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EXPERIMENTAL PAPER

Chemical composition and antifungal activities of *Ziziphora tenuir* and *Z. clinopodioides* essential oils against dermatophytes

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Summary

Introduction: *Ziziphora* species are traditionally used for treatment of different infectious and non-infectious diseases as antiseptic agents.

Objective: The aim of this study was to evaluate the chemical composition of *Ziziphora clinopodioides* and *Z. tenuir* essential oils and their antifungal effects against five strains of dermatophytes.

Methods: GC and GC-MS methods were used for essential oils analyze. The anti-elastase activities were determined by porcine pancreatic elastase assays.

Results: 48 different compounds were identified in these two essential oils, which thymol, *p*-cymene, 1,8-cineole and γ -terpinene were their major components. The anti-dermatophyte activities of essential oils against dermatophytes showed that the essential oils (150 ppm) inhibited the mycelium growth, about 5–100%, which *Z. clinopodioides* essential oil had higher mycelium growth inhibition (28–100%) than that of *Z. tenuir* oil. The MIC and MFC values of essential oils were 0.01–1 μ l/ml. 0.5 μ l/ml essential oils inhibited porcine pancreatic elastase, dose-dependently.

Conclusions: Due to the anti-dermatophyte and anti-elastase effects of *Ziziphora* sp., it can be considered as natural antifungal agent for more clinical and pre-clinical trials.

Key words: *Anti-dermatophyte activity, Ziziphora sp., anti-elastase activity*

INTRODUCTION

Dermatophytosis as one superficial skin infections is caused by dermatophytes. *Microsporum* and *Trichophyton* are the most common dermatophytes, with high incidence in different parts of the world [1]. Produced proteolytic enzymes by dermatophytes as one virulence factor have crucial role in virulence of dermatophytes [2]. Treatment of fungal infections is more difficult than bacterial ones, because of fewer antifungal agents, high adverse effects, and appearance of drug-resistant strains [3]. Therefore, developing the new anti-dermatophyte agents with high efficacy, especially among medicinal plants [4], as natural sources of various metabolites or novel molecules, is essential [5].

Ziziphora genus (*Lamiaceae* family), with common name of "Kakuti" [6] has four species in Iran (*Z. clinopodioides*, *Z. tenuir*, *Z. persica* and *Z. capitata*) [7]. In Iran, *Ziziphora* sp. were used traditionally for treatment of some ailments such as common cold, heart ailments, gastrointestinal disorders, inflammation, depression, diarrhea and wound injuries [8, 9]. Generally, *Ziziphora* sp. essential oils and extracts potentially are known as antibacterial, anthelmintic [6], anti-dermatophyte [10], and antiviral agents [11]. The antibacterial activities of *Ziziphora* sp. essential oils were confirmed against Gram-positive and Gram-negative bacteria [12]. Although, the anti-dermatophyte effects of *Z. clinopodioides* essential oil towards dermatophytes obtained from patients with dermatophytosis [10] was the subject of one study, but there is no article comparing the antifungal activities of *Z. tenuir* and *Z. clinopodioides* essential oils against dermatophytes. Therefore, due to the effect of chemical compositions of essential oil on its antimicrobial activity, the chemical composition of *Z. clinopodioides* and *Z. tenuir* aerial parts essential oils and their antifungal effects were evaluated against four strains of dermatophytes. Furthermore, the potency of these essential oils was evaluated against elastase enzyme as one of virulence factor of dermatophytes.

MATERIAL AND METHODS

Ziziphora sp. essential oil's extraction and chemical composition analysis by GC and GC-MS

Ziziphora clinopodioides and *Ziziphora tenuir* aerial parts at full flowering stages were collected from

Urmia suburb (Urmia Province, Iran) in June 2015.

The samples were identified and confirmed at Agriculture Department, Research Centre of Barij, Kashan, Iran. Each herbarium sample was deposited under the voucher numbers of 234-1 (*Z. clinopodioides*) and 192-1 (*Z. tenuir*).

