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ORIGINAL ARTICLE

Antibacterial activity of *Ziziphora clinopodioides* essential oil against Clinical Isolates of Methicillin Resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Staphylococcus aureus is one of the most important causes of community and hospital acquired infections. methicillin resistant S. aureus (MRSA) is a dominant pathogen in postoperative infections that is also multidrug resistant. Therefore, it is important to introduce new sources of drugs such as traditional medicinal plants with antimicrobial properties. We determined the chemical composition of the essential oil of Ziziphora clinopodioides subsp. bungeana from the Jandagh area and its antibacterial activity against clinical isolates of S. aureus, mostly MRSA isolates as well as standard ATCC cultures. The essential oil of the aerial parts of Ziziphora clinopodioides from Jandagh area in Iran was prepared by hydrodistillation and its chemical constituents were analyzed by a combination of capillary GC and GC-MS. Thirty two components were identified of which, Pulegone (33.17 %), Alpha-terpinyl acetate (11.37%), Thymol (10.31%) and Menthone (7.28 %) were the main constituents. The antibacterial activity of the oil was determined against 10 clinical isolates of Staphylococcus aureus and 6 Gram positive and Gram negative bacterial standards by disc diffusion. Minimum inhibitory and bactericidal concentrations (MIC and MBC) were determined by broth microdilution assay. Considerable inhibitory activity was shown against all test bacteria shown by disc diffusion (except P. aeruginosa) and MIC and MBC values were within the range of 0.66 - 2.64 mg/ml for the susceptible organisms.

Key words: *Antibacterial activity, Staphylococcus aureus, essential oil, Ziziphora clinopodioides, Iran.*

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INTRODUCTION

The genus Ziziphora L. (Lamiaceae) consists of four species (*Z. clinopodioides* Lam., Z. capitata L., Z.persica Bunge. and *Z. tenuior* L.) widespread all over Iran [1]. *Z. clinopodioides* Lam. comprises nine subspecies native to Iran. *Z. clinopodioides* subsp. bungeana (Juz.) grows wild in the eastern parts of Iran [2]. In Iranian folk medicine, Ziziphora species have been used as infusion, decoction and maceration for various purposes such as sedative, stomach tonic, heart disorders, common cold, inflammation, depression, diarrhea, expectorant, coughing, antiseptic, migraine, fever and carminative [3,4]. In Iranian folklore, the dried aerial parts of aforementioned species have been frequently used as culinary and spice in food.

The antibacterial activity of the oil of *Z. taurica* subsp. clenioides and Z. taurica has already been studied [5]. survey showed that the oil of Ziziphora species has been found to be rich in pulegone. The main constituents found in the oil of *Z. vychodceviana* and *Z. persica* collected from Kazakhestan were pulegone (57.5% - 66%) and isomenthone (5.1% - 15.7%) [6]. The major constituent found in the oil of Z. tenuior L. has been reported to be pulegone (87.1%) [7]. The essential oil of Turkish endemic Z. taurica subsp.

clenioides was found to contain pulegone (81.9%), limonene (4.5%) and piperitenone (2.3%) (8). The major constituent found in the oil of Z. clinopodioides subsp. bungeana in Iran were pulegone (65.2%), iso-menthone (11.9%), 1,8-cineole (7.8%) and piperitenone (6.5%) [9].

The antibacterial activity of *Ziziphora clinopodioides* from different regions has been reported against a number of bacteria (5,9). However, in almost all studies, susceptible bacterial standards were employed and resistant clinical isolates which are the main concerns of failure in antibiotic therapy were not considered. Among the bacterial pathogens, *Staphylococcus aureus* is one of the most important causes of community and hospital acquired infections [10]. Since reported in 1961, methicillin resistant *S. aureus* (MRSA) has become a dominant pathogen in postoperative infections. In Iran, MRSA related nosocomial infections range from 36 % to 90 % [11,12]. Due to the fact that MRSA isolates are also multidrug resistant and are among the "difficult" infections to cure, alternative therapy, especially the use of medicinal plants has gained interest. We determined the chemical composition of the essential oil of *Ziziphora clinopodioides* subsp. bungeana from the Jandagh area and its antibacterial activity against clinical isolates of S. aureus, mostly MRSA isolates as well as standard ATCC cultures. To the best of our knowledge, there are a few published reports on antimicrobial effects of the *Ziziphora clinopodioides* subsp. bungeana oil, especially against the clinically important antibiotic resistant microorganisms.

