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In vitro and *in vivo* antihydatid activity of a nano emulsion of *Zataria multiflora* essential oil

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ABSTRACT

In *in vitro* process of this study, protoscoleces of the hydatid cysts were exposed to two concentrations of nano emulsion (NE) of *Zataria multiflora* essential oil (ZMEO) (1 and 2 mg/mL) for 10 and 20 min. Viability of protoscoleces was confirmed using 0.1% eosin staining. For *in vivo* studies, sixteen laboratory mice were infected intraperitoneally by 1500 live protoscoleces. Five months after infection, the infected mice were divided into treatment and control groups. The mice of treatment group received the NE of ZMEO (20 mg/kg) orally *via* their drinking water while the mice of control group received no treatment. Two months after the start of treatment, all of the mice were necropsied and the hydatid cysts were collected. Subsequently, the numbers, sizes and weights of the collected cysts were compared between the mice of two groups. The results of *in vitro* scolicidal assays showed that the scolicidal power of NE of a ZMEO at concentration of 1 mg/mL was 88.01%, and 100% after 10 and 20 min respectively. NE of ZMEO showed 100% scolicidal power at a concentration of 2 mg/mL after 10 min (comparing to 4.46% for the control group). The results of *in vivo* studies revealed that the size of the largest cysts as well as the total number of the cysts were significantly lower in the mice treated with NE of ZMEO ($P < 0.05$). In conclusion, NE of ZMEO may be considered as a natural scolicidal agent and a potential therapeutic tool for treatment of hydatid disease.

1. Introduction

Cystic echinococcosis (CE) is a worldwide zoonotic infection with economic and public health importance in many parts of the world. It is the result of infection of humans and domestic ruminants with the larval stage of *Echinococcus granulosus sensu lato* (Torgerson and Budke, 2003; Dalimi et al., 2005; Moazeni and Alipour-Chaharmahali, 2011; Ahmadnia et al., 2013; Budke et al., 2017). *Echinococcus granulosus sensu lato* is a small tape worm living in the small intestine of the dog. Intermediate hosts such as man, cattle and sheep become infected by ingestion of the parasite's eggs. The liberated embryos in the small intestine of intermediate hosts reach the liver, lungs, *etc. via* the blood circulation and develop to form the larval stage of the parasite, the hydatid cysts. The dogs acquire the infection by eating viscera infected with fertile hydatid cysts, accordingly completing the parasite's life cycle (Larrieu et al., 2001). Depending on the location, size, and number of the cysts, the patients may be surgically operated or be treated by chemical drugs (Nicolao et al., 2014). In surgical treatment of human hydatid disease, various scolicidal agents may be used in the surgery location to inactivate live protoscoleces, preventing the recurrence of infection (Colebrook et al., 2004). Various scolicidal agents such as formalin, povidone–iodine, cetrimide, hypertonic saline, ethyl alcohol, H2O2, silver nitrate, and albendazole have been used to inactivate the hydatid cyst content (Moazeni et al., 2015). Most of the scolicidal agents may be accompanied by adverse side effects (Moazeni and Mohseni, 2012).

Benzimidazole carbamate derivatives (*e.g.* mebendazole and albendazole), are the most effective drugs for chemical treatment of hydatid disease. In humans, these drugs must be administered in high doses for long times, hence, they are accompanied by harmful side effects (Walker et al., 2004). Therefore, it is necessary to investigate for new scolicidal agents and also drugs with more safety and higher effectiveness.

Nanotechnology is one of the most applicable technologies of the 21st century, leading to product innovation. In nanotechnology, the used materials are extremely small in size; this size reduction in the range of nano can change properties of materials comparing to their original size. The technology has enough potential to transform most of the daily consumer products, and a large number of products are already on the market (Chaudhri et al., 2015). Basic essential materials

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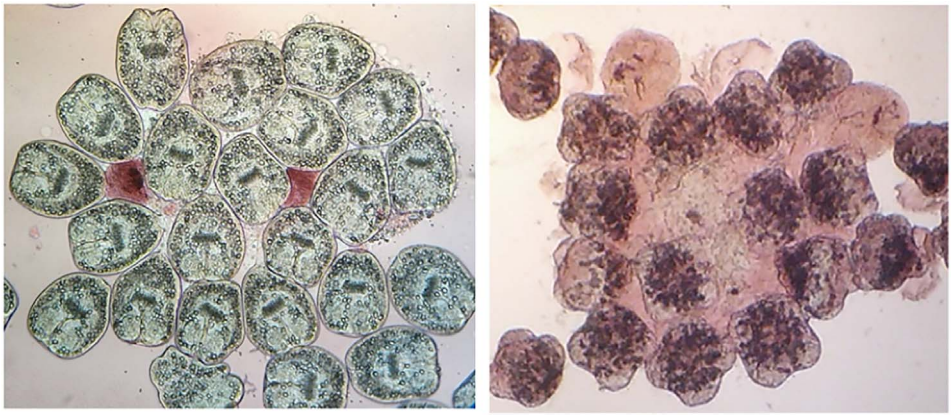