The essential oils were extracted from aerial parts of plants by hydro-distillation method in Clevenger type apparatus. Essential oils were kept in sterile dark vials and stored at 4°C until used in the experiments.

The chemical compositions of essential oils were analyzed by GC-MS using a Thermofinnigan Trace GC/MS single quadruple mass spectrometer with AS 800 auto sampler. Capillary column, DB-5 (30 m × 0.25 mm, film thickness 0.25 μm) were used. The column temperature was kept at 60°C for 5 min and then at 250°C for 10 min. The injection volume was 0.2 μl with split ratio of 1/100. Helium as carrier gas had the flow rate of 1.1 ml/min. MS was taken at 70 eV electron ionization, trap current 150 μA, and source temperature 200°C. Identification was done by comparison of their retention indices (RI), mass spectra fragmentation with those on the stored Wiley 7n.1 mass computer library, and NIST (National Institute of Standards and Technology) [13].

Dermatophytes strains and their preparation

Trichophyton mentagrophytes ATCC 5054, *Trichophyton rubrum* ATCC 5143, *Microsporum canis* ATCC 5069, *Microsporum gypseum* ATCC 5070, *Trichophyton schoenleinii* ATCC 5221, were used. Dermatophytes were cultured on Sabouraud dextrose agar (SDA) medium and incubated at 20-25°C for 5-7 days. Microbial suspensions were prepared by suspending dermatophytes in sterile normal saline solution with 0.05% Tween 80, followed by adjusting its turbidity to 105-106 CFU/ml in RPMI 1640 (Sigma).

Evaluation of antifungal activity

The antifungal evaluations were performed by micro-broth dilution assay and mycelium inhibition techniques as described below:

Micro-broth dilution assay

Determination of minimal inhibitory concentration (MIC) and minimal fungicidal concentration

(MFC) of essential oils were performed by micro broth dilution assay. The essential oils were dissolved in DMSO and serially diluted in RPMI 1640 (sigma) medium at final concentrations in the ranges of 64–0.15 mg/ml. Then, 100 μ l of each concentration of solutions and 100 μ l of diluted fungal suspensions (103–104 CFU/ml) were added to the wells of 96-microtiter plates. The plates were incubated at 20–25°C for 5–7 days. The MICs were reported as the lowest concentration of oils that show no visible microbial turbidity in the wells. The first well that had no fungal growth on agar media was defined as MFC value (mg/ml) [14].

Mycelium inhibition techniques

Ziziphora sp. essential oils were mixed with SDA medium at final concentrations of 150 ppm. In control sets, sterilized water instead of essential oil was used. Then, 6 mm diameter of mycelial discs were cut out from cultures and inoculated on the surface of each prepared agar mediums. Inoculated plates were incubated as above and the observations were recorded on the seventh day [15].

According to $(dc - dt)/dc \times 100$ formula, the percentage of inhibitory effects of *Ziziphora* essential oils against mycelium growth of dermatophytes were calculated (where dc = fungal colony diameter in control sets, dt = fungal colony diameter in treatment sets).

Elastase inhibition effects of essential oils

The activity of *Ziziphora* essential oils on porcine pancreatic elastase inhibition (Type IV, Sigma) were measured using Suc-Ala-Ala-Ala-pNA as the

substrate by spectrophotometrical method. The reaction mixture contained different concentrations of essential oils (1–0.125 μ l/ml) dissolved in 0.2 M Tris-HCl buffer (pH 8.0) and 0.2U porcine pancreatic elastase. The reaction was started by adding the 0.8 mM substrate and production of p-nitroaniline was monitored at 405 nm using a 96-well reader for 20 min at 37°C [16]. The inhibition percent of elastase was calculated according to formula:

$$(1-B/A)*100$$

where A is enzyme activity without essential oil and B is activity in presence of oils.

