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Research Article
Administration of *Zataria multiflora* as a Novel Therapeutic Strategy in Destruction of the Germinal Layer of Hydatid Cyst

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Abstract

Background and Objective: Hydatid cyst is the larval stage of the tapeworm *Echinococcus granulosus* which causes one of the most important zoonotic helminthic diseases worldwide. Using scolicidal agents before surgery may prevent recurrence of infection, however many scolicidal agents may cause serious side effects. Previously it has been proven that methanolic extract of *Zataria multiflora* has scolicidal effect *in vitro* and *in vivo*. However, the exact mechanism by which *Z. multiflora* exerts its scolicidal effect is unknown. **Methodology:** Therefore, the destructive effect of total methanolic extract of *Z. multifora* on the germinal layer of hydatid cyst was investigated. The germinal layer together with laminated layer of hydatid cyst were collected from livers of sheep and were exposed to 10, 20 and 30 mg mL^{-1} concentrations of the methanolic extract of *Z. multiflora* for 20, 30 and 60 min. Viability of the protoscolices was confirmed by 0.1% eosin staining. **Results:** This therapeutic regimen resulted in discontinuity, destruction and irregularity of the cells in the germinal layer, degeneration and destruction of protoscolices, detachment of the germinal layer from the laminated layer and vacuolation of the laminated layer. **Conclusion:** The effects of methanolic extract of *Z. multiflora* were mainly related to its concentration but variation in time of exposure had slight effect on its effect on germinal layer and protoscoleces.

Key words: Hydatid cyst, *Echinococcus granulosus*, germinal layer, scolicidal, *Zataria multiflora*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cystic echinococcosis, caused by the larval stage of *E. granulosus* is considered a public health concern due to the fact that the disease has a global distribution. Moreover, it has the ability to infect humans as well as domestic livestock including cattle, sheep, horses and other herbivores (Eckert and Deplazes, 2004). The adult worm lives in the small intestine of carnivores, as definitive hosts, while the larval form, hydatid cyst is found in the internal organs of mammalian including human (Seimenis, 2003). The metacestode form is responsible for severe tissue damage, reduction in milk and meat and considerable economic loss due to condemnation of infected organs of the herbivorous animals (Oryan *et al*., 2012). The cyst usually lodges in liver and lungs; however it may develops in other organs such as muscles, kidneys, spleen, central nervous system and contributes to in these organs (Spotin *et al*., 2012). High prevalence of this zoonotic infection has been reported in most regions of the world involving Africa, South America, Eurasia, Australia and Middle East (Grosso *et al*., 2012). Hyper endemic incidence of cystic echinococcosis in Iran has been stated previously (Oryan *et al*., 1994; Sadjjadi, 2006).

Until now the therapeutic approach of hydatidosis includes surgery (Rajabi, 2009). However the utility and safety of surgery is limited due to complications including recurrence of echinococcosis and anaphylactic shock after dissemination of the protoscolex-rich fluid on peritoneum and visceral organ (Kilicoglu *et al*., 2008; Moro and Schantz, 2009). Previously different chemical agents have been tried to treat hydatidosis (Ahmadnia *et al*., 2013) however, the clinical use of such drugs are limited because they are severely nephrotoxic and/or hepatotoxic (Sahin *et al*., 2004; Caglar *et al*., 2008). Moreover, using benzimidazole carbamate derivatives, which are currently used for chemotherapeutic treatment of hydatidosis, is associated with adverse side effects because they must be applied in high doses for extended periods of time in human patients (Walker *et al*., 2004). Therefore, a new effective alternative treatment regime is extremely important in today's climate, where species are becoming resistant to many traditional chemical treatment regimes 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).