Fig. 1. Protoscolices of *Echinococcus granulosus* after staining with 0.1% eosin, right: exposured to NE of ZMEO, left: control group. Note the stained protoscolices in the right photograph.

for formulation of nano emulsions (NEs) are oil, aqueous phases, and an emulsifier. In the oil-in-water NEs, oil droplets dispersed in the continuous phase (water) and carries lipophilic active compounds. Mostly, the lipophilic active ingredients are solubilized in the oil phase before to the formation of emulsions. Essential oils (EOs) as oil phase can be formulated with different non-polar compounds (Mason et al., 2006; Odriozola-Serrano et al., 2014).

Previous studies have shown that *Zataria multiflora* has antimicrobial (Moradi et al., 2016), antibacterial (Fatemi et al., 2015), antifungal (Jafari et al., 2015) and antileishmanial (Saedi Dezaki et al., 2016) properties. Recent records implied that EOs may be a valuable source of natural compounds for treatment of hydatid disease (Maggiore et al., 2012). With regard to the medicinal properties of *Z. multiflora*, the present study was undertaken to investigate the scolicidal and antihydatid activity of the NE of. *Z. multiflora* essential oil (ZMEO).

2. Materials and methods

2.1. Preparation of ZMEO

Wild growing *Z. multiflora* was collected at the full flowering stage in the Chahak region of the Neyriz suburb, Fars Province, Iran, in May 2015.The plant species was identified and authenticated and a voucher specimen (24984) has been deposited in the herbarium. Dried samples at room temperature (20–25 °C) of *Z. multiflora* (30 g) were hydrodistillated for 3 h, using an all glass Clevenger-type apparatus according to the method described by the European Pharmacopoeia (1983). The EOs were collected, dried over anhydrous sodium sulfate and stored in sealed vials at 4 °C until use. Approximately 20 g of EO was obtained from 1000 g of dried powder of *Z. multiflora*.

2.2. Preparation of NE of a ZMEO

NE of ZMEO was prepared through low energy system using 96% (v/v) water, 2% (v/v) EO and 2% (v/v) Tween 80. The EO and Tween 80 were stirred at 2400 rpm using a homogenizer for 20 min (Ostertag et al., 2012). Subsequently, water was added slowly to the mixture. The obtained mixture was further stirred at 3000 rpm for 30 min. The resulting NE was stored at 4 °C until use. Approximately 1000 mL of NE of a ZMEO at concentration of 10 mg/mL was obtained from 10 g of EO.

2.3. Collection of protoscoleces

Live protoscoleces for *in vitro* and *in vivo* studies, were aseptically prepared from the liver hydatid cysts collected from the naturally infected sheep slaughtered in Shiraz (South of Iran) and Mashhad (Northeast of Iran) slaughterhouses, respectively. The fluid of hydatid cysts was aseptically aspirated and transferred to glass cylinders and left unmoved for 30 min. The protoscoleces gathered at the bottom of the cylinders. Then the supernatant was removed, and protoscoleces were washed with sterile 0.85% NaCl and stored in RPMI 1640 medium overnight at 37 °C. The viability of protoscoleces was confirmed by their movements under light microscope and 0.1% eosin staining. The live protoscoleces were transferred into dark containers containing normal saline and stored at 4 °C for further use.

