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ORIGINAL ARTICLE

High scolicidal effect of *Zataria multiflora* on protoccoleces of hydatid cyst: an in vitro study

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Abstract Hydatidosis in humans and animals is an economic and public health problem in many parts of the world, and surgery is still the main treatment for hydatid disease. One of the most important endpoints of hydatid cyst surgery is recurrence. The main cause of recurrence is dissemination of protoscolices during the surgical operation. Preoperative destruction of the cyst's contents through instillation of a scolicidal agent into the hydatid cyst is the most commonly used approach to prevent this complication. Various scolicidal agents have been used for inactivation of the cyst content, but most are accompanied by adverse side effects. In the present study, the scolicidal effect of methanolic extract of *Zataria multiflora* is investigated. Protoscoleces were aseptically collected from sheep livers containing hydatid cyst and were used in the experiments. *Z. multiflora* extract was used at a concentration of 10 and 25 mg/ml for 1, 2, and 3 min. Viability of protoscoleces was confirmed by 0.1% eosin staining. *Z. multiflora* extract at a concentration of 10 mg/ml killed 68.9%, 93.7%, and 100% of protoccoleces after 1, 2, and 3 min respectively. The scolicidal effect of this extract at a concentration of 25 mg/ml was 100% after 1 min. The results of present study showed that methanolic extract of *Z. multiflora* has high scolicidal activity and might be used as an effective scolicidal agent. This is the first report on the scolicidal activity of *Z. multiflora*.

Keywords In vitro · Hydatid cyst · Scolicidal · *Zataria multiflora*

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Introduction

Hydatid disease, hydatidosis, and echinococcosis are all terms describing infection with the metacestode of the tapeworm, *Echinococcus* (Thompson 1986; Wen et al. 1993). *Echinococcus granulosus* is a taeniid tapeworm, which occurs in the small intestine of definitive hosts, notably dogs and occasionally other carnivores. The larval stage of *E. granulosus* causes cystic hydatidosis in humans and livestock. Human infection may occur after ingestion of infective eggs passed in the feces from dogs through direct contact or via environmental contamination (Elayoubi et al. 2003). The definitive host becomes infected by ingesting protoscoleces produced by asexual multiplication of the metacestode. There may be several thousand protoscoleces within a single cyst, and each one is capable of developing into a sexually mature adult worm (Thompson 1986; Wen et al. 1993). Hydatidosis is one of the most important zoonotic diseases of man and animals in the world (Sadjjadi et al. 2008). Hydatid disease is prevalent in South America, Australia, the Middle East, and Mediterranean countries. However, with increased travel, isolated cases can be seen anywhere in the world (Aksoy et al. 2001; Mahesh et al. 2000). Although most hydatid cysts are found in the liver and lung, the disease can arise anywhere in the body (Kayaalp et al. 2001). Surgical removal of the intact hydatid cyst is the most preferred method of therapy (Rajabi 2009). One of the major surgical complications of hydatidosis is recurring (secondary) cystic echinococcosis after operation for primary hydatid disease. Dissemination of protoscolex-rich fluid during surgery is a major cause of recurrence and multiple secondary echinococcosis (Kilicoglu et al. 2008; Moro and Schantz 2009). Obviously, use of an effective protoscolicidal adjunct to hydatid surgery is an important procedure which may reduce the recurrence rate (Topcu et al. 2009; Wen et al. 1993).

Scolicidal solutions remain indispensable in the treatment of hydatidosis, and the surgeons need less harmful but more effective drugs in hydatid disease (Adas et al. 2009).

The members of genus *Zataria* are widely distributed in Iran, Afghanistan, and Pakistan (Mahmoudabadi et al. 2007; Shaiq Ali et al. 2000), and *Zataria* is one the most reputable herbal medicines in Iranian traditional medicine (Khalili and Vahidi 2006). This plant is used traditionally in food, especially in yogurt flavoring, is used as a stimulant, condiment, and carminative, and is used for treatment of pre-mature labor pains and rupture (Zargari 1989). This plant has been commonly used in medicine for the treatment of respiratory tract infections as an antiseptic, antitussive, and irritable bowel syndrome treatment (Aynehchi 1991). *Zataria multiflora* has antioxidant (Sharififar et al. 2007) and anti inflammatory (Hosseinzadeh et al. 2000) activities. The tropical application of the hydroalcoholic extract of *Z. multiflora* may present an effective treatment for recurrent aphthous stomatitis (RAS) (Jafari et al. 2003). Since *Z. multiflora* has a number of medicinal properties, in the present study, the scolicidal effect of the methanolic extract of this herbal plant was evaluated.

