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Research Article

Chemical Composition and *in Vitro* Antibacterial Effect of *Ziziphora clinopodioides* Essential Oil

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ABSTRACT

Background: Plants of genus *Ziziphora* are widely used as carminative, stomach tonic, expectorant and antiseptic in different parts of Iran. The aim of the current study was to determine chemical composition of *Ziziphora clinopodioides* essential oil and evaluate its antibacterial activity against common food-borne pathogens (*Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Listeria monocytogenes, Salmonella typhimurium* and *Escherichia coli* O157:H7) with broth micro-dilution and agar disk diffusion methods. ***Methods:*** The chemical composition of the essential oil was identified using gas chromatography coupled with mass spectrometer detector (GC-MS). The antibacterial activity of *Z. clinopodioides* essential oil was evaluated by micro-dilution method in BHI broth medium and agar disk diffusion assay. ***Results:*** According to results of GC-MS analysis, carvacrol (64.2%) followed by thymol (19.2%), *p*-cymene (4.8%) and *γ*-terpinene (4.6%) were the abundant components of the essential oil. The results revealed that the essential oil exhibited strong levels of antibacterial activity against all the tested microorganisms. Regarding the MIC and MBC values, *S. typhimurium* and *E. coli* O157:H7 were more resistance to the essential oil than the other bacteria. ***Conclusion:*** Our findings indicated that *Z. clinopodioides* essential oil had a potential to be applied as antimicrobial agent.

Introduction

Essential oils could be extracted from different parts like leaves, stems, flowers, roots including bushes and trees through distillation. They are effective on a wide range of Gram-negative and positive bacteria such as *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Listeria monocytogenes, Salmonella typhimurium and Escherichia coli* O157:H7.[1,2] In recent years, interest in essential oils has been increased for pharmacological studies which claim that the essential oil have beneficial efficacy for the control and inhibition of human and food-borne pathogens and food spoilage microorganism's growth.[3-5] In addition, essential oils are being used in perfumes, cosmetics and for flavoring of foods including meat and meat products and also milk and dairy products.[6]

Genus *Ziziphora* (known as Kakouti Kouhi in Persian) belongs to the family *Lamiaceae* that is consist of about four species including *Z. clinopodioides* Lam, *Z. capitata* L., *Z. persica* and *Z. tenuior* L which are spread worldwide especially Iran and Turkey. Among them, *Z. clinopodioides* as the most common species have been reported from Iran. Generally, the essential and extracts of this plant possess potential activities as antibacterial, anthelmintic, antifungal and antiviral agents. In Iranian folk medicine, the fresh leaves and stem frequently were applied as appetitive, carminative, antiseptic, wound healing material, sedative, stomach tonic, expectorant and antiseptic. Traditionally, this plant has long been used in Iran as spice in different foods such as meat, cheese and doogh to enhance of their flavor and aroma.[7-8] There are various reports about antifungal and antibacterial of essential oil and extract of *Z. clinopodioides*. Moreover, their chemical composition has been investigated previously and recently. All these studies revealed presence of phenolic constituents including pulegone, 1,8-cineole, thymol, carvacrol, *p*-cymene and limonene as major compounds that have been reported to possess potential activity against various Gram-negative and positive bacteria. Also, methyl acetate, iso-neomenthol, iso-menthone and *α*-pinene have been reported as the most important constitutes.[7-12]

Based on knowledge of author, in comparison to many other pharmaceutical-industrial plants, there is a very little data about chemical composition and antibacterial activity of *Z. clinopodioides* essential oil collected from Kermanshah province, west of Iran. Hence, the aim of the current study was (i): determination of chemical composition of its hydro-distilled essential oil obtained from Gilan Gharb city, Kermanshah province, west of

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Iran by GC–MS, (ii): evaluation of antibacterial activity of the essential oil against common food-borne pathogens (*Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella typhimurium and Escherichia coli* O157:H7) with broth micro-dilution and agar disk diffusion methods.

Materials and Methods

Collection of plant material

The fresh leaves of *Z. clinopodioides* plant were collected during the full flowering period at March-July 2014 from Gilane Gharb city, Kermanshah Province (in the west of Iran) and consequently authenticated by Dr. Seyed Mohammad Masoumi, Faculty of Agriculture, Razi University, Kermanshah, Iran. Voucher specimen (No. 6816) of the plant was deposited in the herbarium of the Research Center of Natural Resources of Tehran, Iran.

