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Original article

Investigation of chemical composition and cytotoxic activity of aerial parts of *Ziziphora clinopodioides* Lam.

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Abstract

Background and objectives: *Ziziphora clinopodioides* is a perennial herb and grows widely in west and northwest of Iran. The aerial parts are used as appetizer, carminative and antiseptic as well as for the treatment of medical conditions such as high blood pressure, asthma hyperhidrosis, palpitation and insomnia **Methods:** The aerial parts of *Ziziphora clinopodioides* Lam. were extracted by ethanol (70%) and fractionated by *n*-hexane. The *n*-hexane fraction was analyzed by GC and GC/MS. This fraction and the total extract were further investigated for *in vitro* cytotoxic activity against HT-29 (colon carcinoma), K-562 (leukemia), T-47D (breast ductal carcinoma) and NIH-3T3 (Swiss mouse embryo fibroblast) cells using MTT assay. **Results**: Nineteen compounds were identified by GC/MS. The main constituents of the *n*-hexane fraction were pulegone (24.35%), menthol (14%) and menthone (9.61%). The results of cytotoxicity evaluation showed that the *n*-hexane fraction strongly exhibited cytotoxic activity against T-47D and K-562 cells with IC_{50} value of 77.41±12.89 and 80±2.56 µg/mL. The total extract did not show considerable activity against any of the cell lines in comparison to the *n*-hexane fraction. **Conclusion**: The presence of compounds such as pulegone, menthol and menthone could explain the cytotoxic activity of the *n*-hexane fraction of *Z. clinopodioides* Lam on K-562, T-47D and HT-29 cell lines.

Keywords: cytotoxic activity, GC/MS, MTT assay, *n*-hexane fraction, *Ziziphora clinopodioides* Lam.

Introduction

The genus *Ziziphora* L. (Labiatae) compromises 4 species (*Z. clinopodioides* Lam., *Z. capitata* L., *Z. persica* Bunge. and *Z. tenuior* L.) in Iran [1]. Among them *Z. clinopodioides* Lam. is a perennial herb and grows widely in west and north-west of Iran [2]. Its aerial parts are used as condiment [3]. In Turkey, the tea prepared from the leaves is used as appetizer, carminative and antiseptic. It is also recommended for wound-healing [3]. In Iranian Traditional Medicine the infusion of aerial parts has been used in common cold and coughs [2,3]. Furthermore, *Z. clinopodioides* has been recommended as a good remedy for the treatment of heart disease, high

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blood pressure, asthma, hyperhidrosis, palpitation, insomnia, bronchitis, lung abscess and some other diseases [4].

So far, the antibacterial [5], antifungal [6], anti-larva [7,8], antioxidant [2] and vasorelaxation activities [9] have been reported from the essential oil of this genus. The investigations about the chemical composition of the essential oil of *Ziziphora* species have revealed that pulegone is the main component of *Z. vychodceviana* (57.5%), *Z. persica* (66%), *Z. tenuior* L. (87.1%), *Z. taurica* subsp. *clenioides* (81.9%) [10], *Z. clinopodioides* (31.86%) [5] and *Z. clinopodioides* subsp. *rigida* from Iran (45.8%) [10]. The main components of the essential oil of *Z. clinopodioides* show some variations in different geographic regions. For example, the main compounds of the essential oil were pulegone (31.86%), 1,8-cineole (12.21%), limonene (10.48%), menthol (9.13%), alpha–pinene (6.88%), menthone (6.73%), piperitenone (5.30%) and piperitone (4.18%) in Turkey [5] while the major constituents of the essential oil were reported as pulegone (30.1%), thymol (21.3%), p-mentha-3-en-8-ol (12.9%), piperitenone (9.3%) and 1, 8-cineol (4.1%) in Lorestan, Iran [2].

Pulegone, a liquid monoterpene ketone practically insoluble in water, is found mostly in the essential oils of the family Labiatae. It has a pleasant odor midway between peppermint and camphor [11]. Several cases of toxicity have been reported in human and experimental animals when ingested in large quantity [12].