Materials and methods

Protoscoleces

Protoscoleces of *E. granulosus* were collected aseptically from liver hydatid cysts of natural infected sheep, slaughtered in Shiraz abattoir in the South of Iran. The hydatid fluid of cysts was aseptically transferred into glass cylinders and left to set for 30 min. The protoscoleces settled at the bottom of the cylinders. The supernatant was then removed, and the yielded protoscoleces were washed three times in normal saline. The viability of protoscoleces was confirmed from their motility characteristics under light microscopy. The live protoscoleces were finally transferred into a dark container containing normal saline and stored at 4°C for further use.

Extraction

The leaves of *Z. multiflora* were dried under shade and powdered mechanically using a commercial electric blender. A total of 900 g of dried powder was extracted. The following method was used for preparation of methanolic extract of *Z. multiflora*: A 100 g of dry powder was added to 400 ml of pure methanol and mixed gently for 1 h using a magnetic stirrer. The obtained solution was left at room temperature for 24 h. The solution was stirred again and filtered, and the solvent was removed by evaporation in a rotary evaporator, and the remaining semisolid material was then freeze dried. The obtained residue was placed in a sterile glass container and stored in the refrigerator at 4°C for later use. A 19.2 g of dried extract from 900 g of dried powder of *Z. multiflora* was obtained

Scolecidal tests

In this study, two concentrations (10 and 25 mg/ml) of the *Z. multiflora* extract were examined for 1, 2, and 3 min. For preparation of *Z. multiflora* extract solution with 10 and 25 mg/ml concentration, 0.1 and 0.25 g of dried extract was dissolved in 10 ml of normal saline, respectively. Two milliliters of each concentration was placed in a test tube, and a drop of protoscolex-rich sediment was added to the tube and mixed gently. The tube was then left at room temperature for 1, 2, and 3 min. The upper portion of the solution was then removed with a pipette avoiding settled protoscoleces. Then 2 ml of 0.1% eosin stain was added to the remaining settled protoscoleces and mixed gently. After 15 min, the upper portion of the solution was discarded. The remaining settled protoscoleces were then smeared on a manual scaled glass slide, covered with a cover glass (24×50 mm), and examined microscopically for viability. The percentages of dead protoscoleces were determined by counting a minimum of 450 (mostly more than 500) protoscoleces. At least 450 protoscoleces with no exposure to *Z. multiflora* extract were considered as control group in each experiment. The experiments were performed in triplicate.

Viability test

In the present study, eosin stain with 0.1% concentration (1 g of eosin powder in 1000 ml of distilled water) was used for the viability test of protoscoleces. After 15 min of exposure, the protoscoleces that did not take the dye in were accepted as potentially viable (Fig. 1) and those done were considered dead (Fig. 2).

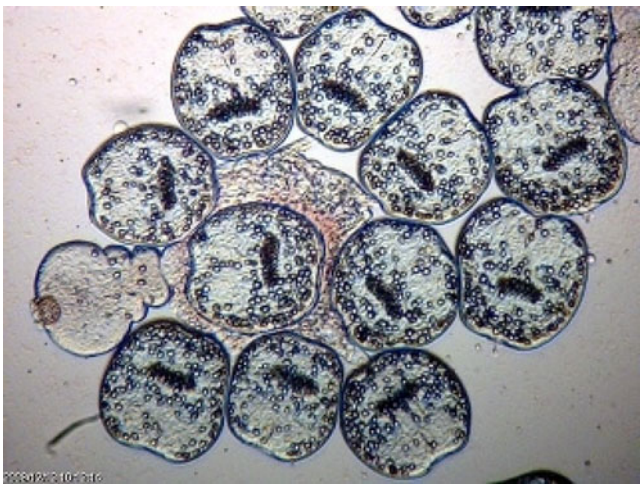

Fig. 1 Live protoscoleces after staining with 0.1% eosin

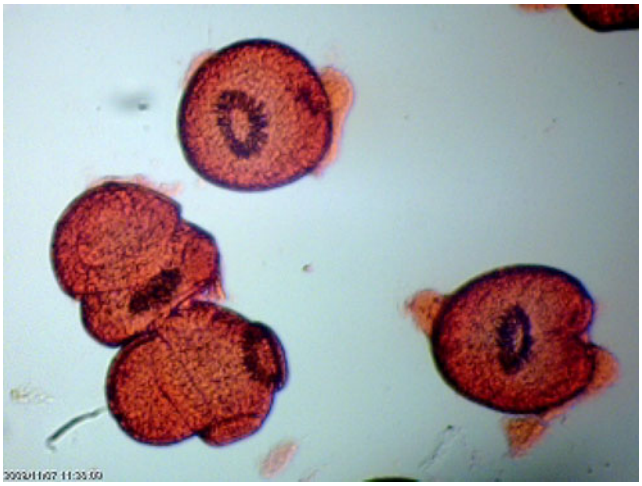

Fig. 2 Dead protoscoleces after exposure to *Z. multiflora* extract and staining with 0.1% eosin