2.4. In vitro scolicidal tests

In the present study, two concentrations of the NE of ZMEO (1 and, 2 mg/mL) were tested for 10 and 20 min. Since the concentration of the stock solution of NE of a ZMEO was 10 mg/mL, to prepare the above concentrations, 1 and 2 mL of the stock solution of NE of a ZMEO was dissolved in 9 and 8 mL of normal saline, in a test tube, respectively. For each experiment, 2 mL of the solution was placed in a small test tube, subsequently one drop of the sediment containing 1000–1600 protoscoleces was added to the tube using a Pasteur pipette. After gently mixing the contents, the tube was incubated at 37 °C. At the end of incubation times (10 and 20 min), the upper part was carefully removed and 1 mL of 0.1% eosin stain (1 g of eosin powder in 1000 mL distilled water) was added to the remaining protoscoleces and mixed gently. After 15 min of incubation, the upper portion of the solution was discarded carefully and the remaining protoscoleces were smeared on a manually scaled glass slide. After covering with a cover glass the specimens were examined under a light microscope. The mortality rate was determined by counting a minimum of 1300 protoscoleces. The protoscoleces in the control group were treated only with normal saline. Protoscoleces with absorbed dye were recorded as dead, otherwise were considered as potentially viable (Fig.1) (Moazeni and Nazer, 2010). The experiments repeated three times.

2.5. In vivo experiments

Sixteen white laboratory female mice (*Mus musculus*), 7 weeks old and weighing 25 to 30 g were infected intraperitoneally by injection of 1500 live protoescolices of *E. granulosus*. The infected mice were kept at 24 to 25 °C on a 12 h light/dark cycle and were fed *ad libitum*, with free access to drinking water. To evaluate the therapeutic effect of NE of a ZMEO on the hydatid cysts, five months after infection, the infected mice were divided into treatment and control groups consisting of 8 animals each. The mice of treatment group received the NE of ZMEO (20 mg/kg) orally *via* their drinking water while the mice of control group received no treatment. Two months after the start of treatment, all of the mice were necropsied and immediately thereafter, the hydatid cysts from each mouse were collected in a petri dish. Then the cysts were counted and their sizes were measured using scaled graph papers and their weights were recorded using a digital scale (AND, Japan). The efficacy of NE of a ZMEO in the treatment of hydatid disease was evaluated through the comparison of numbers, sizes, and weights of

Table 1
Scolicidal effect of the nano emulsion of *Zataria multiflora* essential oil at concentration of 1 mg/mL after 10 and 20 min of application.

Exposure time (min)		Experiments			
		1	2	3	Total
10	Protoscoleces	1539	1467	1574	4580
	Dead protoscoleces	1352	1298	1381	4031
	Mortality rate	**87.84%**	**88.47%**	**87.73%**	**88.01%**
20	Protoscoleces	1491	1338	1375	4202
	Dead protoscoleces	1491	1338	1375	4204
	Mortality rate	**100%**	**100%**	**100%**	**100%**
Control	Protoscoleces	1191	1270	1567	4028
	Dead protoscoleces	73	68	39	180
	Mortality rate	**6.13%**	**5.36%**	**2.56%**	**4.46%**

Table 2
Scolicidal effect of the nano emulsion of *Zataria multiflora* essential oil at concentration of 2 mg/mL after 10 min of application.

	Experiments	Protoscoleces	Dead protoscoleces	Mortality rate
Test	1	1287	1287	100%
	2	1363	1363	100%
	3	1406	1406	100%
	Total	**4056**	**4046**	**100%**
Control	1	1191	73	6.13%
	2	1270	68	5.36%
	3	1567	39	2.56%
	Total	**4028**	**180**	**4.46%**

cysts recovered from the mice of treatment and control groups.

2.6. Statistical analysis

The sizes, weights and numbers of cysts recovered from the mice of treatment and control groups were compared by one way analyses of variance(ANOVA) using the Tukey test. All data are given as the mean ± SE. All statistical analyses were done using the SPSS statistics version 22. Differences of $P < 0.05$ were considered significant.

2.7. Ethics

All experiments were carried out in accordance to the guidelines for the care and use of laboratory animals established by the Ethics Committee of Shiraz University (permit number: 94GCU3M1346) and unnecessary animal suffering was avoided throughout the study.

3. Results

The scolicidal effects of NE of a ZMEO at two concentrations and two exposure times are shown in Tables 1 and 2. Although the mortality rate of protoscoleces in the control group was 4.46%, once protoscoleces were exposed to NE of ZMEO at the concentration of 1 mg/mL, the mortality rate increase to 88.01%and 100% after 10 and 20 min, respectively. NE of ZMEO at the concentration of 2 mg/mL killed 100% of protoscoleces after 10 min. The results of the present study revealed that the NE of ZMEO has high *in vitro* scolicidal activity on the protoscoleces of hydatid cysts and this effect was both dose and time-dependent.