Isolation of essential oil

To extract the essential oil, the shade dried leaves (100g) of *Z. clinopodioides* plant material at room temperature (25±2°C) were subjected to hydro-distillation using a Clevenger-type apparatus until full recovery of essential oil (for 3.5h). Then, the essential oil on top of the distillate was collected and dried over anhydrous sodium sulfate (0.5g) (Merck, Darmstadt, Germany) until the last traces of water removing, kept in dark glass bottle, covered with aluminum foil, filtered and stored at 4±1°C prior GC–MS analysis and further use (antibacterial assay).

Gas chromatography–mass spectrometry (GC–MS) analysis of essential oil

The most volatile chemical constituents of *Z. clinopodioides* essential oil was identified using gas chromatography coupled with mass spectrometer detector (Thermo Quest Finningan, UK) (GC-MS). The GC-MS apparatus was HP-5MS (5% phenyl methyl silicone and 95% dimethylpolysiloxane) was equipped with DB5 capillary column (30m length, 0.25mm ID, 0.25µm stationary thickness). An electron impact (EI) ionization system, with ionization energy of 70eV was employed for detection of oil constituents. Helium (Purity: 99.99%) was the carrier gas at constant flow rate 1.2ml per min, with linear velocity of 29.6 cm/s and split ratio was 1:20. Temperature program for the column included: the initial oven temperature was kept at 50°C for 3 min, then temperature was raised from 50°C to 265ºC at program ramp rate 2.5°C per min. The ultimate temperature was 265ºC and maintained for 6°C. The temperature of the injector was 250°C. The GC-MS analysis of chemical constituents of *Z. clinopodioides* essential oil was done in triplicate.

Identification of chemical compounds

The most volatile chemical compounds of the essential oil were identified by comparison between their retention indices (*RIs*) for n-alkanes (C_6-C_{24}), *retention indices* of published data, Standard Mass Spectral fragmentation pattern (Wiley/NBS) and the National Institute of Standards and Technology (NIST). The GC peak area normalization of the three injections was expressed as mean percentage of individual essential oil composition.

Test microorganisms

In the present study, the antimicrobial activity of *Z. clinopodioides* essential oil was examined against pathogenic bacteria including *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (ATCC 11774), *Listeria monocytogenes* (ATCC 19118), *Salmonella typhimurium* (ATCC 14028) and *Escherichia coli* O157:H7 (ATCC 10536). All bacteria were obtained as a lyophilized culture from the culture collection of the Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. Pure cultures of bacterial strains were stored at -20°C by freezing in BHI broth containing 17% (v/v) glycerol. Before the antibacterial tests, all bacteria were sub-cultured in Brain Heart Infusion broth (BHI; Merck, Darmstadt, Germany) medium in twice and incubated at 37°C for 18h. The needed densities of bacterial cultures for the antibacterial tests were examined using a spectrophotometer at 600nm. The determination of inoculum dose (10^6CFU/mL) also was assessed using plate count on Brain Heart Infusion agar (BHI; Merck, Darmstadt, Germany) medium.

Antibacterial Tests

The antibacterial activity of *Z. clinopodioides* essential oil was evaluated by micro-dilution method in BHI broth medium and agar disk diffusion assay.

Determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)

In order to determine the minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations a broth micro-dilution test according Azizkhani *et al.*,[13] was used with some modification. Briefly, 5% (v/v) dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) as an emulsifier and 0.05% (w/v) agar-agar (Merck, Darmstadt, Germany) as a stabilizer of the essential oil were added to BHI broth medium. After autoclaving the medium, different concentrations of the essential oil (0.015, 0.03, 0.06, 0.12, 0.25, 0.5, 1, 1.5 and 2%) were set up using 96-well sterile micro-dilution plates with U-bottom wells. Then, 180µl BHI broth containing different concentrations of the essential oil and 20µl of the final bacteria inoculums (1×10^6 CFU/ml) were added in to each well. As a positive control, the same amount of BHI broth containing DMSO and bacterium inoculum without essential oil was added in to well. Moreover, in each experiment, negative controls, BHI broth containing DMSO and essential oil was considered. Then, the content of plates were shaken at 250 rpm for 30s and

incubated at 37°C for 24h. The minimum inhibitory concentration is described as the lowest concentration of essential oil that prevents the growth of the microorganisms. In order to determine the minimum bactericidal concentration of the essential oil that reduces 99.99% of population bacteria, 20µl of each well without any invisible growth was cultured on BHI agar at 37°C for 24h.[13,14]

Agar disk diffusion assay

The antibacterial activity of leaves of *Z. clinopodioides* essential oil was also evaluated by agar disk diffusion method according to Bajalan *et al.*,[15] with some modification. For this purpose, 100µl of inoculum of bacteria (1×10^8 CFU/ml) were poured on BHI agar and cultured by surface method and sterile cotton swabs. Then, filter paper discs (6 mm Ø; Whatman#1) impregnated with 20µl of essential oil aseptically was placed on the surface of BHI agar plates. Positive (tetracycline) and negative (only DMSO) controls were considered in the present study. Afterward, the plates were incubated 37°C for 24h. In order to evaluate the antibacterial activity, radius of the inhibition zones was measured.[15,16] The zones of inhibition were compared with zone of inhibition produced by antibiotic.