Statistical analysis

Differences between test and control groups were analyzed by the chi-square test. $P \leq 0.01$ was considered significant. Statistical analysis was performed with GraphPad InStat software.

Results

Results of the effectiveness of different concentrations of *Z. multiflora* extract as a scolicidal agent are shown in Tables 1 and 2. While the death rate in the control group was 14%, scolicidal activity of *Z. multiflora* extract at a concentration of 10 mg/ml was 68.9%, 93.7%, and 100% after 1, 2, and 3 min of application, respectively. Scolicidal effect of *Z. multiflora* extract at concentration of 25 mg/ml was 100% after 1 min of exposure. The scolicidal effect of two concentrations of the methanolic extract of *Z. multiflora* was extremely significant compared to the control groups at all exposure times ($P<0.0001$). The results of the present study indicated that methanolic extract of *Z. multiflora* has high scolicidal activity and might be used as a scolicidal agent in hydatid cyst surgery.

Discussion

Despite some progress in the control of echinococcosis, this zoonosis continues to be a major public health problem in several countries, and in several others, it constitutes an emerging and re-emerging disease (Moro and Schantz 2009). Cystic echinococcosis (CE) is among the most neglected parasitic diseases. Development of new drugs and other treatment modalities receives very little attention, if any, and is slow. Clinical management procedures have evolved over decades without adequate evaluation of important features such as efficacy, effectiveness, rate of adverse reactions, relapse rate, and cost (Junghanss et al. 2008).

To date, many scolicidal agents have been used for inactivation of the hydatid cyst content, but there is no ideal agent that is both effective and safe (McManus et al. 2003). Various experimental studies investigated the effects of formalin, povidone–iodine, alcohol, hypertonic saline, H_2O_2, and cetrimide on the liver and the biliary tree. (Kilicoglu et al. 2008). Among the various scolicidal agents advocated in the past, formalin was the first and most frequently used agent. Despite its effectiveness, it is no longer used because of the associated toxicity and severe hepatobiliary complications (Besim et al. 1998; Topcu et al. 2006). Irrigation of the serosal surface with povidine–iodine caused renal shutdown, sterile peritonitis, sclerosing serositis,

Table 1 Scolicidal effect of *Z. multiflora* extract at concentration of 10 mg/ml after 1, 2, and 3 min of application

Exposure time (min)		Experiments			
		1	2	3	Total
1	Protoscoleces	591	532	512	1,635
	Dead protoscoleces	403	380	344	1,127
	Mortality rate (%)	68.2	71.4	67.2	68.9
2	Protoscoleces	624	521	494	1,639
	Dead protoscoleces	583	501	452	1,536
	Mortality rate (%)	93.4	96.2	91.5	93.7
3	Protoscoleces	583	495	591	1,588
	Dead protoscoleces	583	495	591	1,588
	Mortality rate (%)	100	100	100	100
Control	Protoscoleces	612	483	692	1,787
	Dead protoscoleces	80	93	77	250
	Mortality rate (%)	13	19.2	11.1	14

Table 2 Scolicidal effect of *Zataria multiflora* extract at concentration of 25 mg/ml after 1 min of application

	Experiments	Protoscoleces	Dead protoscoleces	Mortality rate (%)
Test	1	486	486	100
	2	539	539	100
	3	638	638	100
	Total	1,663	1,663	100
Control	1	612	80	13
	2	483	93	19.2
	3	692	77	11.1
	Total	1,787	250	14

