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Scolicidal effect of the aromatic water of *Zataria multiflora*: an in vitro study

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Abstract Hydatidosis is one of the most important parasitic zoonoses with worldwide distribution. Scolicidal agents are usually essential during the surgical therapy of hydatid cysts for prevention of recurrence. There is still no scolicidal agent with high efficacy and low adverse side effects. New and natural scolicidal agents not only may be used for production of new antihydatid drugs, but also they may be useable instead of existing scolicidal agents. In the present study, the scolicidal effect of the aromatic water (AW) of *Zataria multiflora* was investigated. Hydatid cyst protoscolices were collected aseptically from the cysts obtained from the livers of naturally infected sheep. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) were employed to determine the chemical composition of the essential oil (EO) from *Z. multiform* AW. Protoscolices were exposed to *Z.multiflora* AW for 1, 2, 3, 4, and 5 min. Viability of protoscolices was confirmed by 0.1 % eosin stain. Thymol, carvacrol, carvone, neo-dihydrocarveol, and 1,8-cineole were found to be the major EO constituents. The scolicidal power of *Z. multiflora* AW was 93.78, 96.33, 99.16, 99.48, and 100 % after 1, 2, 3, 4, and 5 min, respectively. In the present study, *Z. multiflora* AW showed high in vitro scolicidal power. Since *Z. multiflora* AW is an edible drink, thus, it may be used safely as a natural scolicidal agent. However, further studies will be necessary to determine the feasibility of producing an effective new drug, using the constituents of *Z. multiflora* AW, for the treatment of hydatid disease.

Keywords Hydatid cyst · Echinococcosis · Scolicidal agents · *Zataria multiflora* · Aromatic water

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Introduction

Cystic echinococcosis or hydatidosis is a silent cyclozoonotic infection of humans and domestic animals caused by the larvae of the cestode *Echinococcus granulosus* (Hashemi Tabar et al. 2012). It is a public health problem associated with significant economic losses worldwide (Kumsa and Mohammedzein 2014). According to the WHO-IWGE classification, there are four treatment options for hydatidosis: (1) surgery, (2) PAIR (puncture, aspiration, injection of protoscolicidal agent, reaspiration), (3) chemotherapy with albendazole (ABZ) or mebendazole (MBZ), and (4) watch and wait for inactive, clinically silent cysts (Junghanss et al. 2008; Brunetti et al. 2010). Chemotherapy is the preferred treatment where surgeons are not available or the cysts are too numerous and in inoperable cases, "chemotherapy" is the only option. Chemotherapy has also been used as an adjunct to surgery for prophylaxis against spillage of the cyst contents (Blanton et al. 1998; Arif et al. 2008). Hydatid cyst fluid contains thousands of protoscolices and each one has the potential to grow into a new hydatid cyst (Besim et al. 1998). One of the major surgical complications of hydatidosis is recurring (secondary) cystic echinococcosis after operation for primary hydatid disease. Recurrence ranges from 4.6 to 22.0 % in different studies (Prousalidis et al. 2012). Dissemination of the protoscolex-rich fluid during the surgery is a

major cause of recurrence and multiple secondary echinococcosis (Kilicoglu 2008; Moro and Schantz 2009).

Injection of a scolicidal agent into the hydatid cysts to reduce the risk of spillage of viable protoscolices is an integral part of the surgical technique for many surgeons (Adas et al. 2009). Many scolicidal agents including formalin, cetrimide, povidone–iodine, ethyl alcohol, hypertonic saline, H_2O_2, silver nitrate, and albendazole have been used for inactivation of the hydatid cyst content. Nevertheless, adverse side effects have been reported for formalin, cetrimide, and H_2O_2 (Besim et al. 1998), povidone-iodine (Topcu et al. 2006), ethyl alcohol (Yetim et al. 2005), hypertonic saline (Krige et al. 2002), silver nitrate (Topcu et al. 2006; Rajabi 2009), and albendazole (Adas et al. 2009). Obviously, use of an effective protoscolicidal, adjunct to hydatid surgery, is an important procedure which may reduce the recurrence rate (Arif et al. 2008; Wen et al. 1993); therefore, 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).

