Cucurbita maxima (Pumpkin) seeds: Scolicidal activity and preventive efficacy of its extract on experimental hydatidosis in mice

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Abstract

Introduction: Hydatid Disease (HD), a helminth infection with various clinical complications is a serious economic and public health concern around the world. Nowadays, different agents have been broadly applied as scolicids; these agents have several side effects on patients. Therefore, the aims of this experimental study were to evaluate the scolicidal activity of Cucurbita maxima seeds methanolic extract (ME) against protoscolices of hydatid cyst in-vitro. Also, an in vivo assay was designed to prove the preventive effects of the extract.

Materials and methods: For this work, Two different concentrations (25 and 50 mg/ml) of the C. maxima seeds ME were used in various exposure times (10, 20, 30, 40, 50, and 60 min). Additionally, thirty mice were infected intraperitoneally by injection of 1000 viable protoscolices and were divided into three groups with ten mice in each Group I (treated by Albendazole), group II (received 50 mg/ml of C. maxima seeds ME) and group III (untreated controls).

Results: Overall, C. maxima seeds ME exhibited significant scolicidal effects in compared with control group ($P<0.05$). At a concentration of 50 mg/ml, the mortality percentage increased to 84.7%, 96.1% and 100% after 40, 50 and 60 minutes, respectively. Also, the obtain data in necropsy demonstrated that C. maxima seeds ME have an inhibitory effect on the hydatid cyst development. No hydatid cyst formation observed in mice treated with C. maxima seeds ME (50 mg/ml) versus control group which hydatid cyst occurred in 6 (from 10) mice.

Conclusion: The current investigation indicated that applying C. maxima seeds ME could be considered as a potential scolicidal agent due to being economical, safer and non-toxic compared to the reference chemical drugs. However, further studies on probably active compounds and also to the determination of its mode of action on parasites are required.

Keywords: Hydatid disease, Cucurbita maxima methanolic extracts, Scolicidal, Preventive assay

Introduction

Hydatid Disease (HD) is a major neglected zoonotic disease with various clinical complications caused by the larval stage of *Echinococcus granulosus* (1, 2). HD is still an important public health concern in many countries of the world, such as Iran.

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The disease affects humans as well as domestic animals, including cattle, sheep, camels, pigs, horses and others worldwide (3, 4). HD affects mainly the liver (50-70% of all cysts) but can develop in lung (20-30%) and, less frequently, in spleen, bone, brain, and other organs (5, 6). The preferred method for treatment of HD is surgery, but it increases the risk of intra-operative spillage of protoscolices (6,7,8). This is the major cause of recurrence, which is seen in approximately 10% of the postoperative cases. Operative spillage may also lead to the secondary disseminated intraperitoneal hydatidosis (6, 8). There is a severe need to some safe and more effective scolicids for killing or inactivation of protoscolices in HD surgeries (7, 9). Formalin, hypertonic saline, cetrimide, chlorhexidine, hydrogen peroxide and ethyl alcohol are some of the compounds used as scolicidal, but all of these agents are concentration dependent and their degree of dilution in the cyst contents is quite unpredictable (10), Also, they have different adverse effects such as liver necrosis and biliary tract fibrosis (2). Thus, it is indispensable to develop new scolicidal materials with more efficacious and low adverse effects, which can be implemented during surgery (11). Considerable evidence has demonstrated that some plant derived products (PDPs) can be useful as scolicidal (12). Based on previous in vitro and in vivo data on scolicidal effects of PDPs, *Mallotus philippinensis* (13), *Allium sativum* (Garlic) (6), Zingiber (14), *Zataria multiflora* (11) and *Berberis vulgaris* (15) showed significant scolicidal activity. *Cucurbita maxima* (Pumpkin) (Family Cucurbitaceae) commonly known as Squash is widely used as vegetable and a source of vitamin A, iron, phosphorus and calcium (16). *C. maxima* seeds with considerable safety margin versus commonly consumed chemical drugs (17), is a very rich in terms of amino acids that are building blocks of proteins, especially the essential amino acids such as phenylalanine and methionine which are not produced in the human body(18). Investigation on *C. maxima* (Squash) reported that spinasterol isolated from the flowers of *C. maxima* potentially showed anti-carcinogenic, anti-genotoxic (19), and anti-mutagenic activity (20). The *C. maxima* seeds were used in the treatment of digestive disorders [21], as well as essential oil of *C. maxima* seeds exhibited significant anti helmintic activity (16, 18, 22). Recently, an in vitro study on hydatid cyst protoscolices revealed that 50 mg/ml of *C. maxima* (pumpkin) seeds chloroformic extracts showed scolicidal activity (7). Based on the previous investigations on anthelmintic and scolicidal activity of *C. maxima* seed extracts, present study was conducted in two stages, first was the in vitro activity of *C. maxima* methanolic extracts (ME) on protoscolices of Hydatid Cyst, and also in the next stage in vivo efficacy of this methanolic compound for the prevention of hydatid cysts tested in mice experimentally infected with protoscolices of hydatid cyst.