Zataria is one of the most reputable herbal medicines that gain much attention in recent years (Khalili and Vahidi, 2006). Former studies have revealed that *Zataria multiflora* has antioxidant (Sharififar *et al*., 2007), anti-inflammatory (Hosseinzadeh *et al*., 2000), antibacterial (Misaghi and Basti, 2007; Sharififar *et al*., 2007) antifungal (Gandomi *et al*., 2009) and antiprotozoal (Abdollahy *et al*., 2004) effects. Moazeni and Roozitalab (2012) have shown that methanolic extract of *Z. multiflora* has a high scolicidal effect against hydatid cyst *in vitro* (Moazeni and Roozitalab, 2012). The extract has also therapeutic and preventive effects on formation of the cysts (Moazeni *et al*., 2014a). However, this is not clear how the extract exert its scolicidal effect. This study has been devoted to evaluate the efficacy of the methanolic extract of *Z. multiflora* on destruction of the germinal layer of hydatid cyst in an *in vitro* condition.

MATERIALS AND METHODS

Extraction and preparation of different concentrations of *Z. multiflora* extract: The leaves of *Z. multiflora* were dried under shade and powdered mechanically, using a commercial electric blender. Hundred grams of the dry powder was added to 400 mL pure methanol and stirred gently for 1 h. The solution was left at room temperature for 24 h and then it was stirred again, filtered and the solvent was removed by evaporation in a rotary evaporator. The remaining semisolid material was then freeze dried and transferred to a sterile glass container which was stored at 4°C until its use. Three concentrations, 10, 20 and 30 mg mL^{-1}, of *Z. multiflora* extract in normal saline were used in this study.

Collection and preparation of specimens: Unilocular hydatid cysts were obtained from the livers of 10 naturally infected sheep slaughtered at Shiraz and Marvdasht Slaughter houses in Fars Province, southern Iran. For viability test, the protoscolices were stained by 0.1% eosin and were observed by an ordinary light microscope (Olympus, Tokyo, Japan). The laminated and germinal layers of the cyst's wall were carefully dissected, separated and sectioned to blocks of approximately 1 mm^2.

Effectiveness of *Z. multiflora* extract on specimens: Three concentrations, 10, 20 and 30 mg mL^{-1} of *Zataria multiflora* extract in normal saline were used in this study. The specimens including the germinal layer, while attached to the laminated layer were exposed to different concentrations of *Z. multiflora* extract for 20, 30 and 60 min. The specimens of the control groups were exposed to the normal saline. All the specimens were then fixed in 10% neutral buffered

formalin, dehydrated at graded ethanol, cleared by xylol, embedded in paraffin wax, sectioned at 4-5 µm, stained with haematoxylin and eosin and examined for histopathological changes by an ordinary light microscope (Olympus, Tokyo, Japan) by two pathologists unaware of the treatment protocols.

RESULTS

Efficacy of *Z. multiflora* extract on hydatid cysts was analyzed by investigation of discontinuity of the germinal layer, destruction of germinal layer, destruction of protoscolices and separation of the germinal layer and laminated layer. The scoring was performed so that the least changes received one and the highest changes received three score. The scoring results are shown in Table 1. The degree of discontinuity was higher in germinal layers exposed to higher concentration of *Z. multiflora* extract (Fig. 1). Themethanolic extracts of *Z. multiflora* at concentration of 30 mg mL^{-1} had a severe destructive effect on germinal layer. It was able to disorganize and disintegrate the cells lining this layer at 20, 30 and 60 min. The extract at concentrations of 10 and 20 mg mL^{-1} had mild and sever effect on irregularity of cells. At theses concentrations this effect was higher at 30 and 60 min compared to 20 min (Fig. 2). Two and three percent concentrations of *Z. multiflora* intensely affected the protoscolices after 30 and 60 min. Degradation of protoscolices was mild when exposed to 1% concentration of the methanolic extract (Fig. 3). The germinal layer was completely separated from the laminated layer when the hydatid cyst was exposed to 30 mg mL^{-1} concentration of *Z. multiflora* extract in all three periods as well as 20 mg mL^{-1} concentration at 60 min. Detachment of the germinal layer from laminated layer was moderate after 20 and 30 min at 20 mg mL^{-1} concentration and at all periods at 10 mg mL^{-1} concentration (Fig. 4). Severe vacuolation of the germinal layer exposed to 30 mg mL^{-1} concentration of *Z. multiflora* extract was seen in all three time points while 20 mg mL^{-1} extract had moderate to mild effect and 10 mg mL^{-1} concentration had mild to moderate effect (Fig. 5).