The experiment was performed in triplicate.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSION

Chemical composition of *Ziziphora* sp. essential oils

Ziziphora sp. essential oils were analyzed by GC-MS and resulted in identification of 48 different compounds, representing 99.7% and 100% of total *Z. tenuir* and *Z. clinopodioides* essential oils content, respectively. Thymol (54.4%), (E)-caryophyllene (6.8%), carvacrol methyl ether (5.3%), γ -terpinene (5.1%) and *p*-cymene (4.6%) were the main components of *Z. clinopodioides* essential oil. α -terpineol (19.5%), thymol (13.9%), *p*-cymene

Table 1.

The chemical composition of *Ziziphora clinopodioides* and *Z. tenuir* essential oils

Compound	RI	<i>Z. clinopodioides</i> [%]	<i>Z. tenuir</i> [%]
α -Thujene	926	1.8	–
α -Pinene	933	1.2	3.2
Camphene	948	0.6	1.64
Sabinene	974	–	0.5
β -Pinene	977	0.5	–
β -Myrcene	989	1.9	2.5
α -Phellandrene	1005	0.5	0.7
α -Terpinene	1016	1.9	0.3
<i>p</i> -Cymene	1024	4.6	8.8
Limonene	1028	0.6	0.7
1,8-Cineole	1031	3.5	4.2
β -Trans-ocimene	1045	0.1	3.0
γ -Terpinene	1058	5.1	2.5
Sabinene hydrate	1066	0.3	–
Isoterpinolene	1086	–	0.2

Terpinolene	1088	0.2	0.2
Linalool	1099	1.3	3.8
Nonanal	1102	0.3	-
Camphor	1143	-	1.5
β -Terpineol	1159	-	2.7
Borneol	1170	2.2	2.2
Terpinen-4-ol	1182	0.98	2.3
α -Terpineol	1197	0.1	19.5
α -Terpineol	1206	0.3	-
trans-Dihydrocarvone	1214	0.1	-
cis-Geraniol	1222	0.1	5.83
Thymol methyl ether	1237	0.1	1.0
Carvacrol methyl ether	1247	5.3	-
Thymol	1308	54.4	13.9
Carvacrol	1312	2.6	2.6
Geranial	1320	-	0.8
Germacrene	1363	-	0.8
Geranyl acetate	1383	-	5.2
(E)-caryophyllene	1423	6.8	3.4
Aromandendrene	1441	0.1	0.1
α -Humulene	1455	0.2	0.1
Geranyl propionate	1475	-	0.2
Bicyclgermacrene	1494	-	0.5
Viridiflorene	1496	0.3	-
α -Muurolene	1499	-	0.2
epi-Bicyclosesquiphellandrene	1506	-	0.6
epi-Muurolene	1513	-	0.4
Geranyl isobutyrate	1514	-	0.2
δ -Cadinene	1530	-	0.3
(E)- α -Bisabolene	1543	1.5	0.3
Spathulenol	1580	0.2	1.2
Caryophyllene oxide	1585	0.2	0.2
Farnesol	1689	-	1.5

RI – retention index, (-) not found

(8.8%), cis-geraniol (5.8%), and geranyl acetate (5.2%) were the main constituents of *Z. tenuir* essential oil (tab. 1).

The chemical compositions of *Ziziphora* sp. essential oils were the subject of several studies. In contrast to our results, many of them exhibited that *Ziziphora* sp. (*Z. clinopodioides* subsp. *rigida* and *Z. taurica* subsp. *cleonioides* and *Z. tenuior*) essential oils were rich in pulegone [17-19]. Ozturk *et al.*, indicated that main constituents of *Z. clinopodioides* aerial parts essential oils were pulegone (31.9%), 1,8-cineole (12.2%), limonene (10.5%), menthol (9.1%), β -pinene (6.9%) and menthone (6.7%) [20]. The main constituents of *Z. clinopodioides* ssp. *rigida* essential oils from nine populations of the Lashgardar region (Hamedan Province, Iran) were pulegone [7]. In another study, *Z. tenuir* aerial part essential oil from alpine regions (Kerman province of Iran) contained pulegone