Materials and methods

Collection of protoscolices: Protoscolices were collected from livers of naturally infected sheep, with hydatid disease, slaughtered at Malayer abattoir, Hamadan province, Southwestern of Iran. Protoscolices was isolated from the cysts ,according to the method described previously by Moazeni and Nazer (6). Briefly, the fluid of cysts was transferred into glass cylinders by aspiration, aseptically and left to set for 30 min. The protoscolices settled down at the bottom. The supernatant was discarded and the remaining protoscolices at the bottom were washed three times with normal saline. The viability of the protoscolices was tested by their motility using a light microscope. The protoscolices were transferred into a dark container containing

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normal saline solution and stored at 4°C until use.

Methanolic extract Preparation: The *C. maxima* seeds were purchased from a local market in Hamadan (Iran) and C. maxima seeds ME was obtained as follows: C. maxima seeds, dried under shade, and mechanically powdered using a commercial electric blender. To obtain the methanolic extract, 200 g of dry seeds powder was added to 800 ml of pure methanol and mixed gently for 2h using a magnetic stirrer. The obtained solution was left at room temperature for 24h. The compound was stirred again and filtered and then the solvent was removed by evaporation in a rotating evaporator. The remaining semisolid material was then freeze-dried. The obtained residue was placed into a glass container and stored at 4°C until use (6, 23).

Scolicidal activity: In the present study, we had two concentrations (25 and 50 mg/ml) of the *C. maxima* seeds ME for 10, 20, 30, 40, 50, and 60 min. To obtain the *C.maxima* seeds ME solution at 25 and 50 mg/ml concentrations, 0.25 and 0.5 gr of dried extract was dissolved in 10 ml of normal saline, respectively. Then 2 ml of *C. maxima* seeds ME solution was placed in test tubes, to which drop off protoscolex-rich sediment was added. The contents of the tubes were mixed, gently. The tubes were incubated at 37°C for 10, 20, 30, 40, 50, and 60 min. At the end of each incubation time the upper phase was carefully discarded without disturb the protoscolices. To check the viability of protoscolices, 0.1% Eosin stain (1g of Eosin powder in 1000 ml distilled water) was added to the settled protoscolices and mixed gently. The upper portion of the solution was discarded after 15 min of incubation. The remaining settled protoscolices was then smeared on a manually scaled glass slide, covered with a cover glass, and examined under a light microscope. The percentages of dead protoscolices (which absorbed Eosin and stained red after 15 min of incubation) were calculated by counting a minimum of 500 protoscolices. Remained colorless protoscolices were considered potentially viable (Figure 1). Non-treated protoscolices were considered as a control group in each experiment. The experiments were performed in triplicate (6, 7).

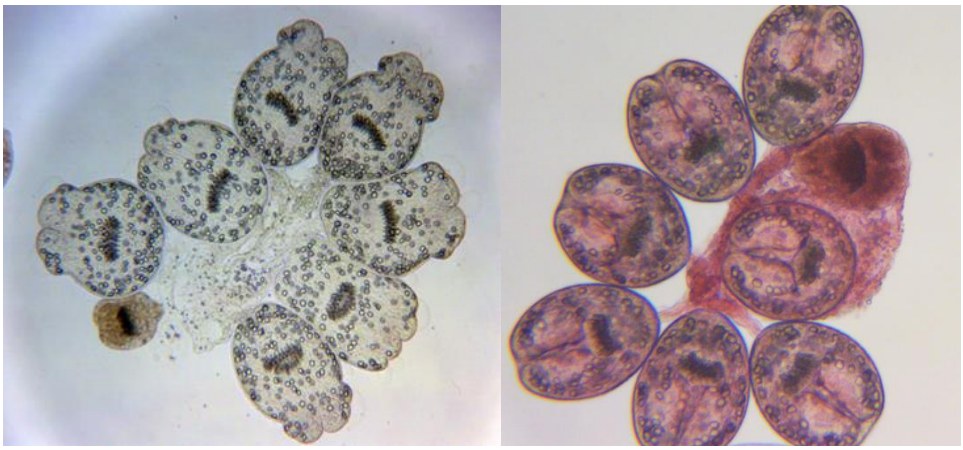


Figure 1. (Left) Live protoscolices after staining with 0.1% eosin. (Right) Dead protoscolices after exposure to *C.maxima* seeds ME and staining with 0.1% eosin.

