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Chemical composition and scolicidal activity of *Zataria multiflora* Boiss essential oil

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ABSTRACT

Zataria multiflora Boiss (Lamiaceae) commonly grows in Iran is a popular medicinal plant with various pharmacological activities mentioned in traditional Iranian medicine and modern phytotherapy. This study was designed to evaluate the chemical composition and scolicidal effects of *Z. multiflora* essential oil on the protoscoleces of hydatid cysts on an *in vitro* model. The components of the *Z. multiflora* essential oil were identified by GC/MS analysis. Protoscoleces were aseptically aspirated from the livers of naturally infected sheep. Various concentrations of essential oil, thymol and carvacrol were used for 5–30 minutes. Eosin exclusion test was used to determine the viability of protoscoleces. The main components were thymol (41.8%), carvacrol (28.8%), and *p*-cymene (8.4%). Findings showed that essential oil at the concentrations of 12.5 and 6.25 µL/mL killed 100% protoscoleces after 5 and 20 minutes of exposure, respectively. In addition, thymol and carvacrol at the concentrations of 100 µg/mL and 100 µL/mL killed 100% protoscoleces after 10 minutes incubation, respectively. Obtained results in this investigation for the first time demonstrated that *Z. multiflora* essential oil and its main components might be a natural source for the production of new scolicidal agents.

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Cystic echinococcosis; prtotoscoleces; essential oil; thymol; carvacrol; GC/MS

Introduction

Since last decades, natural products and their compounds have been the most productive source for new drug development (1). *Zataria multiflora* Boiss (Lamiaceae family.) commonly grows in Iran, Afghanistan and Pakistan. *Z. multiflora* called 'Avishane Shirazi' in Persian is used as a flavor agent (spice) in a variety of foodstuffs in Iran (2). Reviews have reported *Z. multiflora* to have immunostimulant, pain-relieving, antinociceptive, anti-inflammatory, antioxidant, antibacterial, antiviral, antiparasitic and antifungal effects (2, 3). Previous studies have also demonstrated that main constituents of *Z. multiflora* essential oil are phenolic compounds such as carvacrol and thymol (4, 5). However, some factors such as geographical origin of the variety and harvest season could affect on the chemical composition and functional activity of *Z. multiflora* essential oil (6).

Hydatidosis (cystic echinococcosis, CE) is a chronic infection with medical and veterinary importance which is caused by the larval stage of a cosmopolitan parasitic cestode *Echinococcus granulosus* (8). CE is still an important public health and economic concern in many countries of the world, including Iran (9). Surgery is the main treatment modality, although many other options such as chemotherapy with benzimidazoles and PAIR (puncture, aspiration, injection and respiration) are available as alternative treatments for surgery (10). At present, to reduce the risk of intraoperative spillage of the cyst contents (protoscoleces) and subsequently recurrence of CE and secondary infection, which are observed in nearly 10% of the postoperative cases, the use of effective scolicidal agents is essential (11). There are several chemical scolicidal agents including hypertonic saline, Ag-nitrate, cetrimide, and ethanol which have been used for the inactivation of protoscoleces during surgery; but, most of them have demonstrated different side effects such as liver necrosis, sclerosane colangititi, and methaemoglobinaemia (10, 13). Therefore, the development of new scolicidal agents, especially from natural resources with low side effects and more efficacies is an urgent need for hydatid surgery (14).

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To the best knowledge of the present authors, there have been no studies on the scolicidal effects of *Z. multiflora* essential oil. Thus, this work aims to evaluate the chemical composition of *Z. multiflora* essential oil and investigate scolicidal effects of essential oil and its main components against the protoscoleces of hydatid cysts on an *in vitro* model.

Experimental

Chemicals

All the chemicals and solvents used were of the highest purity commercially available. Thymol and carvacrol were purchased from Sigma-Aldrich (St. Louis, MO). Tween 80 and eosin powder were also prepared from Sigma-Aldrich, (St. Louis, MO, USA).

Plant materials

The aerial parts of *Z. multiflora* were collected from rural regions of Kerman district (Kerman province, Iran) in September 2013. The plant materials were identified by a botanist at the Botany Department of Shahid Bahonar University, Kerman, Iran. A voucher specimen of the plant materials was deposited at the Herbarium of Department of Pharmacognosy of School of Pharmacy, Kerman University of Medical Science, Iran (KF1375).

