

Zataria multiflora Boiss: lethal effects of methanolic extract against protoscoleces of *Echinococcus granulosus*

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Abstract The present study was aimed to evaluate the lethal effects of *Zataria multiflora* Boiss (Lamiaceae) methanolic extract against *Echinococcus granulosus* protoscoleces. Protoscoleces were aseptically aspirated from sheep livers having hydatid cysts. Various concentrations of the essential oil (2.5–20 mg/mL) were used for 10–60 min. Viability of protoscoleces was confirmed using eosin exclusion test (0.1 % eosin staining). Obtained results showed that *Z. multiflora* extract at the concentration of 20 mg/mL after 10 min of exposure killed 100 % protoscoleces. The mean of mortality rate of protoscoleces after 20 min of exposure to the concentration of 10 mg/mL was also 100 %. Lower concentrations of *Z. multiflora* extract provoked a delayed protoscolicidal activity. The findings indicated potential of *Z. multiflora* methanolic extract as a natural source for the producing of new scolicidal agent for use in hydatid cyst surgery.

Keywords Cystic echinococcosis ·
Zataria multiflora Boiss · Scolicidal · Protoscoleces

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Introduction

Hydatid cyst (cystic echinococcosis, CE), caused by the larval stage of the dog tapeworm *Echinococcus granulosus*, remains a major public health problem on several continents and is reemerging in several countries (WHO 1996). For many years, surgery was the only option for treatment of hydatid cyst. Moreover, chemotherapy with benzimidazole compounds (albendazole and mebendazole) and, later, treatment by PAIR (cyst puncture, aspiration, injection of chemicals, and re-aspiration) were subsequently introduced, however, and these treatments have increasingly supplemented or even replaced surgery (Eckert and Deplazes 2004). During surgery to reduce the risk of intraoperative spillage of the cyst contents (protoscoleces) and subsequently recurrence of CE and secondary infection, which is observed in nearly 10 % of the post-operative cases, the use of effective scolicidal agents are obligatory (Brunetti et al. 2010). Nowadays, there are several scolicidal agents including hypertonic saline, silver-nitrate, cetrimide, and ethanol which have been used for inactivation of the cyst contents. However, these scolicidal agents are associated with various side effects such as sclerosane colangitis (biliary tract fibrosis), liver necrosis and methaemoglobinaemia (Mahmoudvand et al. 2014a). Thus, enormous efforts have been made to reach new scolicidal agents especially from natural resources with low side effects and more efficacies for hydatid cyst surgery.

Medicinal plants have been used for medical purposes since the beginning of human history and are the basis of modern medicine due to having the high availability, high efficacy, and low side effects (Cosa et al. 2006). *Zataria multiflora* Boiss (Lamiaceae) 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 (Mahboubi and Ghazian Bidgoli 2010). Previous studies have shown immunostimulant, pain-relieving, antinociceptive, anti-inflammatory, antioxidant, antibacterial, antiviral and antifungal effects of *Z. multiflora* (Sharififar et al. 2007; Saei-Dehkordi et al. 2010). This investigation was aimed to evaluate the lethal effects of *Z. multiflora* methanolic extract against hydatid cyst protoscoleces.

Experimental section

Plant materials

The leaves of *Z. multiflora* were collected from rural regions of Kerman district (Kerman province, Iran) in September 2014. 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).

Preparing methanolic extract

Air dried plant materials (100 g) were separately extracted by percolation method with 80 % methanol successively for 72 h in room temperature. The extracts were passed through filter paper (Whatman No. 3, Sigma, Germany) to remove plant debris. The extracts were finally concentrated in vacuum at 50 °C using a rotary evaporator (Heidolph, Germany) and stored at −20 °C, until testing (Mahmoudvand et al. 2014b).

Collection of protoscoleces

The protoscoleces of hydatid cysts were collected from the naturally infected livers of sheep and goats slaughtered at Kerman abattoir, southeastern Iran and carried to the

Parasitology Laboratory at the, Kerman University of Medical Sciences, Iran. The hydatid fluid aspirated by a 50 mL syringe and aseptically transferred into a flask was left to set for 30 min 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 (Sigma Aldrich, St Louis, MO, USA) under a light microscope.