(71.2–85.3%), limonene (0.51–7.8%), thymol (1.0–4.3%), and menthone (0.01–3.7%) [19]. According to our results, the major constituent of *Z. clinopodioides* and *Z. tenuir* essential oils were thymol and α -terpineol, respectively. These data do not correspond with above mentioned reports [7, 17-20].

However, carvacrol (64.2%), thymol (19.2%), *p*-cymene (4.8%) and γ -terpinene (4.6%) were the major components of the *Z. clinopodioides* essential oil from Kermanshah province (in the west of Iran) [6]. Aghajani *et al.* evaluated two samples of *Z. clinopodioides* essential oils from the Lorestan province of Iran and stated that in one sample thymol (53.6%), *p*-cymene (10.5%), carvacrol (8.7%) and γ -terpinene (6.7%) were the major constituents, while in other sample 1,8-cineole (21.6%) and terpinen-4-ol (18.2%) were the major components [21]. Therefore, pulegone was not the main component of essential oils in that literature. These

findings are in agreement with results of our study. The chemical composition of *Z. tenuir* essential oil was not the subject of other studies.

Therefore, we can conclude that pulegone, 1,8-cineole, thymol, carvacrol, *p*-cymene and limonene can be considered as the major compounds of *Ziziphora* sp. [6, 7]. Difference in the essential oils compositions might be arisen from several factors such as collection time, environmental and climatic conditions, and geographical region or plant materials [6, 8].

Anti-dermatophyte activity of *Ziziphora* sp.

The mycelium inhibitory effects of *Ziziphora* oils showed that all tested fungi were inhibited significantly by essential oils. According to table 2, 150 ppm of essential oils showed 5.2–100% of dermatophytes mycelium growth inhibition, while *Z. clinopodioides* essential oil with inhibition percent of 28–100, showed the best inhibitory effect against mycelium growth than that of *Z. tenuir* essential oil (5–23%)

(tab. 2). *T. schoenleinii* and *T. rubrum* showed more sensitivity to *Ziziphora* sp. essential oils.

Table 3 summarizes the anti-dermatophyte effects of *Ziziphora* sp. by micro-broth dilution assay and indicates that the MIC and MFC values the ranged of 0.06–1 and 0.125–1 $\mu\text{l/ml}$ for *Z. tenuir* essential oil and 0.01–0.06 and 0.01–0.06 $\mu\text{l/ml}$ for *Z. clinopodioides* essential oil, respectively, while *Z. clinopodioides* essential oil had better anti-dermatophyte effect than *Z. tenuir* essential oil. *M. gypseum* and *T. schoenleinii* were the most sensitive microorganisms tested by *Z. clinopodioides* essential oils. The MIC and MFC values for *Z. clinopodioides* essential oil against *M. gypseum* and *T. schoenleinii* were 0.01 and 0.01 $\mu\text{l/ml}$, followed by *M. canis*, *T. mentagrophytes*. *T. rubrum* was the less sensitive dermatophyte to *Z. clinopodioides* essential oil (Table 3). *Z. tenuir* essential oil showed the best activity against *T. mentagrophytes* (MIC, MFC = 0.06 and 0.125 $\mu\text{l/ml}$), followed by *M. canis*, *M. gypseum*, *T. rubrum* and *T. schoenleinii*.

Anti-fungal activity of *Ziziphora* essential oils were investigated in limited articles [10, 16].

Table 2.