In the present study, the cytotoxic activity of the total ethanol extract of *Z. clinopodioides* Lam. Has been investigated against three cancerous cell lines H-T29 (colon carcinoma), K-562 (leukemia), T-47D (breast ductal carcinoma) and a normal cell line NIH-3T3 (Swiss mouse embryo fibroblast) using MTT assay. Moreover, the *n*- hexane fraction was prepared from the total extract and its chemical composition was evaluated by GC/FID and GC/MS. Finally, the *n*-hexane fraction was tested for cytotoxic effects and the results were compared with the total extract.

Experimental

Plant material

The dried plant materials (aerial parts) were purchased from local market. The species was identified by professor Gh. Amin and a sample was deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (PMP-305).

Extraction

The aerial parts (500g) were powdered and extracted with ethanol 70% by perculation method. The ethanol extract was dried completely under vacuum (yield 10 g of dried extract). The dried ethanol extract was fractionated by HPLC grade *n*-hexane 3 times. The solvent was evaporated under vacuum without heating. The residue was dried over anhydrous sodium sulfate and kept at 2-8 °C until analysis.

GC/FID and GC/MS analysis of n-hexane fraction

The *n*-hexane fraction analysis was performed on an Agilent 6890 gas chromatography system equipped with flame ionization detector (FID). Column: capillary column HP-5MS, 30 m × 0.25 mm × 0.25 μm film thickness; temperature program: from 50 °C (5 min) to 240 °C at 3 °C/min and to 300 °C at 15 °C/min then kept constant at 300 °C for 3 min; injection temperature: 290°C; injection volume: 1.0 μL; carrier gas: He; injection mode: split (25:1). GC/MS analyses were performed with an Agilent 6890 gas chromatography system.. Column: capillary column HP-5MS, 30 m × 0.25 mm × 0.25 μm film thickness; temperature program: from 50 °C (5 min) to 240 °C at 3 °C/min and to 300 °C at 15 °C/min then kept constant at 300 °C

for 3 min; injection temperature: 290 °C; detector temperature 300 °C; injection volume: 1.0 µL; carrier gas: He; injection mode: split (25:1); MS interface temp.: 220°C; MS mode: EI; detector voltage: 70 eV. Kovats indices were calculated by using retention times of standard normal alkanes that were injected after the oil at the same chromatographic conditions. The identification of each component was confirmed by comparison of their mass spectra and kovats indices with Wiley library and other published references [9].

Cell culture and cytotoxicity assay

Three cancerous cell lines HT-29 (colon carcinoma), K-562 (leukemia), and T-47D (breast ductal carcinoma) and a normal cell line NIH-3T3 (Swiss mouse embryo fibroblast) were purchased from the Pasteur Institute, Tehran, Iran. The cells were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 0.28 units/mL insulin, 100 µg/mL streptomycin, 100 units/mL penicillin, and 0.3 mg/mL glutamine. The cells were grown at 37 °C in a humidified atmosphere of 5% CO2, in air. The cytotoxicity of the total extract and *n*-hexane fraction were assessed using the MTT cytotoxicity assay. The cells (3×10^4) were plated in 500 µL of medium/well in 48-well plates. After an overnight incubation at 37 °C, in 5% CO2, and a humidified atmosphere, the ethanol extract and the *n*-hexane fraction were added to the cells with different concentrations (31.37, 62.75, 125, 250, 500 and 1000 µg/mL). Methotrexate was used as the positive control. The cells were incubated at 37 °C, in 5% CO2, humidified atmosphere, for 48 hours. Then, 50 µL of 5mg/mL MTT (dissolved in PBS) was added to each well. After three hours of incubation, the MTT solution was removed and the cells were washed with 100 µL of PBS, twice. One hundred and fifty microliters of DMSO was added to solubilize the formazan crystals. The optical densities of the wells were then measured at 570 nm (reference 690 nm). By referring to the control (medium with DMSO), the cell survival was assessed [13,14].

Results and Discussion

The GC/MS analysis of the *n*-hexane fraction led to identification of 19 compounds representing 89.72% of the fraction. As shown in table 1, the main components of this fraction were pulegone (24.35%), menthol (14%) and menthone (9.61%). As shown in table 2, the *n*-hexane fraction showed more cytotoxic activity in all cell lines compared to the ethanol extract. The highest cytotoxic activity of the *n*-hexane fraction was observed in T-47D (77.41 ± 12.89 µg/mL) and K-562 (80 ± 2.56 µg/mL) cell lines.