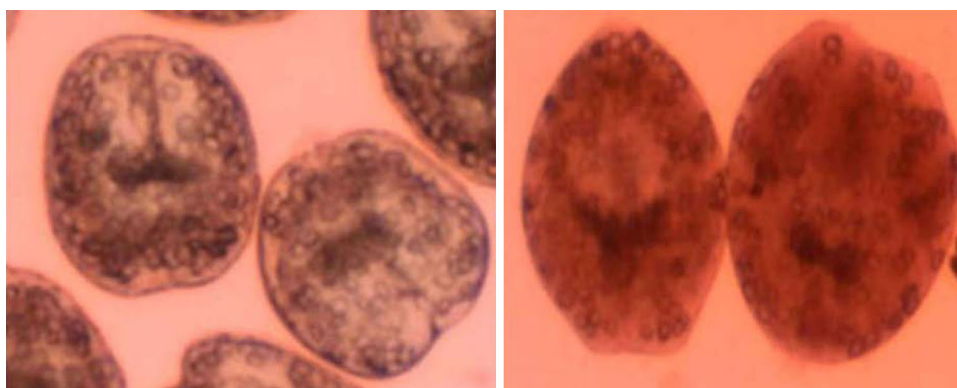
Effect on protoscoleces

To determine scolical effects of *Z. multiflora* extract against protoscoleces of hydatid cysts, various concentrations of the extract (2.5–20 mg/mL) were used for 10, 20, 30, and 60 min 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 was added to each test tube. The contents of the tubes were gently mixed and then incubated at 37 °C for 10, 20, 30, and 60 min. At the end of each incubation time, the upper phase was carefully removed so as not to interrupt the protoscoleces. 50 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 min 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. We also used normal saline and hypertonic saline 20 % as negative and positive control group (Mahmoudvand et al. 2014c).

Viability test

To determine the viability of protoscoleces of hydatid cysts eosin exclusion test was used (Smyth and Barrett 1998).

Fig. 1 Live (*left*) and dead (*right*) protoscoleces of hydatid cysts after exposure with 0.1 % eosin



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, live protoscoleces remained colorless and displayed characteristic muscular movements and flame cell activity; while dead protoscoleces absorbed eosin and colored red (Fig. 1).

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

Table 1 indicates scolical effects of *Z. multiflora* methanolic extract at various concentrations following different exposure times. Obtained findings revealed that *Z. multiflora* extract at the concentration of 20 mg/mL after 10 min of exposure killed 100 % protoscoleces. Similarly, the mean of mortality rate of protoscoleces after 20 min of exposure to the concentration of 10 mg/mL was 100 %. Furthermore, lower concentrations of *Z. multiflora* extract provoked a delayed protoscolicidal effects; so that, at the concentration of 5 mg/mL killed 15.6, 51.3, 92.6, and 100 % of the protoscoleces and at the concentration of 2.5 mg/mL killed 3.3, 26, 58.3, and 86.6 % of the protoscoleces after 10, 20, 30, and 60 min of incubation, respectively. The mortality rate of protoscoleces in the negative and positive control was 5.3 and 100 % after 60 and 10 min exposure, respectively. These results also demonstrated that *Z. multiflora* extract at all of concentrations had significant ($p < 0.05$) scolical effects compared with the control group.

Discussion

Natural products, such as plants extract, either as pure compounds or as standardized extracts, due to having low toxicity, low cost, high efficacy, and high availability provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity (Mahmoudvand et al. 2014d). This study was aimed to evaluate the protoscolicidal effects of *Z. multiflora* methanolic extract on an in vitro model. Results showed that *Z. multiflora* extract at the concentrations of 20 and 10 mg/mL after 10 and 20 min of exposure killed 100 % protoscoleces.