Isolation of the essential oil

Air-dried aerial parts of the plant were subjected to hydro-distillation for 3 hours using an all-glass Clevenger-type apparatus. The essential oil obtained was dried over anhydrous sodium sulfate, and stored in darkness at 4°C in airtight glass vials closed under nitrogen gas until testing (15).

Drug dilutions

For the preparation of dilutions of the essential oil of *Z. multiflora*, 0.1 mL of the essential oil was dissolved in 0.97 mL of normal saline. In addition, to enhance the dispersal of the essential oil in normal saline, 0.03 mL of Tween 20 was added to the test tube. The resulting solution was mixed adequately by a magnetic stirrer. Serial dilution was then made to obtain the essential oil at 3.125, 6.25, 12.5, 25, 50 and 100 µL/mL. Two mg of thymol dissolved in 10 mL of normal saline plus Tween 80 and 0.2 mL of carvacrol dissolved in 2 mL of normal saline plus Tween 20 and serial dilutions were subsequently made to obtain them at the concentrations of 12.5, 25, 50, and 100 µg/mL. The selection of dilutions of the essential oil of *Z. multiflora* and its main compounds was based on initial experiments, which also showed that normal saline plus Tween 80 had no effect on the growth of protoscoleces.

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

In this study, GC analysis was carried out by a Hewlett-Packard 6890 with a HP-5MS column (30 m × 0.25 mm, film thickness 0.25 mm). The column temperature was maintained at 55°C for 3 minutes and programmed to 180°C at a rate of 5°C per minutes, and kept constant at 220°C for 5 minutes. Injector and interface temperatures were 220 and 290°C, respectively. The flow rate of Helium as carrier gas was (1 mL/min C.F). GC/MS analysis was performed using a Thermoquest- Finnigan gas chromatograph equipped with fused silica capillary DB-5 column (30 m × 0.25 mm, film thickness 0.25 mm) coupled with a TRACE mass (Manchester, UK). Helium was used as carrier gas with ionization voltage of 70 eV. Ion source and interface temperatures were 220 and 290°C, respectively. Mass range was from 40 to 400 u. Oven temperature program was the same given above for the GC.

Identification of the essential oil components

The components of the essential oil were identified by calculation of their retention indices under temperature-programmed conditions for *n*-alkanes (C_8 to C_{22}). Identification of individual compounds was made by comparison of their relative retention time and mass spectra with those of standards Wiley 2001 library data of the GC/MS system or with those of reported in the literature data (16).

Collection of protoscoleces

The protoscoleces of *E. granulosus* were obtained from the naturally infected livers of sheep and goats slaughtered at Kerman abattoir, southeastern Iran and carried to the Parasitology Laboratory at the Department of Parasitology and Mycology, Kerman University of Medical Sciences (Kerman, Iran). The hydatid fluid aspirated by a 20 mL syringe and aseptically transferred into a flask was left to set for 30 minutes for protoscoleces to settle down. The supernatant was discarded and the protoscoleces were washed two times with PBS (pH 7.2) solution. The number of protoscoleces per mL was adjusted as 2×10^3 protoscoleces in 0.9% NaCl solution with at least 90% viability rate. The viability of the protoscoleces was confirmed by their flame cell motility and impermeability to 0.1% eosin solution under a light microscope.

Effect on protoscoleces

For evaluation of scolicidal effects of *Z. multiflora*, and its main components against protoscoleces of hydatid cyst, various concentrations of the essential oil, thymol and carvacrol were used for 5, 10, 20 and 30 minutes. At first, 0.5 mL of the protoscoleces (2×10^3/mL) solution was placed in test tubes. Then 0.5 mL of various concentrations of the essential oil, thymol and carvacrol were added to each test tube. The contents of the tubes were gently mixed and then incubated at 37°C for 5, 10, 20 and 30 minutes. At the end of each incubation time the upper phase was carefully removed so as not to interrupt the protoscoleces. Fifty µL of 0.1% eosin stain was then added to the remaining settled protoscoleces and mixed gently. The upper portion of the solution was discarded after 10 minutes of incubation. The remaining pellet of protoscoleces was then smeared on a glass slide, covered with a cover glass and examined under a light microscope. The percentages of dead protoscoleces were determined by counting 300 protoscoleces (17). In addition, normal saline containing Tween 20 and 20% hypertonic saline were used as negative and positive control, respectively.

