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Original Article

Subcutaneous Hydatid Cyst in Laboratory Mice: Is it a Suitable Method for Evaluating Therapeutic Agents against Hydatid Cyst?

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ABSTRACT

Hydatid disease is an economic and public health concern in many countries. Currently, surgery is the main treatment option for hydatid disease. In the surgical treatment of hydatidosis, the use of scolicidal agents is very important due to inactivating live protoscoleces and preventing the recurrence of infection. Therefore, it is necessary to investigate new scolicidal agents and novel medications with higher safety and efficacy. In the previous *in vitro* studies, the scolicidal effects of the methanolic extracts and aromatic water of *Zataria multiflora* (*Z. multiflora*) have been demonstrated. Consequently, in this study, the impact of the nanoemulsion of *Z. multiflora* essential oil on subcutaneous hydatid cysts was compared with albendazole (ABZ). Fifty laboratory male mice were inoculated with 300 viable protoscoleces subcutaneously on the two sides of the abdomen. Following five months of infection, the remaining infected mice (n=42) were allocated into two treatment and one control (without treatment) groups containing fourteen animals each. Group A received ABZ at the dose of 50 mg/kg for 60 days, group B received the nanoemulsions of Z. *multiflora* at the dose of 50 mg/kg in drinking water for 60 days, and group C was considered as the control group. All the infected mice were euthanized and necropsied two months post-intervention. Afterwards, the cysts were cautiously collected and their number, size, and weight were compared between the mice of different groups. The mean number of hydatid cysts indicated that the nanoemulsion of *Z. multiflora* essence had a relative superiority to ABZ. On the other hand, the therapeutic effect of ABZ was higher than the nanoemulsion of *Z. multiflora* essential oil in terms of the mean weight and mean size of hydatid cysts. However, no significant difference was observed between the groups ($P>0.5$). Overall, the number, weight, and size of cysts were not significantly different between the groups in this investigation. The lack of satisfactory therapeutic results in this study might be due to the location of hydatid cysts in the subcutaneous space.

Keywords: Albendazole, Essential oil, Nanoemulsion, Subcutaneous hydatid cyst, *Zataria multiflora*

Kyste Hydatique Sous-Cutané chez les Souris de Laboratoire: Existe-t-il une Méthode Appropriée pour Évaluer les Agents Thérapeutiques Contre le Kyste Hydatique?

Résumé: L'hydatidose est un problème sanitaire et économique grave dans de nombreux pays. Actuellement, la chirurgie est le principal traitement de la maladie hydatique. Il est très important d'utiliser des agents scolicides dans le traitement chirurgical de l'hydatidose, en raison de l'inactivation des protoscoléces vivants et de la prévention de la récurrence de l'infection. Par conséquent, il est nécessaire d'étudier de nouveaux agents scolicides ainsi que l'efficacité de nouveaux médicaments plus sûrs et plus efficaces. Des études in vitro antérieures ont montré les effets scolicides des extraits méthanoliques et du distillat aromatique de *Zataria multiflora* (*Z. multiflora*). Cette étude a comparé l'impact sur les kystes hydatiques sous-cutanés d'une nano-émulsion d'huile essentielle de *Z. multiflora* à celui de l'albendazole (ABZ). Au total, 50 souris mâles de laboratoire ont été inoculées avec 300 protoscoléces viables par voie sous-cutanée des deux côtés de l'abdomen.

Après cinq mois d'infection, les souris infectées (n=42) ont été réparties en deux groupes de traitement et un groupe témoin (sans traitement) contenant chacun 14 animaux. Le groupe A a reçu ABZ à la dose de 50 mg/kg pendant 60 jours, le groupe B a été traitée avec une nano-émulsion de *Z. multiflora* à la dose de 50 mg/kg dans l'eau potable pendant 60 jours, et le groupe C a été considéré comme le groupe témoin. Toutes les souris infectées ont été euthanasiées et autopsiées deux mois après l'intervention. Ensuite, les kystes des souris de différents groupes ont été collectés avec précaution et leur nombre, taille et poids ont été comparés. Le nombre moyen de kystes hydatiques a indiqué que la nano-émulsion d'huile essentielle de *Z. multiflora* avait une efficacité relative supérieure à ABZ. Cependant, l'effet thérapeutique de l'ABZ, en termes de poids moyen et de taille moyenne des kystes hydatiques, était supérieur à celui obtenu pour la nano-émulsion d’huile essentielle de *Z. multiflora*. Il est à noter qu'aucune différence significative n'a été observée entre les groupes (P>0.5). En général, le nombre, le poids et la taille des kystes n'étaient pas significativement différents entre les différents groupes de cette étude. L'absence de résultats thérapeutiques satisfaisants dans cette étude pourrait être dû à la localisation des kystes hydatiques dans l'espace sous-cutané.