Nowadays, an ideal scolical agent explained by its potency at lower doses, high efficacy in a shorter time of

Table 1 Protoscolicidal effects of *Zataria multiflora* extract against protoscoleces of hydatid cyst at various concentrations following various exposure times. Data are expressed as the mean \pm SD (n = 3)

Concentration (mg/mL)	Exposure time (min)	Mean of mortality rate (%)
20*	10	100 \pm 0.0
	20	100 \pm 0.0
	30	100 \pm 0.0
	60	100 \pm 0.0
10*	10	57.6 \pm 5.5
	20	100 \pm 0.0
	30	100 \pm 0.0
	60	100 \pm 0.0
5*	10	15.6 \pm 2.5
	20	51.3 \pm 4.5
	30	92.6 \pm 6.5
	60	100 \pm 0.0
2.5*	10	3.3 \pm 0.5
	20	26.0 \pm 2.5
	30	58.3 \pm 4.0
	60	86.6 \pm 5.5
Normal saline	10	1.6 \pm 0.5
	20	2.6 \pm 0.5
	30	3.2 \pm 0.5
	60	5.3 \pm 0.5
20 % Hypertonic saline*	10	100 \pm 0.0
	20	100 \pm 0.0
	30	100 \pm 0.0
	60	100 \pm 0.0

* Difference is significant ($p < 0.05$) compared with control group

exposure, stability in the presence of cystic fluid, scolical ability inside a cyst, lower toxicity, higher availability, and ability for rapid preparation (WHO 1996). However, existing scolical agents are associated with serious adverse effects and their efficacy is controversial (Mahmoudvand et al. 2014e). Our findings revealed that *Z. multiflora* extract had remarkable scolical activity which is comparable with the existing scolical agents such as 20 % hypertonic saline (15 min), 20 % silver nitrate (20 min), 0.5–1 % cetrimide (10 min), H₂O₂ 3 % (15 min), and 95 % ethyl alcohol (15 min). Thus, findings of present investigation supported the idea that *Z. multiflora* extract could be a natural source for the production of a new scolical agent for use in hydatid cyst surgery. Main mechanisms of scolical effects of *Z. multiflora* are not clear and further studies are needed to elucidate these mechanisms. However, it has been previously 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 (Moshayedi et al. 2012). Moreover, Kavooosi et al. reported that *Z. multiflora* essential oil, thymol, and carvacrol significantly reduced activities of nitric oxide and H₂O₂ production as well as NO synthase and NADH oxidase in LPS-stimulated murine. Thus, exact mechanisms of scolical activity (Kavooosi et al. 2012).

In the regard to toxicity effects of *Z. multiflora*, Hosseinzadeh et al. reported that LD₅₀ of the infusion and maceration *Z. multiflora* extract was 3.85 and 3.47 g/kg, respectively (Hosseinzadeh et al. 2000). In addition, Malekinejad et al. showed *Z. multiflora* had no toxicity on Chinook salmon (*Oncorhynchus tshawytscha*) embryo (CHSE-214) cells (Malekinejad et al. 2012). According to a toxicity classification, the methanolic extract of *Z. multiflora* had no significant toxicity and is safe for mammalian cells (Loomis 1968).

Conclusion

Findings of the present study demonstrated that *Z. multiflora* extract might be a natural source for the production of new scolical agents to reduce the risk of protoscoleces spillage during hydatid cyst surgery. However, further studies will be needed to confirm these results by checking the extract in a clinical setting as a new scolical agent.

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Conflict of interests The author declares that there is no conflict of interests in this study.