The 20 and 30 mg mL^{-1} concentrations of the methanolic extract of this plant had severe harmful effects on protoscoleces of hydatid cysts after 60 min and the changes represented as increase in degradation of these structures at longer durations and higher concentrations. The germinal layer was completely separated from the laminated layer when the hydatid cyst was exposed to 30 mg mL^{-1} concentration of *Z. multiflora* for 60 min.

DISCUSSION

Germinal layer has an important role in the growth and production of protoscolices and infectivity of hydatid cyst, hence destruction of this layer is an effective approach in treatment of the infection (Yalcin *et al*., 2010). Our experiment has revealed the interruption and disappearance of germinal layer as well as damage to protoscolices after exposure to methanolic extract of *Z. multiflora*. Such a result has been previously achieved by application of high-intensity focused

Table 1: Effects of the methanolic extract of *Zataria multiflora* on hydatid cyst

		Exposure time (min)		
Index	Concentration (mg mL^{-1})	20	30	60
Discontinuity of the germinal layer	10	*	*	*
	20	*	*	**
	30	**	**	***
Destruction and irregularity of the cells in the germinal layer	1	*	**	**
	2	**	***	***
	3	***	***	***
Degeneration and destruction of protoscolices	1	**	**	**
	2	**	***	***
	3	***	***	***
Detachment of the germinal layer from the laminated layer	1	**	**	**
	2	**	**	***
	3	***	***	***
Vacuolation of the laminated layer	1	*	**	**
	2	**	**	***
	3	***	***	***

*Mild, **Moderate and ***Severe

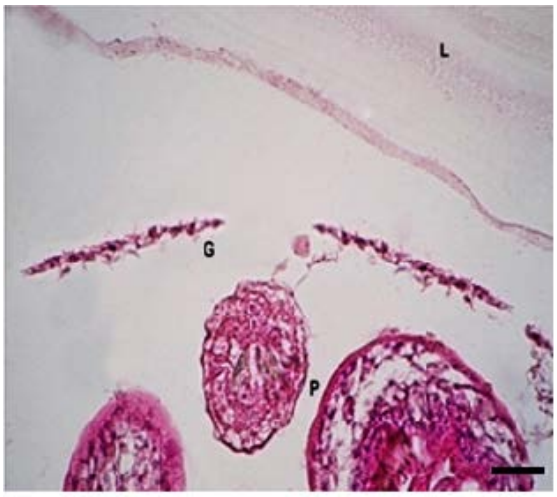

Fig. 1: Section from the germinal layer and protoscoleces of hydatid cyst exposed to 20 mg mL^{-1} concentration of the methanolic extract of *Zataria multiflora* for 30 min. Severe discontinuity of the germinal layer is evident in this section, scale bar: 200 µ, G: Germinal layer, P: Protoscoleces and L: Laminated layer

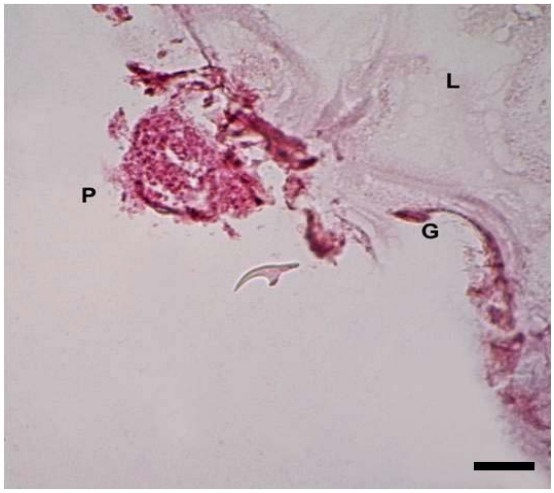

Fig. 3: Section from the germinal layer and protoscoleces of hydatid cyst exposed to 30 mg mL^{-1} concentration of the methanolic extract of *Zataria multiflora* for 60 min. Degeneration and destruction of protoscoleces is evident in this section, scale bar: 200 µ, G: Germinal layer, P: Protoscoleces and L: Laminated layer