MATERIAL AND METHODS

Plant material

The aerial parts of *Ziziphora clinopodioides* were collected at full flowering stage (April/May 2009) from Jandagh in Isfehan Province at an altitude of 1230m. A voucher specimen was deposited at the Herbarium of Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran.

Essential oil preparation

The essential oil of the air-dried sample (100g) was isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia (1988). The distillated oil was dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4°C until analysis.

Essential oil analysis

GC-FID analysis of the oil was conducted using a Thermoquest-Finnigan instrument equipped with a DB-5 fused silica column (60 m x 0.25mm i.d., film thickness 0.25 μm). Nitrogen was used as carrier gas at the constant flow rate of 1.1ml/min. The spilt ratio was 1/50. The oven temperature was raised from 60°C to 250°C at a rate of 5°C/min. The injector and detector (FID) temperatures were kept at 250°C and 280°C, respectively. GC-MS analysis was carried out on a Thermoquest-Finnigan Trace GC-MS instrument equipped with the same column and temperature programming as mentioned for GC. The transfer line temperature was 250°C. Helium was used as the carrier gas at a flow rate of 1.1ml/min with a split ratio equal to 1/50. The constituents of the essential oil were identified by calculation of their retention indices under temperature-programmed conditions for n-alkanes (C6-C24) and the oil on a DB-5 column under the same conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library (Wiley 7.0) or with those of authentic compounds and confirmed by comparison of their retention indices with those reported in the literature [13,14]. Semi-quantification data was obtained by FID area percentages without the use of correction factors.

Bacterial strains

Sixteen bacterial strains were employed including 10 clinical isolates *S. aureus* (8 methicillin resistant and 2 methicillin sensitive) and 6 reference bacterial strains (Bacillus subtilisATCC 465, Enterococcus faecalis ATCC 29737, *Staphylococcus aureus* ATCC 25923, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC10031 and *Pseudomonas aeruginosa* ATCC85327).

Antibacterial activity of *Ziziphora clinopodioides* measured by disc diffusion and broth microdilution

The antibacterial activity of the essential oil and its main components were determined by the disc diffusion method (15). Briefly, 0.1 ml of a suspension of each test microorganism (10^8 cells/ml) was spread on Mueller-Hinton agar plates and discs containing 4.4, 6.5 or 8.7 mg essential oil/disc were placed on the microbial lawns. For preparation of the discs, dried oil was reconstituted in Dimethlyl Sulfoxide (DMSO, Merck) (w/vol) before placing the appropriate amounts onto 6 mm sterile blank discs. For MIC and MBC determinations, the distilled dry oil was reconstituted in DMSO to make the starting stock before preparing the dilutions in Muller Hinton Broth (MHB, Merck) within the range of 64-125mg/ml.

Antibiotic discs (Padtan Teb, Tehran) including penicillin (10 U), erythromycin (15 µg) and gentamycin (10 µg) were also employed. The tests were carried out in triplicate and plates were incubated at 37°C for 24 h. The diameters of inhibition zones were measured following the incubation period and reported in mm. For quantitative determination of susceptibility, minimum inhibitory concentrations (MIC) were measured according to the CLSI protocol by the microdilution broth assay using serial two-fold dilutions of the essential oil in Mueller-Hinton broth (MHB) containing 0.5 % Tween 80 (16). Minimum bactericidal concentrations (MBC) were determined by spreading 100 ml of the contents of all MIC wells that showed no bacterial growth over nutrient agar plates and incubated at 37°C for 24 h. The highest dilution showing at least 99 % inhibition (less than 5 colonies) was recorded as MBC.

RESULTS AND DISCUSSION

Hydrodistillition of *Ziziphora clinopodioides* aerial parts gave an oil yield of 0.5 % (w/w), based on the plant dry weight. Table I shows that the *Ziziphora clinopodioides* essential oil contained 32 components, of which, Pulegone constituted 33.17 % followed by Alpha-terpinyl acetate (11.37 %), Thymol (10.31%) and Menthone (7.28 %) representing 98.59 % of the total oil. Other studies on chemical composition of *Ziziphora clinopodioides have* reported different amounts of these components depending on the ecological regions the plants were collected from, since the environmental conditions affect plant chemical contents [5-9].