Results and Discussion

Yield and analysis of Ziziphora clinopodioides essential oil

The oil yield of the *Z. clinopodioides* essential oil was 0.65%, calculated on the fresh leaves of plant. The results of the oil yield were in accordance with Behravan *et al.*,[9] which they reported the yield of their essential oil 0.75%, but higher levels of essential oil yields (1.1% and 0.98%) was reported by Amiri[8] and Morteza-Semnani *et al.*,[11] respectively.

Analysis of the obtained essential oil from leaves of *Z. clinopodioides* by GC-MS leads to the identification of eighteen components, representing 99.65% of the total essential oil (Table 1). Regarding the chemical constituents, their relative percentage of the total chromatogram area and Kovats index as it had been shown in Table 1, the oxygenated monoterpenes afforded a main portion of the essential oil (86.1%) with carvacrol (64.2%) and thymol (19.2%) as the major abundant constituents. Monoterpene hydrocarbons the second major class of compounds constituted (11.9%) of the essential oil with *γ*-terpinene (4.6%) and *p*-cymene (4.8%) as the main components, whereas, sesquiterpene hydrocarbons and oxygenated sesquiterpenes comprised 1% and 0.4%, respectively. Chemical composition of the essential oils of plants and spices can vary depending upon genetic and growth stage, age of the plant, part of the plant, seasonal and environmental condition, geographical location, the method used in extraction of the essential oil and other factors.[3,13,17,18]

Table 1. Essential oil composition of *Z. Clinopodioides* identified by GC-MS.

No	Compound name	Composition%	Retention time (min.)	KI[a]
1	*α*-Thujene	0.2	11.33	927
2	*α*-Pinene	0.2	11.71	934
3	Camphene	0.1	12.61	952
4	Myrcene	0.5	14.62	992
5	*α*-Phellandrene	0.1	15.58	1010
6	*α*-Terpinene	0.7	16.11	1021
7	***p*-Cymene**	**4.8**	**16.62**	**1030**
8	Limonene	0.1	16.77	1033
9	*β*-Phellandrene	0.1	16.89	1036
10	***γ*-Terpinene**	**4.6**	**18.31**	**1063**
11	Linalool	0.1	20.5	1105
12	Borneol	0.6	24.36	1183
13	Terpinene-4-ol	0.4	24.7	1190
14	**Thymol**	**19.5**	**29.61**	**1293**
15	**Carvacrol**	**65.2**	**30.57**	**1315**
16	*E*-Caryophyllene	1	35.47	1427
17	Spathulenol	0.1	42.10	1590
18	Caryophyllene oxide	0.3	42.30	1595
	Total	99.65		

*Expressed as percentage of the total peak area
** The dominant compounds are indicated in bold
[a] Kovats index

In case of *Z. clinopodioides* essential oil, chemical composition has been subjected to several studies in different parts of Iran.[7-10] In contrast with our results, some authors reported pulegone, imonene and methyl acetate as the major abundant of their essential oil content.[10] Ozturk and Ercisli, reported that the abundant compounds of *Z. clinopodioides* essential oil collected from the Erzurum–Palandoken mountain of Turkey were the phenolic compounds pulegone (31.8%), 1,8-cineole (12.2%), limonene (10.4%), menthol (9.1%), *β*–pinene (6.8%), menthone (6.7%), piperitenone (5.3%) and piperitone (4.1%).[10] As well as, Behravan *et al.*, found pulegone (44.5 %), terpineol (14.5%), methyl acetate (10.9%), iso-neomenthol (7.1%) and 1, 8-cineole (4.1%) to be the main components of the essential oil obtained from Mashhad, Khorasan Razavi province (North East of Iran).[9] The greatest content of phenolic compounds was reported by Aghajani *et al.*,.[7] Based on their results, carvacrol (8.7%) and thymol (53.6%) were the major compounds of the essential oil of *Z. clinopodioides* plant harvested from the Lorestan provine of Iran. This finding was in agreement with the results of the present study. Moreover, our results are in accordance with Schulz et *al.*,.[19] In contrast with our results, Sonboli *et al.* reported (0.7–44.5%), 1,8-cineole (2.1–26.0%), neomenthol (2.5–22.5%), 4-terpineol (0.0–9.9%), 1-terpineol (0.0–13.2%), neomenthyl acetate (0.0–7.1%), and piperitenone (0.0–5.4%) were the most chemical

composition of *Z. clinopodioides* plant collected from Lashgardar protected region (Hamedan Province, Iran).[12] Morteza-Semnani *et al.*, investigated chemical composition of *Z. clinopodioides* essential oil collected from the suburb of Sari, Mazandaran province, north of Iran and reported that 1,8-cineole (10.4%), iso-menthone (6.0%) and α-pinene (5.6%) were the major components.[11] As described above, variability and diversity in the major constituents depending on numerous factors. Hence, further studies are required to investigate the chemical composition of *Z. clinopodioides* essential oil which grows in different parts of Iran.