Mots-clés: Albendazole, Huile essentielle, Kyste hydatique sous-cutané, Nanoémulsion, *Zataria multiflora*

INTRODUCTION

Cystic echinococcosis or hydatid disease is considered as an important zoonotic disease throughout the world resulting from infection by the larval stages of several species belonging to the genus *Echinococcus* (Moro and Schantz, 2009; Otero-Abad and Torgerson, 2013; Moazeni et al., 2014a). Six species of *Echinococcus* have been recognized; however, four species, including *Echinococcus granulosus* (Batsch, 1786), *E. multilocularis* (Leuckart, 1863.), *E. vogeli* (Rausch and Bernstein, 1972), and *E. oligarthrus* (Diesing, 1863) are of public health concern and can cause severe to fatal disease in humans (Moro and Schantz, 2009; Otero-Abad and Torgerson, 2013). Furthermore, the number of cases in certain areas of the world is increasing. As a result, it could be considered as an emerging or re-emerging disease (Otero-Abad and Torgerson, 2013). The prevalence of hydatidosis has found to be higher in humans and animals in temperate countries (Ito, 2017). In the endemic regions, human incidence rates may reach > 50 cases per 100000 and the prevalence may be as high as 5–10%. Although the disease can arise anywhere in the body, hydatid cysts are commonly found in the liver and lungs (Kayaalp et al., 2003; Laura et al., 2015). According to the classification of the World Health Organization Informal Working Group on Echinococcosis, four treatment modalities are applied for hydatidosis. These four entail surgery as the standard treatment, the puncture-aspiration-injection-reaspiration (PAIR) technique using protoscolicidal agents, chemotherapy with benzimidazole (BZD) compounds, namely albendazole (ABZ) or mebendazole (MBZ), in addition to the Watch and Wait approach for inactive, clinically silent cysts (Junghanss et al., 2008; Brunetti et al., 2010). Although surgery has remained the most influential treatment, chemotherapy is the preferred practice where surgeons are not available (Moazeni et al., 2015). The BZDs are extensively utilized for treating hydatid disease. Nonetheless, chemotherapy with BZDs has some limitations, such as low solubility, poor absorption, long-term therapies, as well as some side effects and improper results (Daniel-Mwuambete et al., 2003; El-On, 2003). In surgical hydatidosis treatment, the usage of scolicidal agents is of remarkable importance because these agents inactivate live protoscoleces and prevent the recurrence of infection (Moazeni et al., 2017). Formalin, cetrimide, hypertonic saline, povidone-iodine, ethyl alcohol, H_2O_2, silver nitrate, and ABZ have been used as scolicidal agents inactivating hydatid cyst content (Moazeni et al., 2015). However, most of these scolicidal agents may be accompanied by adverse effects (Moazeni and Roozitalab, 2012). Therefore, it is essential to investigate new medicines and scolicidal agents with higher safety and effectiveness. Nanotechnology represents one of the most capable technologies of the 21st century that involves the understanding, design, and fabrication of materials at atomic and molecular scales. Applications of nanotechnology in medicine are commonly related to the diagnosis and treatment of diseases. Nanoemulsions are thermodynamically stable oil-in-water (o/w) dispersions with a drop size of 10-100 nm. Enhanced uptake of nanoemulsions (lipid emulsion) by the cells of the phagocytic system has been reported leading to their potential role as a novel antimicrobial agent (Chaudhri et al., 2015; Rai and Kon, 2015). *Zataria multiflora* is a useful medicinal herb belonging to the Lamiaceae family with numerous pharmacological and therapeutic properties (Shokri and Sharifzadeh, 2015). The *Z. multiflora* Boiss is a spicy plant that geographically grows only in Iran, Pakistan, and Afghanistan. It is used as a flavor agent or spice in a variety of foodstuffs in Iran (Mahboubi and Ghazian Bidgoli, 2010). Moreover, the literature showed immunostimulating, pain-relieving, anti-inflammatory, antinociceptive, antioxidant, antibacterial, antifungal, and antiviral effects for *Z. multiflora* (Saei-Dehkordi et al., 2010; Moazeni et al., 2017). With this background in mind, this experimental study evaluated the *in vivo* efficacy of the nanoemulsions of *Z. multiflora* essential oil and ABZ in the treatment of subcutaneous hydatid cysts in experimentally infected laboratory mice.