Disc diffusion results showed that *Ziziphora clinopodioides essential* oil was highly active against all clinical isolates as well as the ATCC standards in comparison with the antibiotic discs (Table II). The inhibition zones obtained were 16 - 31 mm for the lowest oil concentration (4.4 mg /disc) followed by 23 - 37 mm (6.5 mg/disc) and 25 - 44 mm (8.7 mg/disc) against the ATCC standards except for *P. aeruginosa*. The same results were observed against the clinical isolates of *S. aureus* and inhibition zones of 21 - 29 mm were obtained for the lowest oil concentration. The MIC and MBC values (Table II) were within the range of 0.66 - 2.64 mg/ml for all test organisms except for P. aeruginosa (42.2 mg/ml). The essential oil was equally active against methicillin resistant and sensitive isolates of S. aureus. This is the fact that MIC and MBC values were similar, shows the bactericidal activity of the oil. It may also explain that the target of the active oil components is the cell membrane. Pulegone has a similar structure to carvone which has been shown to affect the cell membrane by dissipation of pH gradient and membrane potential of cells [17]. To our knowledge, this is the first report on anti MRSA activity of *Ziziphora clinopodioides* oil.

Table I. Chemical composition of *Ziziphora clinopodioides* essential oil

Compound	RI*	%	Compound	RI*	%
piperitenone	1226	0.67	Alpha-pinene	939	1.47
Pulegone	1237	33.17	Camphene	954	0.71
Geraniol	1253	7.42	Sabinene	975	0.23
Thymol	1290	10.31	Beta-pinene	979	0.38
Carvacrol	1299	1.16	Myrcene	991	0.94
Alpha-terpinyl acetate	1348	11.37	Alpha Ttrepinene	1017	0.3
Eugenol	1359	0.28	Limonene	1029	2.38
Beta-bourbonene	1388	0.45	1,8-cineol	1031	2.46
Beta-caryophyllene	1419	1.34	Gama-terpinene	1060	1.05
Germacrene-D	1485	1.71	Linalool	1087	0.87
Beta-bisbolene	1506	0.93	Terpinolene	1089	1.17
Gama-cadinene	1514	0.29	Menthone	1141	7.28
Delta-cadinene	1523	0.27	Iso menthone	1164	1.49
Nerolidol	1563	1.18	Menthol	1172	1.28
Spathulenol	1578	0.98	Octanol	800	1.96
Epi alpha cadinol	1646	1.14	Total		98.53

* Retention Index

Table II. Antibacterial activity of *Ziziphora clinopodioides essential* oil against clinical isolates

Microorganism	Inhibition Zone (mm)[a] Oil (mg/disc)						MIC[b])mg/ml(	MBC[c])mg/ml(
	4.36	6.54	8.72	P	Em	Gm		
B. subtilis[*]	28	35	42.3	24	23	23	1.32	1.32
E. faecalis[*]	16	21.7	30	6	19	6	2.64	2.64
S. aureus[*]	309	36.8	43.5	29	24	17	0.66	1.32
E. coli[*]	20.8	29	37	6	20	6	0.66	0.66
K. pneumoniae[*]	23.7	23	25	6	6	20	1.32	1.32
P. aeruginosa[*]	6	6	10	6	6	20	42.2	42.2
S.aureus EH12	21	nt[d]	nt	10	25	17	1.32	1.32
S.aureus EH18	21.3	nt	nt	11	25	13	0.66	0.66
S.aureus EH24	25	nt	nt	6	10	6	1.32	1.32
S.aureus EH27[**]	25.3	nt	nt	23	26	11	2.64	2.64
S.aureus EH29	28	nt	nt	6	6	6	1.32	1.32
S.aureus EH35[**]	27	nt	nt	29	27	17	2.64	2.64
S.aureus EH42	27	nt	nt	6	10	6	1.32	1.32
S.aureus EH48	29	nt	nt	6	6	6	2.64	2.64
S.aureus EH50	24.5	nt	nt	6	24	18	2.64	2.64
S.aureus EH53	26	nt	nt	6	17	11	1.32	1.32

[a] No inhibition zones (6 mm); resistant, 10-14; intermediately resistant and >15; sensitive.
[b] Minimum inhibitory concentration
[c] Minimum bactericidal concentration
[d] Not tested
[*] ATCC standard cultures
[**] Methicillin sensitive isolates
►Values are means of 3 repeats

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