Zataria multiflora is an important aromatic plant belonging to the Lamiaceae family, which distributed in Iran, Afghanistan, and Pakistan (Ali et al. 2000; Mahmoudabadi et al. 2007) and used frequently in the traditional Iranian medicine. It has been shown that *Z. multiflora* has anti-inflammatory (Hosseinzadeh et al. 2000), antioxidant (Sharififar et al. 2007), antibacterial (Misaghi and Akhondzadeh Basti 2007), antifungal (Gandomi et al. 2009), and antiprotozoal (Abdollahy et al. 2004) properties. Since *Z. multiflora* has a number of medicinal properties, in this experimental study, the scolicidal effect of the aromatic water of this herbal plant was evaluated in an in vitro study.

Materials and methods

Protoscolices collection

Protoscolices of hydatid cysts were collected aseptically from livers of naturally infected sheep obtained from the slaughterhouse of Ahwaz, southwest of Iran. Hydatid fluid was aspirated from the cysts and transferred into the glass cylinders and was allowed to settle for 30 min. The supernatant was then removed and the sedimented protoscolices were washed several times with normal saline under aseptic conditions. The live protoscolices were maintained in normal saline in a dark container and stored at 4 °C for further use.

Aromatic water extraction

Aerial parts of *Z. multiform* Boiss were collected from wild-growing plants at the full flowering stage in the Chahak region of Neyriz suburb, Fars province, Iran, in May, 2012. The plant species was identified and authenticated at Shiraz University Herbarium, Shiraz, Iran. Voucher specimen (24,984) has been deposited in the Herbarium. One hundred kilograms of collected plant material were hydrodistillated with 400 l water for 3 h, using an industrial apparatus of Shirin Osare factory. This is the most ancient and versatile method of distillation in Iran and some other countries for producing edible aromatic water (AW) and essential oil. In this method, plant materials are fully submerged in water. The water is heated to produce steam carrying the most volatile chemicals. The steam is then chilled (by a condenser) and the resulting distillate collected. The essential oil will normally float on top of the hydrosol (AW) and then is separated off the AW. The resulted AW was used for GC and GC/MS analysis.

Extraction of essential oil from aromatic water

To separate essential oil (EO) from AW, 300 ml of the sample was put in a decanter funnel and active ingredients were extracted by diethyl ether (20 ml of solvent, four times). Finally, the solvent was removed with a stream of nitrogen gas. The resulting essential oil was dried over anhydrous sodium sulfate and kept in sealed vial at low temperature (4 °C) until gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) analysis. The yield of essential oil of the AW was 0.12 % (wt/*v*).

Essential oil analysis

GC analysis was performed using an Agilent gas chromatograph series 7890-A with a flame ionization detector (FID) to analyze EO obtained from AW. The analysis was carried out on a fused silica capillary HP-5 column (30 m by 0.32 mm inside diameter [i.d.]; film thickness, 0.25 m). The injector and detector temperatures were kept at 250 and 280 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 1 ml/min. The oven temperature was programmed to increase from 60 to 210 °C at a rate of 4 °C/min, and then the oven was programmed to reach 240 °C at a rate of 20 °C/min and finally held isothermally for 8.5 min. The split ratio was 1:50. GC-MS analysis was carried out by use of an Agilent gas chromatograph equipped with a fused silica capillary HP-5 MS column (30 mby 0.25 mm i.d.; film thickness, 0.25 m) coupled with a 5975-C mass spectrometer. Helium was used as a carrier gas with an ionization voltage of 70 eV. The ion source and interface temperatures were 230 and 280 °C, respectively. The mass range was from 45 to 550 atomic mass units (amu). The oven temperature program was the same given above for GC.

Identification of compounds

The constituents of the EOs were identified by calculation of their retention indices under temperature-programmed conditions for n-alkanes (C8 to C25) and the oil on a HP-5 column under the same chromatographic conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectrum library or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those reported in the literature (Adams 2007). For quantification purposes, relative area percentages obtained by FID were used without correction factors.