MATERIAL AND METHODS

Plant. Aerial parts of *Z. multiflora* Boiss were collected from wild-growing plants at the full flowering stage in the Chahak region of Neyriz suburb, Fars Province, Iran. The plant species were identified and authenticated in the Herbarium department of Shiraz University, Shiraz, Iran.

Preparation of Nano-emulsions of *Z. multiflora* Essential Oil. Nanoemulsions of *Z. multiflora* essential oil was prepared by a low-energy system using water (96% v/v), EO (2% v/v), and Tween 80 (2% v/v). Tween 80 and EO were stirred at 2400 rpm for 20 min by the means of a homogenizer (Ostertag et al., 2012) and water was added gently to the mixture. The obtained mixture was further stirred at 3000 rpm for 30 min. The resultant nanoemulsions were stored at 4°C until use. Approximately 1000 mL of the nanoemulsions of *Z. multiflora* essential oil at the concentration of 10 mg/mL was obtained from 10 g of essential oil (Ostertag et al., 2012).

Collection of Protoscoleces. Protoscoleces of *E. granulosus* were aseptically collected from hydatid cysts obtained from the liver of naturally infected sheep slaughtered in Shiraz abattoir, South of Iran. The hydatid fluid of cysts was aseptically aspirated and transferred into glass cylinders and left aside for 30 min. Protoscoleces settled at the bottom of cylinders and the supernatant was discarded. Next, the settled protoscoleces were removed through washing three times by normal saline. The viability of protoscoleces was confirmed based on their motility under a light microscope. Finally, the live protoscoleces were transferred into a dark bottle containing normal saline and were stored at 4 °C until use.

Infection of Mice. Fifty 12-week-old laboratory male mice weighing 34-40 g were infected by the subcutaneous injection of 300 protoscoleces dissolved in 0.5 ml of RPMI 1640 medium. Protoscoleces were injected into the two sides of the mice abdomen. Infected animals were allocated to the intervention and control groups. All animals in the study were kept at 24-25 °C, fed *ad libitum*, and given tap water.

Therapeutic Trials. After 5 months of infection, the remaining infected mice (*n*=42) were allocated into two treatment and one control groups with fourteen animals each. Group A received ABZ at the dose of 50 mg/kg/day for 60 days, group B received the nanoemulsions of *Z. multiflora* in drinking water at the dose of 50 mg/kg for 60 days, and group C was considered as the control group. All the remaining mice (n=39) were euthanized at the end of the treatment period and necropsy was performed immediately. At necropsy, the skin of the abdominal region was opened and the region was observed for hydatid cysts. Next, all the hydatid cysts of each mouse were carefully collected and counted. The sizes of the cysts were determined by a scaled ruler and Adobe Photoshop CS6. In addition, we recorded the weights of the cysts using a digital scale (Kern & Sohn GmbH, Balingen, Germany). The efficacy of the nanoemulsions of *Z. multiflora* was evaluated by comparing the number, size, and weight of cysts between the three groups.

Statistical Analysis. Following the evaluation of homogeneity, the means of the size of the cysts were compared between different groups by the Tukey test [one-way analysis of variance (ANOVA)]. Moreover, the mean numbers of the cysts and mean weights were compared by the Mann-Whitney U test. All the data were analyzed using the SPSS software version 22. $P < 0.05$ was considered significant for all the tests.