Viability test

Eosin exclusion test was used to investigate the viability of protoscoleces (18). Eosin solution with a concentration of 0.1% (1 g of eosin powder in 1000 mL distilled water) was used. After exposure to the stain, alive protoscoleces remained colorless and showed characteristic muscular movements and flame cell activity (Figure. 1), whereas dead protoscoleces absorbed eosin and colored red (Figure. 2).

Statistical analysis

All the tests were performed in triplicate. Data analysis was carried out by using SPSS statistical package version 17.0 (SPSS Inc., Chicago, IL, USA). Differences between test and control groups were analyzed by *t*-test. In addition, $p < 0.05$ was considered statistically significant.

Results

GC/MS analysis of Z. multiflora essential oil

In this study, yellow-colored essential oil (yield 3.1% v/w) was obtained by hydro-distillation method and analyzed using GC/MS. Table 1 indicates the results obtained by GC–MS analysis of *Z. multiflora* essential oil. Twenty-two compounds were identified, representing 96.9% of the total oil. The main components were thymol (40.8%), carvacrol (27.8%), and *p*-cymene (8.4%).

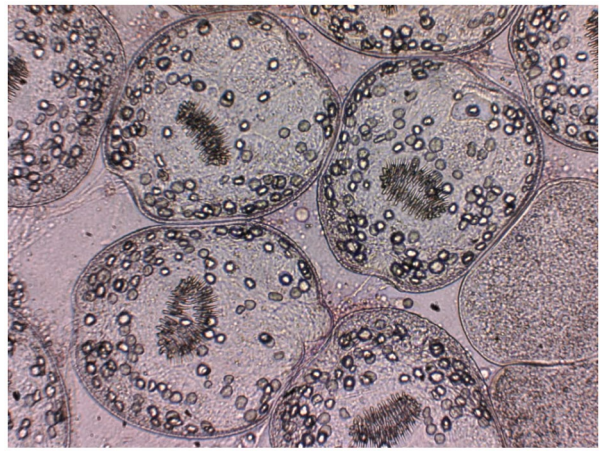

Figure 1. Live protoscolex of hydatid cysts after exposure with 0.1% eosin.

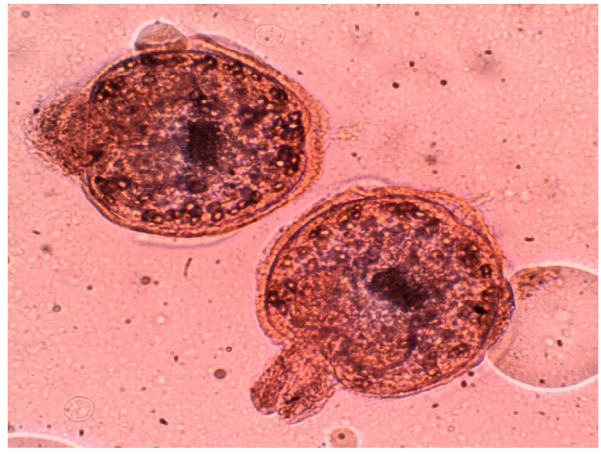

Figure 2. Dead protoscolece of hydatid cysts after exposure to *Z. multiflora* essential oil with 0.1% eosin.

Scolicidal effects on hydatid cyst protoscoleces

According to the results given in Tables 2, *Z. multiflora,* essential oil had a potent scolicidal activity against the protoscoleces of hydatid cyst. Findings showed that essential oil at the concentrations of 25 and 12.5 µL/mL killed 100% protoscoleces after 5 minutes of exposure. Similarly, all of the protoscoleces were killed after 20 and 30 minutes of exposure to 6.25 and 3.125 µL/mL concentration of essential oil, respectively. As shown in Tables 3 and 4, thymol and carvacrol as the main components of *Z. multiflora* essential oil at the concentrations 100 µg/mL and 100 µL/mL killed 100% protoscoleces after 5 minutes incubation, respectively. In contrast, the mortality rate of protoscoleces in the negative and positive controls was 4.3% after 30 minutes and 100% after 5 minutes of exposure, respectively. These results also demonstrated that all the concentrations of essential oil, thymol, and carvacrol had significant ($p < 0.05$) scolicidal effects compared with the control group.