Scolocidal test

One milliliter of *Z. multiflora* AW was placed in a test tube; then, 0.1 ml protoscolex-rich sediment was added to the tube and mixed slowly. Then, the test tube was placed at room temperature for the desired times (1, 2, 3, 4, and 5 min). At the end of the exposure times, the supernatant of the solution was discarded carefully by a pipette without any disturbance of sedimented protoscolices. After proper washing of protoscolex-rich sediment with normal saline (to remove the remaining aromatic water), the volume of the solution raise to 1 ml by adding normal saline. Then, the same volume of 0.1 % eosin stain was added to the test tube and mixed gently. After an incubation time of 15 min, the upper phase of solution was discarded. The remaining sedimented protoscolices was smeared on a manually scaled glass slide, covered with a cover glass (24×50 mm), and examined under a light microscope and the mortality rate of protoscolices was calculated. The mortality rate of protoscolices was determined by counting a minimum of 500 protoscolices. The protoscolices did not take the dye, were considered as potentially live, and otherwise were accepted as dead protoscolices (Fig. 1). The experiments were performed in triplicate.

Statistical analysis

All statistical calculations were performed with SPSS version 11.5 package. Differences between the test and control groups were analyzed by one-way ANOVA test. The p values less than 0.05 were considered to be significant.

Results

Chemical compositions of EO from *Z. multiflora* AW are shown in Table 1. Thymol (66.9 %), carvacrol (15.2 %), carvone (7.3 %), neo-dihydrocarveol (2 %), and 1,8-cineole (1.6 %) were found to be the major EO constituents. Other constituents were present in very low concentrations. Scolicidal effect of *Z. multiflora* AW at different exposure times are presented in Table 2. As shown in Table 2, the death rate of protoscolices was 3.74 % in the control group. Scolicidal power of *Z.multiflora* AW was 93.78, 96.33, 99.16, 99.48, and 100 % after 1, 2, 3, 4, and 5 min of exposure time, respectively. The scolicidal effect of Z. *multiflora* AW was extremely significant compared to the control groups at all exposure times ($p<0.0001$). The results of this study showed that the aromatic water of *Z. multiflora* has high scolicidal activity and could be considered as a natural scolicidal agent .

Discussion

Despite some progress in the control of echinococcosis, this zoonoses 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). Few chemotherapeutic agents are available for the medical management of hydatid disease (Blanton et al. 1998).

We have previously studied several medicinal plant extract or essential oils and found the methanolic extract of *Z. multiflora* as the most effective agent against protoscolices of hydatid cyst (Moazeni and Nazer 2010; Moazeni et al. 2012; Moazeni and Roozitalab 2012). In this study, we investigated the scolicidal effect of *Z. multiflora* AW because its preparation is different and very easier than the preparation of *Z. multiflora* extract. Furthermore, *Z. multiflora* AW is an edible and delicious drink. The results of our study showed

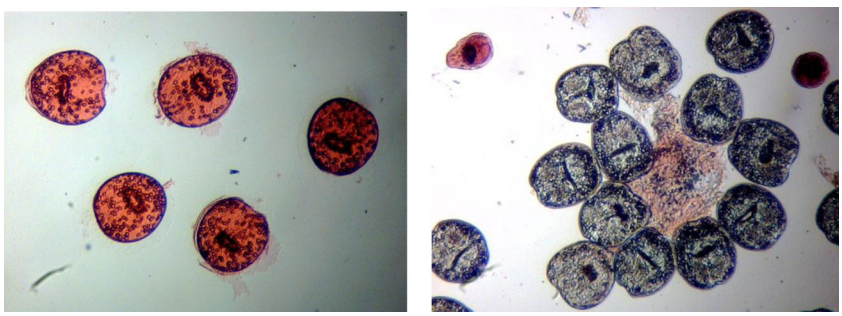

Fig 1 Live and dead protoscolices of hydatid cyst after exposure to aromatic water of *Z. multiflora* and staining with 0.1 % eosin. Live protoscolices did not take the dye in and have normal color (*right*). Dead protoscolices take the dye in and are *red* (*left*)

Table 1 Essential oil chemical components of *Zataria multiflora* aromatic water identified by gas chromatography–mass spectroscopy

Components	Percentage	RI	Identification method
Thymol	66.9	1294	RI, MS
Carvacrol	15.2	1302	RI, MS
Carvone	7.3	1242	RI, MS
neo-Dihydro carveol	2	1192	RI, MS
1,8-Cineole	1.6	1029	RI, MS
Pulegone	1.2	1238	RI, MS
cis-Dihydro carvone	1.2	1195	RI, MS
a-Terpineol	0.8	1189	RI, MS
Piperitenone	0.6	1339	RI, MS
Terpinene-4-ol	0.6	1175	RI, MS
Linalool	0.4	1098	RI, MS
iso-Menthone	0.3	1162	RI, MS
Borneol	0.3	1163	RI, MS