Inhibition percent of *Ziziphora* essential oils by mycelium inhibition technique

Essential oil	Species				
	<i>M. canis</i>	<i>T. rubrum</i>	<i>M. gypseum</i>	<i>T. schoenleinii</i>	<i>T. mentagrophytes</i>
<i>Z. clinopodioides</i>	59.1%	60.9%	48.8%	100%	28.2%
<i>Z. tenuir</i>	5.7%	15.2%	5.2%	23.6%	14.3%

Table 3.

Anti-dermatophyte activity of *Ziziphora* essential oils by micro broth dilution assay

Essential oil	Dermatophyte			
	<i>Z. tenuir</i> [$\mu\text{l/ml}$]		<i>Z. clinopodioides</i> [$\mu\text{l/ml}$]	
	MIC	MFC	MIC	MFC
<i>M. canis</i>	0.125	0.125	0.01	0.03
<i>M. gypseum</i>	0.25	0.5	0.01	0.01
<i>T. mentagrophytes</i>	0.06	0.125	0.03	0.03
<i>T. rubrum</i>	0.25	0.5	0.06	0.06
<i>T. schoenleinii</i>	1	1	0.01	0.01

Khosravi et al. [10] evaluated anti-fungal properties of some essential oils including *Z. clinopodioides* by micro-broth dilution assay and have been stated that this essential oil had antifungal activity against *A. fumigates* and *A. flavus* with MIC₉₀ ranging from 0.25 to 1.5 mg/ml and also against some dermatophytes (*T. mentagrophytes*, *T. rubrum*, *E. floccosum*, *M. gypsum* and *M. canis*) in the range of 1–2 mg/ml (MIC) and 2–4 mg/ml (MFC) [10]. In another research, the anti-dermatophytes evaluation of *Z. clinopodioides* essential oil with high content of pulegone (44.5%) by mycelium inhibition technique showed that 120, 250 ppm of essential oil inhibited the mycelium growth about 53.5% and 88.4% for *Trichoderma reesei* [16], while 150 ppm *Z. clinopodioides* essential oil from our study with high content of thymol (54.4%) had the better anti-dermatophyte effects than the latter study.

Generally, obtained data showed that essential oils exhibited discrepancy levels of anti-dermatophyte activities. The principles of these differences in sensitivity may be related to difference in chemical composition of essential oils, intrinsic tolerance of microorganisms or measurement methods [8, 10]. Therefore, it is important to perform future studies on the impact of each of these factors alone or in combination.

Anti-elastase activity of *Ziziphora* sp. essential oils

Elastase is the only enzyme that is capable of breaking down elastin, which determines the mechanical

properties of connective tissue [22]. Furthermore, certain microorganisms produce extracellular enzymes with collagenolytic, elastinolytic or keratinolytic activities that are responsible for fungal pathogenicity on tissue invasion. It is seemed that these proteinases play multiple roles in pathogenesis on host, including degradation of the host structural barriers. The role of these proteolytic enzymes as virulence factors is identified in *Sporothrix schenckii*, dermatophytes, *Aspergillus* and candidiasis [23]. Several researches have been made in order to investigate compounds which inhibit elastase and can reduce the severity of diseases [16]. Natural compounds or plant extracts have been studied for their effects on inhibiting elastase activity, but no similar research have been done for impact of *Ziziphora* sp. essential oils on elastase enzymes. As it is shown figure 1, 0.5 μ l/ml of *Z. clinopodioides* and *Z. tenuir* essential oils can inhibit elastase enzyme dose dependently in same manner.