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

No	Components	[a]RI	RI (Literature)	% Composition
1.	3-Octanone	956	965	0.6
2.	α-Pinene	973	978	0.4
3.	β-Myrcene	987	983	0.4
4.	3-Octanol	993	993	0.5
5.	Δ-3-Carene	1017	1023	0.4
6.	1,8-Cineole	1026	1031	0.3
7.	Limonene	1031	1029	0.3
8.	*ρ*-Cymene	1035	1032	8.4
9.	γ-Terpinene	1060	1059	4.0
10.	trans-Sabinene hydrate	1086	1096	0.3
11.	Linalool	1097	1099	1.7
12.	2-Nonanol	1101	1098	0.1
13.	Borneole	1179	1166	0.3
14.	α -Terpineol	1184	1189	1.1
15.	α-Terpinolene	1196	1201	1.3
16.	Decenal,4Z	1199	1192	0.1
17.	Thymol methyl ether	1234	1234	1.3
18.	Thymol	1288	1290	40.8
19.	Carvacrol	1297	1300	27.8
20.	Thymol acetate	1354	1356	0.5
21.	β-Caryophyllene	1428	1420	2.0
22.	Aromadendrene	1448	1440	0.9
	Total			93.5

Note: [a]RI, retention index on an HP-5 column.

Table 2. Scolicidal effects of *Z. multiflora* essential oil against protoscoleces of hydatid cyst at the various concentrations following various exposure times.

Concentration (µl/mL)	Exposure time (min)	Mean of mortality rate (%)
3.125	5	16.6 ± 1.50
	10	43.3 ± 2.51
	20	78.6 ± 5.05
	30	100 ± 0.00
6.25	5	31.6 ± 5.03
	10	65.6 ± 6.02
	20	100 ± 0.00
	30	100 ± 0.00
12.5	5	100 ± 0.00
	10	100 ± 0.00
	20	100 ± 0.00
	30	100 ± 0.00
25	5	100 ± 0.00
	10	100 ± 0.00
	20	100 ± 0.00
	30	100 ± 0.00
Normal saline + Tween 20	5	0.0 ± 0.00
	10	1.3 ± 0.50
	20	2.6 ± 1.15
	30	4.3 ± 1.15
20% Hypertonic saline	5	81.3 ± 3.50
	10	100 ± 0.00
	20	100 ± 0.00
	30	100 ± 0.00

Table 3. Scolicidal effects of thymol against protoscoleces of hydatid cyst at the various concentrations following various exposure times.

Concentration (µg/mL)	Exposure time (min)	Mean of mortality rate (%)
12.5	5	14.6 ± 1.50
	10	32.3 ± 2.15
	20	68.6 ± 5.05
	30	98.6 ± 7.50
25	5	26.3 ± 2.15
	10	58.6 ± 5.02
	20	100 ± 0.00
	30	100 ± 0.00
50	5	53.3 ± 4.20
	10	100 ± 0.00
	20	100 ± 0.00
	30	100 ± 0.00
100	5	100 ± 0.00
	10	100 ± 0.00
	20	100 ± 0.00
	30	100 ± 0.00
Normal saline + Tween 20	5	0.0 ± 0.00
	10	1.3 ± 0.50
	20	2.6 ± 1.15
	30	4.3 ± 1.15
20% Hypertonic saline	5	81.3 ± 3.50
	10	100 ± 0.00
	20	100 ± 0.00
	30	100 ± 0.00

Table 4. Scolicidal effects of carvacrol against protoscoleces of hydatid cyst at the various concentrations following various exposure times.

