investigated for antioxidant activity with DPPH free radical scavenging assay. *Z. clinopodioides* methanol extract had the highest activity with IC_{50}=37.73±1.18 μg/mL in the studied plants (Gursoy et al., 2009). In our study, aerial parts of *Z. clinopodioides* extract showed moderate DPPH free radical scavenging activity with IC_{50}=94.27±0.65 μg/mL value and better activity than root extract (Table 3). Our results confirmed those from the literature survey.

Table 3. DPPH free radical and ABTS cation radical activities of *Ziziphora clinopodioides* extracts.

Samples	IC_{50} values (μg/mL)*	
	DPPH Free Radical	ABTS Cation Radical
ZCH	94.27±0.65	40.69±0.19
ZCR	124.51±0.66	45.61±1.01
α-TOC	16.30±0.79	10.20±0.05
BHA	7.88±0.20	2.74±0.03
BHT	58.86±0.50	3.16±0.06

*Values expressed are means ± standard deviation of three parallel measurements; ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

Based on the literature review, there has not been a study about ABTS cation radical scavenging activity and cupric reducing antioxidant capacity (CUPRAC) assays of *Ziziphora* species previously . This is the first study of *Z. clinopodioides* ABTS cation radical scavenging activity and CUPRAC assay. Ethanol extracts of aerial and root parts of *Z. clinopodioides* have shown moderate inhibition of ABTS cation radicals with 40.69±0.19 and 45.61±1.01 μg/mL IC_{50} values, respectively (Table 3). The extracts did not show any activity in *β*-carotene linoleic acid test system. In CUPRAC assay, ethanol extracts of aerial and root parts of *Z. clinopodioides* have shown moderate inhibition with similar absorbance values that are 1.74±0.083 and 1.74±0.090 at 100 μg/mL concentration, respectively (Table 4).

Enzyme inhibition activities

Z. clinopodioides aerial parts extract showed only very low tyrosinase inhibitory activity. No activity was observed against other enzymes; including acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and urease enzymes (Table 5).

There are almost no *in vitro* anti-Alzheimer activity studies of *Ziziphora* species in the literature except the study of Ozdemir et al. (2013). In the study of Ozdemir et al., *Z. clinopodioides* extract was prepared with sodium phosphate buffer inhibited AChE of erythrocytes dose dependant manner. There was approximately 40% inhibition of AChE of erythrocytes at 200 μg/mL concentration (Ozdemir, Turkoglu, & Demir, 2013). In our study there were no acetylcholinesterase and butyrylcholinesterase inhibition activities. This difference is observed due to the variance in the chemical compositions of different extracts.

Table 4. CUPRAC activity of *Ziziphora clinopodioides* extracts*.

Samples	10 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL
ZCH	0.28±0.0105	0.59±0.019	1.05±0.048	1.74±0.083
ZCR	0.32±0.074	0.53±0.0225	1.05±0.0427	1.74±0.0898
α-TOC	0.48±0.017	1.04±0.020	1.94±0.042	3.41±0.136
BHA	1.23±0.025	2.03±0.021	2.98±0.106	3.86±0.064
BHT	1.40±0.053	2.47±0.075	3.01±0.112	3.95±0.102

*The results are given as absorbance value (Values expressed are means ± standard deviation of three parallel measurements); ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

Table 5. Enzyme inhibitory activities of *Ziziphora clinopodioides* extracts.

Samples	AChE (Inhibition %)[a]	BChE (Inhibition %)[a]	Tyr (Inhibition %)[a]	Urease (Inhibition %)[a]
ZCH	NA	NA	8.60±0.87	NA
ZCR	NA	NA	NA	NA
Galanthamine[b]	78.92±1.04	78.22±0.58	-	-
Kojic acid[b]	-	-	95.26±0.23	-
Tiyourea[b]	-	-	-	88.61±1.16

*Values expressed are means ± standard deviation of three parallel measurements; [a]200 µg/mL, [b]Standard compound , NA: Not Active, Not tested; ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

According to the literature , there have been no reports about any tyrosinase and urease inhibition activities on *Z. clinopodioides* extracts. The ethanol extract of aerial parts of *Z. clinopodioides* showed very weak activity against tyrosinase with 8.60±0.87 inhibition %.