CONCLUSIONS

In this research, the anti-dermatophyte effects of *Z. tenuir* and *Z. clinopodioides* essential oils was evaluated against dermatophytes. *Ziziphora* sp. essential oils especially *Z. clinopodioides* essential oil with high content of thymol had better anti-dermatophyte effects against skin infections. Our findings also highlight the novel property of *Ziziphora* as anti-elastase agent for control of superficial dermatophytes. Although, the chemical composition of *Z. clinopodio-*

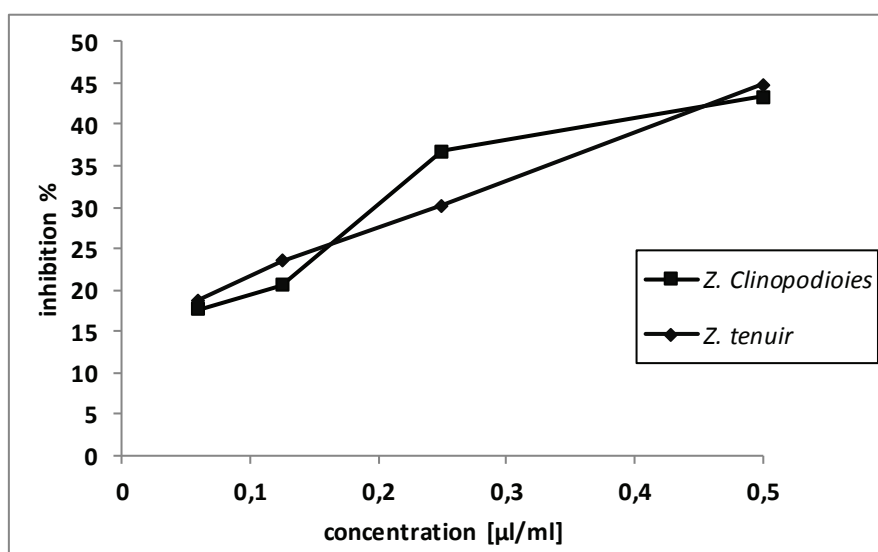


Figure 1.

Anti-elastase activity of *Ziziphora* sp. essential oils against elastase substrate

ides essential oil, not *Z. tenuir* essential oil was the subject of some studies, but there was many discrepancies between the results. So, for the first time, we compared the chemical composition of *Z. tenuir* and *Z. clinopodioides* essential oils from Urmia, then, for the first time, the anti-dermatophyte and anti-elastase effects of these essential oils were compared.

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Conflict of interest: Authors declare no conflict of interest.

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Skład chemiczny i działanie przeciwgrzybicze olejków eterycznych z *Ziziphora tenuior* i *Z. clinopodioides* przeciw dermatofitom

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Streszczenie

Wstęp: Rośliny z rodzaju *Ziziphora* są tradycyjnie stosowane w leczeniu różnych chorób infekcyjnych i nie-infekcyjnych ze względu na swe działanie antyseptyczne.

Cel: Celem pracy było określenie składu chemicznego olejków eterycznych *Ziziphora clinopodioides* i *Z. tenuir* oraz działania przeciwgrzybiczego przeciwko pięciu dermatofitom.

Metody: Skład chemiczny olejków badano za pomocą GC i GC-MS. Działanie antyelasazowe badano z wykorzystaniem świńskiej trzustkowej elastazy.

Wyniki: W dwóch olejkach zidentyfikowano 48 różnych składników, wśród których głównymi były tymol, *p*-cymen, 1,8-cyneol i γ -terpinen. Badanie aktywności antydermatofitowej olejków pokazało, że olejki eteryczne (150 ppm) hamowały wzrost mycelium o około 5–100%. Olejek eteryczny z *Z. clinopodioides* bardziej hamował wzrost mycelium (28–100%) niż olejek z *Z. tenuir*. Wartości MIC i MFC olejków eterycznych wynosiły 0,01–1 μ l/ml. Stężenie 0,5 μ l/ml olejków eterycznych hamowało elastazę trzustki świńskiej w sposób uzależniony od dawki.

Wnioski: Z powodu działania antydermatofitowego i antyelasazowego *Ziziphora* może być uważana za naturalny środek przeciwgrzybiczy, który wymaga dalszych badań przedklinicznych i klinicznych.

Słowa kluczowe: *działanie antydermatofitowe, Ziziphora, działanie antyelasazowe*