Experimental infection of mice: Preventive trial in the present study was designed according to the method described previously (11) with some modification as follows: To confirm the preventive effects of *C. maxima* seeds ME on hydatid cyst formation, an in vivo assay was carried out on 30 healthy laboratory mice, weighing 20 to 30 g, 6-8 weeks old.

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Field application and study design: The mice were infected intraperitoneally by injection of 1000 protoescolices per animal, dissolved in 0.5 ml of distilled water. They were randomly divided into three groups of ten animals each. The experimental groups were kept in cages separately with free access to food and water. Group I (10 mice) was treated by Albendazole at 150 mg/kg body weight/day for 10 days, the group II (10 mice) received 50 mg/ml of *C.maxima* seeds ME in drinking water for 6 months, and group III (10 mice) were the untreated controls. Albendazole and *C. maxima* seeds ME each was administered by oral gavage. All mice (from groups I, II and III were euthanized at the end the experiment (after 6 months) by Ether, and a necropsy was carried out immediately. At necropsy, the body cavity was opened, and the internal organs were observed for hydatid cysts (11).

Statistical analysis

The scolicidal trial was repeated three times and their average of death protoscolices were calculated and considered for each extract. Statistical analysis was performed using SPSS software version 20. Differences between the control and treatment groups were analyzed by Chi-square test. Values were considered statistically significant at $p<0.05$.

Results

Scolicidal effects of *C. maxima* seeds ME: The mortality rate of hydatid cyst protoscolices after exposure to different concentrations of the *C. maxima* seeds ME following various exposure times are presented in (Table 1). It could be observed that the *C. maxima* seeds ME in 50 mg/ml concentrations exhibited significant scolicidal effects in compared with control group ($P<0.05$). All protoscoleces were killed after 60 min of exposure to 50 mg/ml concentration of the agent. Also, after 60 min exposure time, the scolicidal activity of *C. maxima* seeds ME at a concentration of 25 mg/ml was 83.4%. The results showed a time dependent manner in mortality rate of hydatid cyst protoscolices, so that with the increasing in time mortality percentage was increased, dramatically (Figure 2). When protoscolices were exposed to the *C. maxima* seeds ME at a concentration of 50 mg/ml, the mortality percentage increased to 84.7%, 96.1% and 100% after 40, 50 and 60 minutes, respectively. The mortality rate of hydatid cyst protoscolices after exposure to 25 mg/ml concentration of *C. maxima* seeds ME at all times was lower versus 50 mg/ml of its. The results of our study confirmed that *C. maxima* seeds ME showed a high scolicidal activity in vitro.

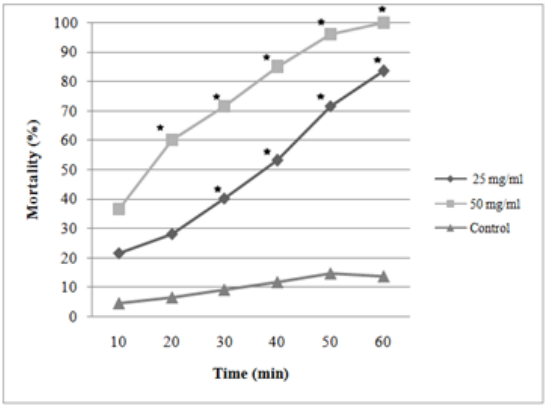


Figure 2. Mortality percentage of protoscolices in different concentrations of the C.maxima seeds ME and various times. Values are presented as Mean of mortality rate (%) of protoscolices.

Preventive effects of *C. maxima* seeds ME: We used 50 mg/ml concentration of *C. maxima* seeds ME in Preventive trial, which exhibited higher scolicidal activity in vitro. Based on necropsy evidences, *C. maxima* seeds ME showed a preventive effect on formation of hydatid cyst in mice. The obtain data in necropsy showed that no hydatid cyst formation observed in mice treated with *C. maxima* seeds ME (50 mg/ml in drinking water for 6 months) and Albendazole (150 mg/kg body weight/day for 10 days). Additionally, hydatid cyst

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occurred in 6 (from 10) mice of control group, and the number of developed cysts in this group were more than four in two mice.

Table1. Scolicidal activity of C.maxima seeds ME in different concentrations and various exposure times.