Table 1. Composition of the *n*-hexane fraction of *Z. clinopodioides* Lam.

No	compounds	RRI[a]	%
1	alpha-pinene	929	2.37
2	ortho-cymene	1001	2.17
3	limonene	1008	7.47
4	1,8-cineole	1013	1.2
5	gamma-terpinene	1026	1.03
6	menthone	1147	9.61
7	neomenthol	1147	2.45
8	menthol	1168	14
9	pulegone	1176	24.35
10	carvone	1240	1.19
11	piperitone	1251	1.42
12	menthyl acetate	1280	1.16
13	thymol	1284	2.58
14	piperitenone	1296	4
15	pentadecane	1463	0.98
16	hexadecane	1546	2.06
17	n-heptadecane	1645	3.35
18	n-octadecane	1670	4.31
19	n-nonadecane	1843	4.15
	Total identified	-	89.72

Notes: [a] Relative Retention Indices, calculated against *n*-alkanes. %, calculated from FID

Z. clinopodioides Lam. has been used in traditional medicine as carminative, sedative, stomachic, antibacterial and antioxidant [2,5]. In

the present study, the effect of its ethanol extract and the *n*-hexane fraction on proliferative response of the three cancerous cell lines HT-29 (colon carcinoma), K-562 (leukemia), T-47D (breast ductal carcinoma) and a normal cell line NIH-3T3 (Swiss mouse embryo fibroblast) were investigated by treating the cells with different concentration of each extract and significant decrease in cell line proliferation was observed. According to the results, the *n*-hexane fraction indicated stronger cytotoxic activity on the tested cell lines in comparison to the ethanol extract. The effect of this fraction on K562 and T47D was stronger than on HT-29. Thus this fraction may contain certain compounds that can significantly inhibit the proliferation of breast carcinoma and leukemia cell lines.

Table 2. Cytotoxic activity of the total extract and *n*-hexane fraction of *Ziziphora clinopodioides* Lam. using MTT assay

Sample	IC_{50}(µg/mL)			
	HT-29	K-562	T-47D	NIH-3T3
Total extract	>1000	319.48±11.2	633.29±3.1	572.1±6.5
***n*-hexan fraction**	128.1±13.4	80±2.56	77.41±12.8	162.2±9.9
Methotrexate	0.23±0.0	-	0.16±0.1	0.24±0.0

To identify the chemical composition of the *n*-hexane fraction, GC and GC/MS methods were used. The results revealed that pulegone (24.35%) was the main component in the *n*-hexane fraction of this plant. Pulegone has also been reported as the major component of the essential oil of *Z. clinopodioides* growing in east and west part of Turkey and USSR (31.86%, 21.9 % and13.2%, respectively) [3]. Moreover, menthone was identified in high amount in the above mentioned species (6.73%, 4.6 and 5.44%, respectively) as well as in the *n*-hexane fraction (9.61%). The amount of menthol (14%) in the tested fraction was higher than east Turkish plant (9.13%) [3]. Although, 1, 8-cineole was one of the main constituent of the east Turkish species (12.21%), but it was found in small amount in the *n*-hexane fraction (1.2%) [3].

So far, various biological properties have been reported from pulegone including antibacterial, antifungal, antihistaminic, antipyretic, anti treponemal, convulsant, spontaneous activity reducing, hepatotoxic, hypercholesterolemic, cytochrome P-450 inhibitory and lysozymal enzyme inhibitory activities [11]. Also the investigation of cytotoxic activity of pulegone and its metabolites like piperitenone, piperitone, menthofuran and menthone demonstrated their cytotoxic activity against rat (MYP-3) and human (1T1) urothelial cell lines [15]. Previous studies have shown that menthol has induced cytotoxicity against murine leukemia WEHI-3 cells *in vitro* in a dose dependent manner [16]. Menthol has inhibited the DNA topoisomerase I, II alpha and beta and increased NF-kappa B expression in human gastric cancer SNU-5 cells [14]. Menthol has also induced human promyelocytic leukemia HL-60 cell death due to the Ca^{2+} release from the endoplasmic reticulum [16]. In conclusion, the presence of compounds such as pulegone, menthol and menthone could explain the cytotoxic activity of the *n*-hexane fraction of *Z. clinopodioides* Lam against K562, T47D and HT-29 cell lines.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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