RI retention indices on HP-5 column, *MS* mass spectroscopy

that *Z. multiform* AW is able to kill all protoscolices of hydatid cyst after 5 min of application. Our results revealed that the scolicidal power of *Z. multiform* AW after 5 min of application is equal to the scolicidal power of 0.5–1 % cetrimide (10 min) (Frayha et al 1981), 95 % ethyl alcohol (15 min), 10 % povidone–iodine (15 min), 3 % H_2O_2 (15 min) (Besim et al. 1998), 100 μg/ml albendazole sulfoxide (15 min) (Erzurumlu et al. 1998), and 20 % silver nitrate (20 min) (Caglar et al. 2008).

To have a better understanding about the anthelmintic effects of any compound, especially natural derivatives from traditional medicinal herbs, the chemical composition profile of used material is an important aspect. Therefore, in the current study, we analyzed the chemical components of the EO of *Z. multiflora* AW. Our results of EO analysis of AW showed that *Z. multiflora* EO is a rich source of thymol (66.9 %).

Thymol has been known to have acaricidal (Ellis and Baxendale 1997), insecticidal (Pandey et al. 2009), anthelmintic (Mathew et al. 2008), and scolicidal (Elissondo et al. 2008) properties. Elissondo et al. (2008) incubated the protoscolices of hydatid cyst in thymol at a concentration of 10 μg/ml for 4, 12, 42, and 80 days and reported 100 % scolicidal activity after 80 days of incubation. We observed 100 % scolicidal activity for *Z. multiform* AW after 5 min of application. Some studies have shown that the whole essential oil has a stronger antiseptic activity than an individual major component (Gill et al. 2002; Mourey and Canillac 2002),

Table 2 Scolicidal effect of *Zataria multiflora* aromatic water at different exposure time

Exposure time (min)	Experiments	Number of protoscolices	Dead protoscolices	Mortality rate (%)
1	1	576	535	92.88
	2	583	543	93.13
	3	515	492	95.53
	Total	1674	1570	93.78
2	1	622	587	94.37
	2	515	508	98.64
	3	498	480	96.38
	Total	1635	1575	96.33
3	1	707	698	98.72
	2	672	672	100
	3	533	526	98.68
	Total	1912	1896	99.16
4	1	658	654	99.39
	2	595	592	99.49
	3	503	501	99.60
	Total	1756	1747	99.48
5	1	672	672	100
	2	546	546	100
	3	683	683	100
	Total	1901	1901	100
Control	1	683	16	2.34
	2	700	28	4
	3	672	33	4.91
	Total	2055	77	3.74

demonstrating that the minor constituents are also important to the anti-microbial activity and may have a synergistic influence (Burt 2004).

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 scolices in the cyst, being non-toxic, having low viscosity, and being readily available and easily prepared as well as being inexpensive (WHO 1996). Indeed, there is no ideal scolicidal agent that is both effective and safe (McManus et al. 2003). Therefore, a search for new scolicidal agents is needed and the introduction of new drugs could help patients suffering from cystic echinococcosis (Walker et al. 2004). New scolicidal agents not only may be applicable for preparation of new anti hydatid drugs, but also may be useful in surgery and also PAIR procedure.

Zataria multiform AW is traditionally used as a delicious drink and is well adapted to animals and human nature. *Z. multiflora* has been used in pregnant BALB/c mice and showed no pathogenic effect on the fetus digestive system (Monsefi et al. 2007). Furthermore, this herbal plant could act as a stimulator of innate and acquired immunity in experimental animals (Khosravi et al. 2007; Shokri et al. 2006).

In conclusions, this study confirmed that the AW of *Z. multiflora* has clear destructive effect on the protoscolices of hydatid cyst. Since *Z. multiform* AW is an edible drink and immunostimulatory agent, thus, it may be used safely as a natural scolicidal agent. However, further studies should focus on the in vivo scolicidal efficacy of *Z. multiform* AW and also its major EO constituents. In addition, the possible cytotoxicity of *Z. multiflora* AW and its main components remain to be more investigated in future in vivo studies. Further studies will also be necessary to determine the feasibility of producing an effective new drug using the constituents of *Z. multiflora* AW, for the treatment of hydatid disease.

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