Antimicrobial activity

The MIC values of the extracts and standard compounds are summarized in Table 6. The in-vitro antimicrobial activities were evaluated against different Gram-positive and Gram-negative bacterial strains in addition to the antifungal activities. The results showed that both extracts possessed moderate activity against *C. tropicalis* with MIC values of 39.06 µg/mL. The results reveal that there is no difference in antimicrobial activity between the ethanol extracts of aerial parts and roots, as the antifungal activity of the extracts were able to induce appreciable growth inhibitory activity against *Candida* spp.

Cytotoxic activities

The cytotoxic activity results of the extracts on Renal and Colon cell lines were presented in Table 7. According to the results, the extracts did not show any activity against Renal (A498 and UO-31) and Colo (COLO205 and KM12) cell lines.

Metastatic potential

The metastatic potential result of the extracts was given in Table 8. The results showed that the extracts do not have any metastatic potential against high and low metastatic osteosarcoma cell lines (MG63.3 and MG63).

Table 6. Antimicrobial activity of *Ziziphora clinopodioides* extracts.

Microorganisms	MIC values of the extracts (µg/mL)	
	ZCH	ZCR
P. aeruginosa ATCC 27853	NA	NA
E. coli ATCC 25922	NA	NA
K. pneumoniae ATCC 4352	NA	NA
P. mirabilis ATCC 14153	NA	NA
S. aureus ATCC 29213	625	NA
S. epidermidis ATCC 12228	1250	312.5
E. faecalis ATCC 29212	1250	1250
C. albicans ATCC 10231	NA	NA
C. parapsilosis ATCC 22019	312.5	NA
C. tropicalis ATCC 750	39.06	39.06

NA: No Activity. Standards; Cefuroxime-Na: 1.2 µg/mL for *S. aureus* ATCC 29213, Cefuroxime 9.8 µg/mL for *S. epidermidis* ATCC 12228, Amikacin 128 µg/mL for *E. faecalis* ATCC 29212, Ceftazidime 2.4 µg/mL for *P. aeruginosa* ATCC 27853, Cefuroxime-Na: 4.9 µg/mL for *E. coli* ATCC 25922 and *K. pneumoniae* 4352, Cefuroxime-Na 2.4 µg/mL for *P. mirabilis* ATCC 14153, Clotrimazole 4.9 µg/mL for *C. albicans* ATCC 10231, Amphotericin B 0.5 µg/mL for *C. parapsilosis* ATCC 22019, Amphotericin B 1 µg/mL for *C. tropicalis* ATCC 750; ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

Table 7. Cytotoxic activity results of *Ziziphora clinopodioides* extracts.

Extracts	Cell viability (%)			
	Renal Cells		Colo Cells	
	A498	UO-31	COLO 205	KM12
ZCH	84.53	80.01	77.51	>99.00
ZCR	63.32	95.94	92.79	>99.00

ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

Table 8. Metastatic potential results of *Ziziphora clinopodioides* extracts.

Extracts	Cell viability (%)	
	Osteosarcoma cells	
	MG 63.3	MG 63
ZCH	99.17	90.55
ZCR	99.33	97.33

ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

CONCLUSION

Ziziphora clinopodioides is an edible plant which is widespread in mainly in central and eastern Turkey. It is traditionally used for its stomachic, carminative and antimicrobial activities. In this study, an LC-MS/MS analysis was performed for the first time to determine the chemical composition of the ethanol extracts of the aerial parts and the roots of the plant which was collected from Ardahan. It has been shown that the plant is rich in terms of phenolic compounds. Phenolic compounds are known to be beneficial to human health. Due to the rich mixture of these phenolic compounds, the extracts showed biological activities including anti-tyrosinase and antimicrobial activities *in vitro* as expectedly**.** *Furthermore* , the extracts have not shown any cytotoxic effects, which means it can be considered safe to use as food ingredients. In conclusion, this study provides evidence that *Z. clinopodioides* is a safe plant for consumption and has important potential medical uses that will benefit from further investigations.

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