Antibacterial activity

In the current study, the results of antibacterial activity of leaves essential oil against six bacterial strains (*S. aureus*, *B. subtilis*, *B. cereus*, *L.monocytogenes*, *S. typhimurium* and *E. coli* O157:H7) by the broth micro-dilution and agar disc diffusion assays were shown in Table 2 and 3.

Table 2. Antibacterial activity of *Z. clinopodioides* essential oil indicated as Minimum Inhibitory/Bactericidal Concentrations-MIC/MBC (µl/ml).

Bacteria	Essential oil		Tetracycline	
	MIC	MBC	MIC	MBC
Gram-positive				
S. aureus	0.0025	0.0025	2	2.5
B. subtilis	0.0012	0.0012	2.5	2.5
B. cereus	0.0012	0.0012	2	2
L. monocytogenes	0.0012	0.0012	2.5	2.5
Gram-negative				
S. typhimurium	0.0025	0.0025	2.5	2.5
E. coli O157:H7	0.0025	0.0025	2.5	2.5

Table 3. Antibacterial effect of *Z. clinopodioides* essential oil by agar disk diffusion assay.

	Bacteria					
	S. aureus	*B. subtilis*	*B. cereus*	*L. monocytogenes*	*S. typhimuriu*	*E. coli* O157:H7
Essential oil	30mm	23mm	23mm	28mm	22mm	26mm
tetracycline	10mm	0.5mm	14mm	13mm	12mm	10mm

As shown in these Tables, the essential oil exhibited strong levels of antibacterial activity against the test microorganisms. Regarding the MIC and MBC values *S. typhimurium* and *E. coli* O157:H7 were more resistance to the essential oil than the other bacteria. Among all bacteria, *S. aureus* exhibited best zone of inhibition (30mm) for the essential oil (Table 2). In general, tested Gram-negative bacteria were more resistant than the Gram-positive bacteria. This phenomenon could be attributed to intrinsic tolerance and compounds of microorganisms especially the presence of hydrophobic lipopolysaccharide outer membrane structure of Gram-negative bacteria which is especially impermeable to essential oil molecules and the nature and combinations of phytocompounds present in the essential oil.[20,21] In other *in vitro* antibacterial investigations it had been reported that *Z. clinopodioides* essential oil had strong antibacterial activity but their effectiveness varied. For example, Ozturk and Ercisli, evaluated the microbial growth inhibition of *Z. clinopodioides* essential oil by disc diffusion method and MIC and reported that the essential oil had high antibacterial activity against *B. subtilis*, *B. cereus* and *L. monocytogenes.*[10] The results of the present study were in agreement to a certain degree with the traditional uses of *Z. clinopodioides*. This plant seems to be a valuable source for antibacterial drugs. Sonboli *et al.*,[12] and Behravan *et al.*,[9] showed the essential oil of *Z. clinopodioides* collected from Khorasan province had strong antibacterial activity against *Staphylococcus epidermidis*, *S. aureus*, *E. coli* and *B. subtilis*. This finding showed a similarity with the results of the present study. It is believed the higher amounts of phenolic compounds exhibited higher antimicrobial properties against microorganisms. The most important reason of the strong antibacterial activities of carvacrol and thymol could be the acidic nature of their hydroxyl group and involvement in the formation of hydrogen bonds.[22]

Conclusion

We can concluded from the present study that the oxygenated monoterpenes followed by monoterpene hydrocarbons, sesquiterpene hydrocarbons and oxygenated sesquiterpenes were the predominate part of the *Z. clinopodioides* essential oil. The essential oil possessed strong antibacterial activity against all tested microorganisms, whereas *S. typhimurium* and *E. coli* O157:H7 showed the most resistance to the essential oil among all the tested bacterial strains. The antibacterial activity of the essential oil was due to the presence of various active compounds. Hence, the phytochemical compounds responsible for the antibacterial effects of bacteria can be subjected to isolation of the therapeutic antimicrobials.

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Conflict of Interest

The author report no conflicts of interest.

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