References

- Brunetti E, Kern P, Vuitton DA (2010) Writing panel for the WHO-IWGE expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. *Acta Trop* 114(1):1–16
- Cosa P, Vlietinck AJ, Berghe DV, Mae L (2006) Anti-infective potential of natural products: how to develop a stronger in vitro ‘proof-of-concept’. *J Ethnopharmacol* 106:290–302
- Eckert J, Deplazes P (2004) Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. *Clin Microbiol Rev* 17:107–135
- Hosseinzadeh H, Ramezani M, Salmani G (2000) Antinociceptive, anti-inflammatory and acute toxicity effects of *Zataria multiflora* Boiss extracts in mice and rats. *J Ethnopharmacol* 73(3):379–385
- Kavooosi G, Teixeira da Silva JA, Saharkhiz MJ (2012) Inhibitory effects of *Z. multiflora* essential oil and its main components on nitric oxide and hydrogen peroxide production in lipopolysaccharide-stimulated macrophages. *J Pharm Pharmacol* 64:1492–1500
- Loomis TA (1968) *Essential of toxicology*. Lea and Febige, Philadelphia
- Mahboubi M, Ghazian Bidgoli F (2010) Antistaphylococcal activity of *Zataria multiflora* essential oil and its synergy with vancomycin. *Phytomedicine* 17:548–550
- Mahmoudvand H, Fasihi Harandi M, Shakibaie M, Aflatoonian MR, Makki MS, Jahanbakhsh S (2014a) Scolical effects of biogenic selenium nanoparticles against protoscoleces of hydatid cysts. *Int J Surg* 12(5):399–403
- Mahmoudvand H, Sepahvand A, Jahanbakhsh S, Ezatpour B, Ayatollahi Mousavi SA (2014b) Evaluation of antifungal activities of the essential oil and various extracts of *Nigella sativa* and its main component, thymoquinone against pathogenic dermatophyte strains. *J Mycol Med* 24(4):155–161
- Mahmoudvand H, Sharififar F, Saedi Dezaki E, Ezatpour B, Jahanbakhsh S, Fasihi Harandi M (2014c) Protoscolecal effect of *Berberis vulgaris* root extract and its main compound, berberine in cystic echinococcosis. *Iran J Parasitol* 9(4):26–34
- Mahmoudvand H, Tavakoli R, Sharififar F, Minaie K, Ezatpour B, Jahanbakhsh S, Sharifi I (2014d) Leishmanicidal and cytotoxic activities of *Nigella sativa* and its active principle, thymoquinone. *Pharm Biol* 4:1–6
- Mahmoudvand H, Dezaki ES, Kheirandish F, Ezatpour B, Jahanbakhsh S, Harandi MF (2014e) Scolical effects of black cumin seed (*Nigella sativa*) essential oil on hydatid cysts. *Korean J Parasitol* 52(6):653–659
- Malekinejad H, Bazargani-Gilani B, Tukmechi A, Ebrahimi H (2012) A cytotoxicity and comparative antibacterial study on the effect of *Zataria multiflora* Boiss, *Trachyspermum copticum* essential oils, and Enrofloxacin on *Aeromonas hydrophila*. *Avicenna J Phytomed* 2(4):188–195
- Moshayedi SH, Shahrz F, Schaffner DW, Khanlarkhani A, Shojaei-Aliabadi S, Shahnia M, Khaksar R (2012) In vitro control of *Enterococcus faecalis* by *Zataria multiflora* Boiss, *Origanum vulgare* L and *Mentha pulegium* essential oils. *J Food Saf* 4:1745–4565
- Saei-Dehkordi SS, Tajik H, Moradi M, Khalighi-Sigaroodi F (2010) Chemical composition of essential oils in *Zataria multiflora* Boiss. From different parts of Iran and their radical scavenging and antimicrobial activity. *Food Chem Toxicol* 48:1562–1567
- Sharififar F, Moshafi MH, Mansouri SH, Khodashenas M, Khoshnoodi M (2007) In vitro evaluation of antibacterial and antioxidant activities of the essential oil and methanol extract of endemic *Zataria multiflora* Boiss. *Food Control* 18:800–805
- Smyth JD, Barrett NJ (1998) Procedures for testing the viability of human hydatid cysts following surgical removal, especially after chemotherapy. *Trans R Soc Trop Med Hyg* 74:649–652
- World Health Organization (1996) Informal working group on echinococcosis. *Bull WHO* 74:231–242