		Dead protoscoleces /total treated protoscoleces (%)					
Concentration	Experiment	10 min	20 min	30 min	40 min	50 min	60 min
25 mg/ml	1	127/500(21.9)	117/420(27.8)	255/560(45.6)	280/480(58.4)	387/500(77)	462/550(84)
	2	100/500(20)	152/540(28.2)	189/510(37)	258/520(49.6)	295/490(60)	384/460(83.4)
	3	117/520(22.5)	95/340(28)	176/480(36.7)	235/460(51)	305/390(78)	324/390(83)
Total		344/1600(21.5)	364/1300(28)	620/1550(40)	773/1460(53)	987/1380(71.5)	1170/1400(83.4)
50 mg/ml	1	182/480(37.9)	368/600(61.3)	434/600(72.3)	323/380(85)	412/430(96)	590/590(100)*
	2	201/540(37.2)	252/420(60)	294/420(70)	522/600(87)	472/500(94.5)	450/450(100)*
	3	160/460(34.7)	281/480(58.5)	403/560(72)	443540(82)	518/530(97.8)	600/600(100)*
Total		543/1480(36.6)	901/1500(60)	1131/1580(71.5)	1288/1520(84.7)	1402/1460(96.1)	1640/1640(100)
Control	1	23/520(4.5)	38/580(6.5)	46/500(9.2)	46/380(12)	74/510(14.5)	84/600(14)
	2	26/480(5.4)	22/420(5.2)	23/290(8)	45/450(10)	63/460(13.7)	61/510(12)
	3	13/370(3.5)	27/360(7.5)	47470(10)	64/490(13)	91/600(15.2)	54/360(15)
Total		62/1370(4.4)	87/1360(6.4)	116/1260(9)	155/1320(11.7)	228/1570(14.5)	199/1470(13.6)

Values are presented as a fraction or percent of killed protoscolices.
*Significant difference at P<0.05.

Discussion

Hydatid cyst as a larval stage (metacestode) of *E. granulosus* is still a serious economic issue and public health concern worldwide. Few strategies are available for the treatment of hydatid disease in human (3). Surgery is the most common method for particular WHO stage disease. Chemotherapy with benzimidazoles and also PAIR (puncture, aspiration, injection and reaspiration) are recommended as alternative strategies to surgery, in some patients who cannot tolerate surgery (13). Removal of the cysts with chemotherapy using benzimidazoles before and after surgery is the best and selective method. But, there are evidences of resistance to synthetic anthelmintic agents and the adversity of numerous side effects, including thrombocytopenia, leukopenia and hepatotoxicity (4, 24). Therefore, investigations of appropriate alternative and safer compounds with a different mode of action to develop new treatment strategies are necessary. Several experimental studies conducted for finding such scolicidal, previously. For instance, hypertonic saline (25), silver nitrate (26), and chlorhexidine gluconate (27) were tested. Recently, natural compounds such as PDPs were introduced as good candidates to safer control agents that may provide scolicidal activity and few drawbacks. A number of researches describe the scolicidal effects of different herbs and spices and their volatile components. Recently, Moazeni et al. in some in vitro investigations, reported that herbal extracts including *Sumac* (*Rhus coriaria L.*) (28), Zingiber officinale R (14). Have a significant scolicidal activity. *C. maxima* seeds (Pumpkin) (Family Cucurbitaceae) which commonly known as Squash has been used in different parts of the world as traditional medicine for treatments of gastrointestinal parasites as anthelmintic. Also, anthelmintic and in vitro scolicidal activity of *C. maxima* seeds was confirmed, recently (7, 18, 29). This work for the first time describes the scolicidal effects of *C. maxima* seeds ME against protoscoleces of hydatid cysts on in vitro model. Additionally, preventive effects of *C. maxima* seeds ME on hydatid cysts was evaluated in a mouse model. Our results showed that *C. maxima* seeds ME potentially have a scolicidal activity, especially at concentrations 25 and 50 mg/ml (83.4 and 100% mortality rate) after 60 min of application, respectively. Scolicidal effects of *C. maxima* seeds ME against protoscoleces of hydatid cysts in previous studies which conducted by Eskandarian (7) was lower than present data, it could be due to differences in the

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extraction methods and concentrations. In addition to the confirmation of the Scolicidal effects of *C. maxima* seeds ME, especially in the present study, we also confirmed our data in vivo assay. Based on in vivo data no hydatid cyst formation observed in mice treated with *C. maxima* seeds ME (50 mg/ml in drinking water for 6 months) versus control group. Our experimental study proved the preventive effects of *C. maxima* seeds ME for hydatid cyst development. Although spinasterol is an active compound which isolated from flowers of *C. maxima* [19], *further* studies on probable other active compounds of *C. maxima* seeds ME to the determination of its mode of action on parasites are needed. Obviously, we demonstrated that *C. maxima* seeds ME (at 50 mg/ml concentration) exhibited a highly significant scolicidal and preventive activity in vitro and in vivo trials, respectively. Based on our present data *C. maxima* seeds ME with considerable safety margin versus chemical drugs commonly consumed to treatment of HD (17), could be a logical candidate to use as an effective scolicids and preventive agent against cystic echinococcosis.

Conclusion

The current investigation indicated that applying C. maxima seeds ME could be considered as a potential scolicidal agent due to being economical, safer and non-toxic compared to the reference chemical drugs. However, further studies on probably active compounds and also to the determination of its mode of action on parasites are required.

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