RESULTS

The mean number, weight, and size of the subcutaneous hydatid cysts in the mice of ABZ, *Z. multiflora* essential oil, and control groups are summarized in tables 1, 2, and 3 respectively. As shown in table 1, the means of number, weight, and size of the subcutaneous hydatid cysts after 2 months of treating the experimentally infected laboratory mice with ABZ were 23.57±4.14, 1.28±0.28, and 4.47±0.34 respectively. The mentioned values for the mice treated by the nanoemulsion of *Z. multiflora* essential oil were 18.92±3.07, 1.68±0.31, and 5.34±0.4, respectively (Table 2). Furthermore, the means of number, weight, and size of the subcutaneous hydatid cysts in the mice of the control group were 36.91±9.74, 1.89±0.41, and 5.42±0.64, respectively (Table 3). The therapeutic effects of ABZ and *Z. multiflora* essential oil on subcutaneous hydatid cyst in the experimentally infected laboratory mice are demonstrated in table 4. According to the data presented in table 4, in terms of the number of hydatid cysts, the nanoemulsion of *Z. multiflora* essential oil imposed a better therapeutic effect, compared to ABZ. However, the latter difference was not statistically significant (P>0.05). Although the number of hydatid cysts was considerably lower in the group receiving the nanoemulsion of *Z. multiflora* essential oil, in comparison with the control group, the difference was not significant (P>0.05). In terms of the means of the weight and size of the hydatid cysts, ABZ was reported to have more therapeutic influences than the nanoemulsion of *Z. multiflora* essential oil. Nonetheless, this difference was not found to be significant (P>0.05). However, neither the nanoemulsion of *Z. multiflora* essential oil nor ABZ caused a significant reduction in the mean weight or mean size of the hydatid cysts, compared to the control group (Table 4). Overall, the findings of the present study revealed that regarding the mean number of hydatid cysts, the nanoemulsion of *Z. multiflora* essential oil had a relative superiority to ABZ. On the other hand, the therapeutic impact of ABZ was more prominent than the nanoemulsion of *Z. multiflora* essential oil regarding the mean weight and mean size of the hydatid cysts.

Table 1. Number, weight, and size of the hydatid cysts in the mice treated by albendazole (50 mg/kg)

Mouse number	Number of cysts	Weight of cysts (g)	Size of cysts (mm)
1	8	1.02	7.21
2	6	0.8	6.66
3	18	0.64	3.67
4	7	0.39	5.5
5	9	0.3	4.88
6	12	0.45	4.7
7	15	0.57	4.2
8	28	1.55	2.73
9	55	2.9	3.46
10	36	2.42	4.87
11	46	3.77	4.06
12	36	1.16	3.56
13	27	1.67	4.2
14	27	0.41	2.9
Mean±SD	**23.57±4.14**	**1.28±0.28**	**4.47±0.34**

DISCUSSION

Herbal treatments can be used as alternative therapies depending on their conditions and efficacy. Herbal remedies do not have the complications of chemical treatments and are acceptable in terms of sustainability and compatibility with the environment (Elissondo et al., 2008).

Table 2. Number, weight, and size of the hydatid cysts in the mice treated by the nanoemulsion of *Zataria multiflora* essential oil (25 mg/kg)

Mouse number	Number of cysts	Weight of cysts (g)	Size of cysts (mm)
1	28	1.36	3.67
2	11	0.68	**5.13**
3	8	0.81	**5.81**
4	24	1.64	**4.45**
5	9	1.73	**6.27**
6	14	0.74	**4.82**
7	35	2.9	**4.82**
8	31	2.61	**3.91**
9	30	0.98	**3.75**
10	8	0.46	**7.3**
11	32	4.47	**4.25**
12	10	2.3	**8.15**
13	6	1.2	**7.1**
Mean±SD	**18.92±3.07**	**1.68±0.31**	**5.34±0.4**

Table 3. Number, weight, and size of the hydatid cysts in the mice of the control group

Mouse number	Number of cysts	Weight of cysts (g)	Size of cysts (mm)
1	19	1.11	3.92
2	24	1.6	7.2
3	61	1.68	5.59
4	15	0.57	3.63
5	14	1.18	5.64
6	7	0.53	6.08
7	53	5.7	6.35
8	11	0.65	5.31
9	117	2.77	3.15
10	4	1.9	11.12
11	50	2.93	4.23
12	68	2.06	2.89
Mean±SD	**36.91±9.74**	**1.89±0.41**	**5.42±0.64**

Table 4. Mean number, weight, and size of the hydatid cysts in the mice of the test and control groups