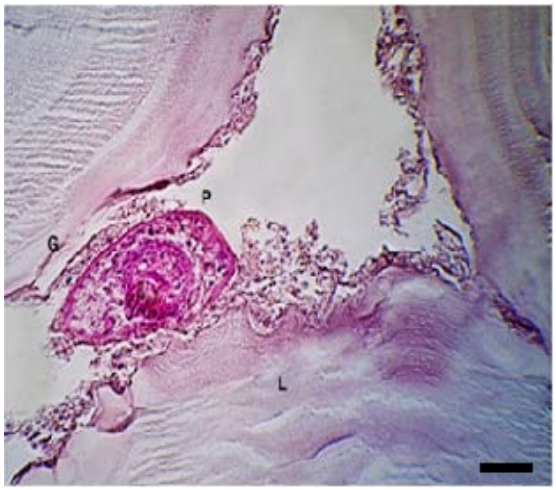

Fig. 2: Section from the germinal layer and protoscoleces of hydatid cyst exposed to 30 mg mL^{-1} concentration of methanolic extract of *Zataria multiflora* for 60 min. Destruction and irregularity of the cells in the germinal layer is evident in this section, scale bar: 200 µ, G: Germinal layer, P: Protoscoleces and L: Laminated layer

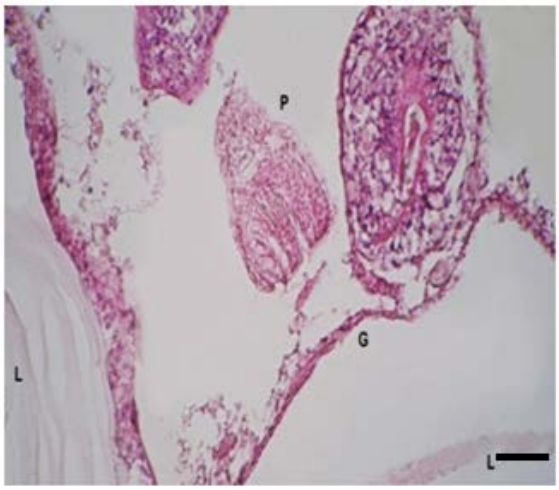

Fig. 4: Section from the germinal layer and protoscoleces of hydatid cyst exposed to 20 mg mL^{-1} concentration of the methanolic extract of *Zataria multiflora* for 30 min. Detachment of the germinal layer from the laminated layer is evident in this section, scale bar: 200 µ, G: Germinal layer, P: Protoscoleces and L: Laminated layer

ultrasound (Wang *et al*., 2007, 2009). The other effect of *Z. multiflora* on hydatid cyst was discontinuation of germinal layer.

Exposure of hydatid cyst layers to the methanolic extract of *Z. multiflora* resulted in detachment of the germinal layer of the hydatid cyst from the laminated layer. Since the