Therapeutic effect of NE of a ZMEO on hydatid cysts in experimentally infected laboratory mice are shown in Table 3. As demonstrated in this table, the mean size of the largest cysts, as well as the total number of the cysts, were significantly decreased ($P < 0.05$) upon treatment with NE of ZMEO. The mean weight of the largest cysts as well as the total weight of cysts were considerably, but not significantly lower in the mice treated with NE of ZMEO (20 mg/kg) in comparison to those of the control group (Table 3).

4. Discussion

Echinococcus granulosus sensu lato is the etiological agent for CE in animals and humans. Based on phenotypic characters and both mitochondrial and nuclear gene sequences, there are currently five clades of *E. granulosus sensu* lato including *E. granulosus sensu stricto* (G1–3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6–10) and the lion strain, *Echinococcus felidis* (Alvarez Rojas et al., 2014; Romig et al., 2015; Spotin et al., 2017). The great majorities of CE in man and domestic animals in different areas of Iran, are caused by *sensu stricto* (G1–G3) particularly the G1 genotype of *E. granulosus* (Alvarez Rojas et al., 2014; Moazeni et al., 2016; Kamelli et al., 2016; Sharbatkhori et al., 2016; Spotin et al., 2017). G1 genotype of *E. granulosus* which is transmitted by sheep, has the widest distribution all around the world and is the main causing agent for human CE (Alvarez Rojas et al., 2014).

Many advances have occurred in the surgical treatment of CE, nevertheless, leakage of protoscoleces may occur during surgical operation. Recurrence rates have been reported to be 2–12% and 10–30% after surgical treatment of pulmonary and hepatic hydatid disease respectively. Consequently, scolicidal agents appear to have an important role in prevention of secondary hydatid disease (Ciftci et al., 2007; Arikan et al., 2007). Even though a lot of efforts have been carried out to discover effective and safe scolicidal agents in the last 150 years, more experimental and clinical studies are still required to identify and evaluate the efficacy and safety of new scolicidal agents (Arikan et al., 2007; Sadjjadi et al., 2008).

In the present study, we obtained 100% *in vitro* scolicidal activity using the NE of ZMEO at a concentration of 1 and 2 mg/kg after 20 and 10 min of exposure respectively. The methanolic extract of *Z. multiflora* at concentrations of 10 and 25 mg/mL has been reported to kill 100% of protoccoleces after 3 and 1 min respectively (Moazeni and Roozitalab, 2012). One hundred scolicidal power for *Z. multiflora* EO has been reported with 12.5, 6.25 and 3.125 μL/mL after 5, 20 and 30 min of exposure respectively (Mahmoudvand et al., 2017). The scolicidal power of pure *Z multiflora* aromatic water has been reported to be 100% after

Table 3
Therapeutic effect of nano emulsion of *Zataria multiflora* essential oil on hydatid cysts in laboratory mice.

Group	The largest cyst size	The largest cyst WT	Total number of cysts	Total weight of cysts
	(mm) (Mean ± SE)	(gr) (Mean ± SE)	(Mean ± SE)	(gr) (Mean ± SE)
NE of ZM EO (20 mg/kg)	16.25 ± 1.081^{b}	2.41 ± 0.49^{a}	32.37 ± 4.77^{b}	10.53 ± 3.13^{a}
Control	19.87 ± 0.91^{a}	2.73 ± 0.12^{a}	84.25 ± 14.24^{a}	18.21 ± 2.96^{a}

SE: Standard error.
NE: Nanoemulsion.
ZM: *Zataria multiflora.*
EO: Essential oil.
Columns with different letters indicate a significant difference ($P < 0.05$) between groups.

Table 4
One hundred scolicidal activity obtained by different types of *Zataria multiflora* products.

Scolicidal agent	Concentration	Exposure time	Control viability (%)	Reference
Zataria multiflora (ME)	10, 25 mg/mL	3, 1 min	86	Moazeni and Roozitalab (2012)
Zataria multiflora (EO)	17.5 µg/mL	10 min	97.9	Kavoosi and Mahammadi Purfard (2013)
Zataria multiflora (EO)	3.125 µL/mL	30 min	94.6	Mahmoudvand et al., 2017
Zataria multiflora (EO)	6.25 µL/mL	20 min	96.3	Mahmoudvand et al., 2017
Zataria multiflora (EO)	12.5 µL/mL	5 min	100	Mahmoudvand et al., 2017
Thymol	10 µ g/mL	80 days	50	Elissondo et al. (2008)
Thymol	250 µg/mL	10 min	100	Elissondo et al. (2013)
Thymol	50 µg/mL	5 days	89.5	Yones et al. (2011)
Thymol	25 µg/mL	20 min	96.3	Mahmoudvand et al., 2017
Thymol	50 µg/mL	10 min	98.0	Mahmoudvand et al., 2017
Thymol	100 µg/mL	5 min	100	Mahmoudvand et al., 2017
Zataria multiflora (AW)	Pure	5 min	96.3	Moazeni et al., 2015

ME: Methanolic extract.
EO: Essential oil.
NE: Nano emulsion.
AW: Aromatic water.