Group	Number of cysts (Mean±SD)	Weight of cysts (g) (Mean±SD)	Size of cysts (mm) (Mean±SD)
Albendazole	23.57±4.14[a]	1.28±0.28[b]	4.47±0.34[c]
Z. multiflora	18.92±3.07[a]	1.68±0.31[b]	5.34±0.4[c]
Control	36.91±9.74[a]	1.89±0.41[b]	5.42±0.64[c]

Herefore, many studies have been carried out to investigate the use of medicinal plants for treating bacterial, viral, and parasitic diseases. The *Z. multiflora* is a plant well suited to the nature of humans and animals. It significantly improves the intrinsic and acquired immune function in laboratory animals and acts as a stimulator of the immune system (Shokri et al., 2006; Khosravi et al., 2007). In the previous *in vitro* studies, the scolicidal effects of the methanolic extracts and aromatic water of *Z. multiflora* were demonstrated (Moazeni and Roozitalab, 2012; Moazeni et al., 2015). In addition, recent *in vivo* investigations demonstrated the therapeutic impacts of the methanolic extract and aromatic water of *Z. multiflora* on hydatid cysts (Moazeni et al., 2014a; Moazeni et al., 2014b; Moazeni et al., 2017). The BZD compounds, especially ABZ, are commonly utilized for the treatment of hydatid disease (Casado et al., 1996). In the present study, the efficacy of ABZ and the nanoemulsion of *Z. multiflora* essential oil on the subcutaneous hydatid cyst was investigated. Although the nanoemulsion of *Z. multiflora* essential oil reduced the number of hydatid cysts, compared to ABZ, the difference between the effect of the two agents was not significant (P>0.05). Furthermore, *Z. multiflora* essential oil diminished the number of hydatid cyst to about half of the control group. However, the latter difference was not statistically significant (P>0.05). Results of the current study indicated that neither *Z. multiflora* essential oil nor ABZ imposed significant decreasing impact on the mean weight and size of the hydatid cysts, compared to the control group. Moazeni et al. (2017) used the nanoemulsion of *Z. multiflora* essential oil (20 mg/kg) to treat the mice that were intraperitoneally infected by hydatid disease. They observed a significant decline in the size and number of hydatid cysts of the test group, in comparison with the control group. Moreover, they reported that the weight of cysts considerably decreased in the intervention group. Nonetheless, the mean weight of the test group was not significantly lower than the control group (Moazeni et al., 2017).

According to the literature, thymol is the main compound of *Z. multiflora* essential oil (Saei-Dehkordi et al., 2010; Sajed et al., 2013; Moazeni et al., 2014b). On the other hand, the killing effect of thymol on the germinal layer of hydatid cysts has been formerly reported in mouse models (Elissondo et al., 2008). Nanoemulsions can be more easily received by the target organ due to the small size of their particles. Moreover, these compounds have high stability, are more soluble in water, and have greater ability in passing through the biological membranes (Ghosh et al., 2013; Odriozola-Serrano et al., 2014). Overall, our findings demonstrated that the number, weight, and size of the cysts were not significantly different between the two treatment and control groups. The lack of satisfactory therapeutic results in the present study could be attributed to the location of hydatid cysts in the subcutaneous space.

In the previous studies, favorable outcomes have been obtained with the cysts located in the internal organs or peritoneal cavity of infected mice. Consequently, the intraperitoneal route is recommended for the establishment of hydatid disease in laboratory mice in order to test the efficacy of the nanoemulsions of essential oils against hydatid disease.

Ethics

The present study was approved by the Ethics Committee of the Aja University of Medical Sciences.

Research Involving Human Participants and/or Animals

This article does not contain any studies with human subjects. All institutional and national guidelines concerning the care and use of laboratory animals were followed.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Authors' Contribution

Study concept and design: Dr. Moazeni, Dr. Shaddel
Acquisition of data: Dr. Moazeni
Analysis and interpretation of data: Dr. Moazeni, Dr. Shaddel, Amin Ahmadi
Drafting of the manuscript: Amin Ahmadi
Critical revision of the manuscript for important intellectual content: Dr. Moazeni, Amin Ahmadi
Statistical analysis: Dr. Moazeni, Amin Ahmadi
Administrative, technical, and material support: AJA University of Medical Sciences & Shiraz University

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