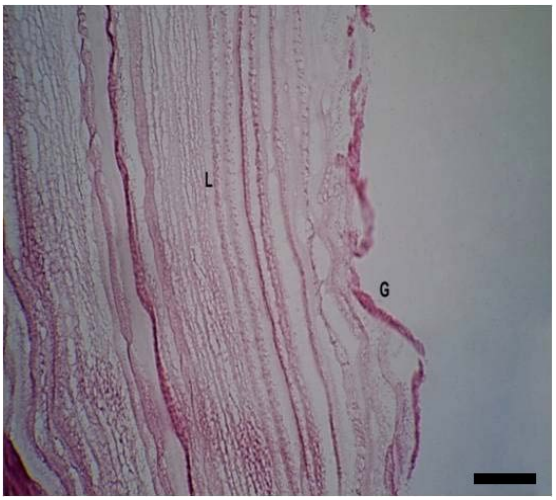

Fig. 5: Section from the germinal layer and protoscoleces of hydatid cyst exposed to 30 mg mL^{-1} concentration of the methanolic extract of *Zataria multiflora* for 30 min. Vacuolation of the laminated layer is evident in this section, scale bar: 200 µ, G: Germinal layer, P: Protoscoleces and L: Laminated layer

laminated layer provides nutrients for germinal layer, detachment of these two layers may contribute in deprivation of germinal layer from such nutrients which was associated with other findings of the present experiment i.e., destruction and irregularity of the cells in the germinal layer. Such approaches have previously been used by Zeghir-Bouteldja *et al*. (2009) on human hydatid cyst. They have used nitric oxide *in vitro* to reach this approach; however the clinical use of nitric oxide was not studied (Zeghir-Bouteldja *et al*., 2009). Combination of albendazole sulfoxide and praziquantel has also led to stronger effect associated by changes in germinal layer including disappearance of cellular integrity as well as loss of cytoplasm and nucleus in cells lining of germinal layer (Palomares *et al*., 2006). Moreover, exposure of the cysts to the extract of *Z. multiflora* resulted in vacuolation of laminated layer. Since laminated layer prevents direct contact between host cell and germinal layer and activation of complement system (Ferreira *et al*., 2000) detachment of these layers and vacuolation of laminated layer as seen in the present study, may affect this prevention. Such changes in the germinal layer and laminated layer may exerted scolicidal effects so that the protoscolices exposed to 20 and 30 mg mL^{-1} concentrations of *Z. multiflora* extract were degenerated. Moazeni and Roozitalab (2012) have reported the scolicidal effect of the methanolic extract of *Z. multiflora* *in vitro*. Another study by Moazeni *et al*. (2014a) has shown that the methanolic extract of *Z. multiflora* has preventive and therapeutic effects on hydatidosis in Balb/C mice so that its preventive and therapeutic effects was very close to albendazol. Moreover, their scanning ultramicroscopic investigations have confirmed the destructive effect of *Z. multiflora* on germinal layer. Similar results have been obtained after administration of *Z. multiflora* aromatic water in Balb/C mice (Moazeni *et al*., 2014b). The effect of *Z. multiflora* extract on the germinal layer is comparable with that of other chemical therapeutic agents used in treatment and prevention of hydatid cyst. Albendazol and aqueous extract of huaier have been shown to be effective against the larval stage of *Echinococcus granulosus* by reducing in cell numbers in the germinal layer (Lv *et al*., 2013). Manouras *et al*. (2007) have used albendazole to detach the germinal layer from laminated layer before surgery. This procedure has resulted in safe removal of the cyst during surgery without any recurrence of infection.

The methanolic extract of *Zataria multiflora* contains phenolic compounds including quercetin, caffeic acid, catechin and gallic acid (Moazeni *et al*., 2014a). Phenolic compounds have antiseptic, antibacterial, anti-fungal, antiparasitic and anti-noceceptive activities (Hajhashemi *et al*., 2002). However, identification of the effective compounds, present in *Z. multiflora* on hydatid cyst was not the aim of our study. Our study has revealed that the effect of *Z. multiflora* extract on the germinal layer was mainly depended on concentration.

CONCLUSION

From this study it was concluded that the methanolic extract of *Z. multiflora* exerts scolicidal effects by affecting the germinal layer. It causes discontinuity of the germinal layer as well as destruction and irregularity of the cells in this layer and also detachment of the germinal layer from the laminated layer. The effects of *Z. multiflora* extracts on hydatid cysts, clearly correlated with concentration of the extract.

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