and constructive pericarditis (LeVeen et al. 1993). Severe hepatobiliary complications have been reported for ethyl alcohol (Yetim et al. 2005). No scolicidal effect can be shown with a concentration of less than 10% saline at 5 min. The lowest concentration of saline should be 20% but severe hepatobiliary complications have been reported for 10–20% NaCl (Besim et al. 1998; Topcu et al. 2006). H_2O_2 has been found to be 90.3% effective on scolices, but because of the side effects, it is not used in many fields today (Adas et al. 2009). Cetrimide is a potent disinfectant and effective scolicidal agent. Low concentrations of cetrimide (0.1–0.5%) have been used by many surgeons. Although cetrimide is effective in very low concentrations, it is not devoid of complications. Metabolic acidosis and methemoglobinemia were the two other reported complications due to cetrimide installation into hydatid cysts (Besim et al. 1998). An ideal scolicidal agent is defined as being potent in low concentrations, acting in a short period of time, being stable in cyst fluid, not affected by dilution with the cyst fluid, being able to kill the scolex in the cyst, being non-toxic, having low viscosity, and being readily available and easily prepared, as well as being inexpensive (Puryan et al. 2005; WHO 1996). New effective alternative treatment is extremely important in today's climate, where species are becoming resistant, and there is resurgence in the use of natural alternative therapies, instead of synthetic pharmaceuticals that often have severe side effects (Harris et al. 2000). Since the mid 1970s, significant advances have been made in the chemotherapy of the metacestode stage of *Echinococcus*. In vitro and in vivo techniques for drug testing are now available, and considerable clinical, pharmacological, and parasitological knowledge has accumulated. Experiences from animal experiments and human trials provide an encouraging basis for future studies, which should include research into new formulations of benzimidazoles, combinations of existing anti-hydatid drugs, and a continued search for new and effective agents (Wen et al. 1993)

The antimicrobial properties of plant essential oils are well established against the wide spectra of microbes including bacteria and fungi, using a variety of experimental methods. Most of the antimicrobial activity in essential oils, derived from plants, appears to derive from phenolic compounds. The main constituents of the essential oil of *Z. multiflora* are phenolic compounds such as carvacrol and thymol (Akhondzadeh Basti et al. 2007). The chemical composition of hydrodistilled essential oils (comprising 84.9% of the oil) of *Z. multiflora* are thymol (37.59%), carvacrol (33.65%), para-cymene (7.72%), c-terpinene (3.88%), and b caryophyllene (2.06%) (Sharififar et al. 2007). The hexane soluble part of *Z. multiflora* afforded six including multiflotriol, multiflorol, p-hydroxy benzoic acid, dihydroxyaromadendrane, luteolin and a-tocopherolquinone (Shaiq Ali et al. 2000). Other chemicals of *Z. multiflora* extract include zatrinal, oleanolic acid, betulic acid, rosmarinic acid and monoterpenoids, sesquiterpenoids, p-cymene and y-terpinene (Mahmoudabadi et al. 2007). Scientific reports show that *Z. multiflora* has antibacterial (Fazeli et al. 2007; Misaghi and Akhondzadeh Basti 2007; Moosavy et al. 2008; Rasouli and Rezaei 2001; Shams Alizadeh et al. 2009; Sharififar et al. 2007), antifungal (Gandomi et al. 2009; Mahmoudabadi et al. 2007), and antiprotozoal (Abdollahy et al. 2004) properties.

In the present study, we investigated the effectiveness of methanolic extract of *Z. multiflora* on the protoscoleces of hydatid cyst. The results of our study showed that *Z. multiflora* extract at a concentration of 10 and 25 mg/ml can kill all protoscoleces after 3 and 1 min of application, respectively. According to the results of our study, the scolicidal activity of *Z. multiflora* extract at a concentration of 10 mg/ml (3 min) and 25 mg/ml (1 min) is comparable with scolicidal power of formalin (Erzurumlu et al. 1998), 95% ethyl alcohol (15 min) (Erzurumlu et al. 1998), 20% hypertonic saline (45 min) (Caglar et al. 2008), 3% H_2O_2 (15 min) (Besim et al. 1998), and 0.5–1% cetrimide (10 min) (Frayha et al. 1981). *Z. multiflora* is an edible plant; therefore, it is well adapted to animals and human nature. *Z. multiflora* is safe and nonpathogenic on the fetus digestive system when used in pregnant BALB/c mice (Mokhberi et al. 2007). Furthermore, this herbal plant can remarkably stimulate innate and acquired immunity function in experimental animals, and it may be used as an immunostimulatory agent (Khosravi et al. 2007; Shokri et al. 2006). The results of the present study allowed us to suggest that *Z. multiflora*

is likely a source of new compounds that could be used as an effective scolicidal agent. Further studies will be necessary to identify and isolate these active compounds. The results of this study also open the possibility of more investigations of in vivo scolicidal effect of this traditional medicine. This is the first report on the scolicidal activity of *Z. multiflora*. This is the first report on the scolicidal activity of methanolic extract of *Z. multiflora*.

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