Concentration (µl/mL)	Exposure time (min)	Mean of mortality rate (%)
12.5	5	12.6 ± 0.50
	10	28.3 ± 2.15
	20	53.6 ± 3.05
	30	91.3 ± 6.50
25	5	21.3 ± 2.51
	10	52.6 ± 6.50
	20	96.3 ± 7.50
	30	100 ± 0.00
50	5	53.3 ± 4.20
	10	97.3 ± 6.50
	20	100 ± 0.00
	30	100 ± 0.00
100	5	100 ± 0.00
	10	100 ± 0.00
	20	100 ± 0.00
	30	100 ± 0.00
Normal saline + Tween 20	5	0.0 ± 0.00
	10	1.3 ± 0.50
	20	2.6 ± 1.15
	30	4.3 ± 1.15
20% Hypertonic saline	5	81.3 ± 3.50
	10	100 ± 0.00
	20	100 ± 0.00
	30	100 ± 0.00

Discussion

Medicinal plants are the oldest medicines used by humans. Their increasing use in recent years is a clear piece of evidence for the public interest in alternatives to conventional drugs (19). *Z. multiflora* is a thyme-like plant that broadly grows in the central and southern parts of Iran. This plant is used in traditional medicine to treat a wide range of diseases, such as infectious ones (2). This work was designed to evaluate the chemical composition and scolicidal activities of essential oil of *Z. multiflora* on an *in vitro* model.

At present, an ideal scolicidal agent is defined by its potency at lower concentrations, high efficacy in a shorter time of exposure, stability in the presence of cystic fluid, scolicidal ability inside a cyst, lower toxicity, higher availability, and ability for rapid preparation (20). Up to now, in the various studies the scolicidal effects of hypertonic saline, silver nitrate and mannitol, cetrimide, ethyl alcohol (95%), H_2O_2 and 10% providone iodine, chlorhexidine gluconate, selenium nanoparticles, honey and some plant extracts (21–23). have been demonstrated. However, it has been shown that existing scolicidal agents are associated with adverse effects and their efficacy is controversial (10). Current findings showed that *Z. multiflora* essential oil with the concentrations of 12.5 and 25 µL/mL killed 100% protoscoleces of hydatid cyst after 5 minutes of exposure. Moreover, we found that thymol and carvacrol as the main components of *Z. multiflora* essential oil at the concentrations 100 µg/mL and 100 µL/mL killed 100% protoscoleces after 5 minutes incubation, respectively. These findings were comparable with the scolicidal activity of 20% hypertonic saline (15 minutes), 20% silver nitrate (20 minutes), 0.5–1% cetrimide (10 minutes), H_2O_2 3% (15 minutes), and 95% ethyl alcohol (15 minutes). Therefore, this study supported the idea that *Z. multiflora* might be a natural source for the production of a new scolicidal agent for use in hydatid cyst surgery. However, main mechanisms of scolicidal effects of *Z. multiflora* are not clear and further studies are needed to elucidate these mechanisms, particularly on *in vitro* models. It has been formerly proven that *Z. multiflora,* due to having higher content phenolic compounds especially thymol and carvacrol, acts on the cell membrane microorganisms and causes damage and depletion of the contents of the cells (24). Moreover, Kavoosi et al reported that *Z. multiflora* essential oil, thymol, and carvacrol significantly reduced activities of nitric oxide and H_2O_2 production as well as NO synthase and NADH oxidase in LPS-stimulated murine. Thus, exact mechanisms of scolicidal activity of *Z. multiflora* are not obvious and further studies are required to elucidate these mechanisms (25).

Composition of *Z. multiflora* essential oil, which has been previously analyzed by other research groups, was shown to depend on species, climate, and time of collection along with growth stage (26), thereby altering the biological activities studied (27). In the previous studies, the main components of the essential oil have been only reported to be thymol (5–56%) and carvacrol (5–78%), both at a high percentage and with few other compounds. However, in this study, the main components were found to be thymol (41.81%), carvacrol (28.85%), and *p*-cymene (8.36%). Thus, the plant analyzed in this research was a new chemotype of *Z. multiflora*. In the case of cytotoxicity effects of *Z. multiflora*, Malekinejad et al showed *Z. multiflora* had no toxicity on Chinook salmon (*Oncorhynchus tshawytscha*) embryo (CHSE-214) cells. Therefore, it can be suggested that the *Z. multiflora* derivatives are safe for mammalian cells (28).

In conclusion, the results obtained in this study for the first time showed that essential oil of *Z. multiflora* might be a natural source for the production of new scolicidal agents for use against hydatid cyst surgery. However, further clinical studies are required to evaluate exact biological activity of *Z. multiflora* and its main compounds in animal models.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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