5 min of exposure (Moazeni et al., 2015).

New and more effective therapeutic tools are required to optimize the treatment of hydatid disease (Elissondo et al., 2008). Nowadays, only albendazole and mebendazole, are licensed for treatment of human echinococcosis, therefore, the search for new drugs more suitable for the treatment of this disease is indispensable. In the present study, we observed a significant decrease in the size and number of hydatid cysts obtained from the infected mice treated with NE of ZMEO at 20 mg/kg ($P < 0.05$). The weights of total cysts were also considerably, but not significantly lower in treated mice in comparison to those of the control group. *Zataria multiflora* aromatic water (40 ml/L in drinking water for 30 days) and the methanolic extract of *Z. multiflora* (8 g/L in drinking water for 30 days) have been reported to significantly reduce the weights and sizes of hydatid cysts in experimentally infected laboratory mice (Moazeni et al., 2014a, 2014b).

It has been previously reported that thymol is the main compound of *Z. multiflora* EO (Saleem et al., 2004; Saei Dehkordi et al., 2010; Sajed et al., 2013; Moazeni et al., 2014a). Aditionally, the scolicidal activity (Elissondo et al., 2008, 2013; Yones et al., 2011; Mahmoudvand et al., 2017) and destructive effect of thymol on the germinal layer of hydatid cysts (Elissondo et al., 2013) have been formerly documented. As shown in Table 4, scolicidal effect of thymol, the main compound of *Z. multiflora* EO, seems to be controversial. While Yones et al. (2011) reported 100% scolicidal activity for thymol at 50 µg/mL after 5 days of exposure, Mahmoudvand et al. (2017) obtained 100% scolicidal activity with this concentration after 10 min of exposure. It may be related to temperature at which the specimens were incubated. Since, they carried out their experiments at 30 °C and 37 °C respectively. However, all previous studies have shown that, the scolicidal power of the all products of *Z. multiflora* is time and dose dependent (Table 4). In the present study also, NE of ZMEO showed time and dose dependent scolicidal activity (Tables 1 and 2).

Several chemical agents have been used as scolicides against hydatid cysts. The benefits of *Z. multiflora* over the other chemical or even herbal scolicidal agents are its immunostimulatory (Shokri et al., 2006; Khosravi et al., 2007; Soltani et al., 2010) antioxidant (Babaie et al., 2007; Sharififar et al., 2007; Saei Dehkordi et al., 2010; Akrami et al., 2015; Moradi et al., 2016; Mohammadi et al., 2016), and hepatoprotective (Sakhaee et al., 2011; Shokrzadeh et al., 2015) activities. Additionally, this herbal plant has no toxic or severe adverse effects following the consumption at pharmacologically relevant doses and thymol as the main ingredient of ZMEO, can be considered as a safe compound (Sajed et al., 2013).

Optimal scolicidal agents are those that are nontoxic and destroy the scolices during a short time and at a low concentration (Sahin et al., 2004). According to required dose for full scolicidal power, and in terms of cost, among all products of *Z. multiflora*, it seems more advantageous to use NE of ZMEO. Furthermore, NEs may reach the target organ more easily because of their small size. Long-term stability, higher water solubility and enhanced ability to penetrate across the biological membranes, are the other privileges of nonoemulsions (Ghosh et al., 2013; Odriozola-Serrano et al., 2014).

In conclusions, we obtained high scolicidal effect and significant reduction in the mean size, number and weight of the cysts in experimentally infected mice following treatment with NE of ZMEO. Therefore, it is most probably that NE of a ZMEO not only maybe used as a scolicidal agent during surgery, but also it may be applied as a therapeutic drug for treatment of human echinococcosis. However, more *in vivo* studies for determination of the safety of NE of a ZMEO on hepatic and biliary systems in animals should be done before recommendation of this type of treatment for human beings.

Conflicts of interest

None.

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