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Research Article

The Effect of Alcoholic and Aqueous Extracts of Malva Sylvestris L. on the Vitality of Protoscolices in *Echinococcus Granulosus* (*In Vitro* and *In Vivo*)

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A B S T R A C T

Introduction: Medicinal plants are an important source of compounds that contain effective biological substances for the treatment of serious diseases all over the world.

Aim: The study aimed to determine the effect of aqueous and alcoholic extracts of *Malva sylvestris L*. leaves on the vitality of protoscolices *in vitro* and *in vivo*.

Results: The results of this study showed that the alcoholic and aqueous extracts had an inhibitory effect on protoscolices compared with the control group in all concentrations. Their vitality was measured using dye eosin, which is based on changing the green colour of living protoscolices to red after killing them. The results of this study showed that the concentration of 60 mg/ml for both alcoholic and aqueous extracts showed the highest reduction in the vitality of the protoscolices, by killing them (100% at all exposure times (60, 40, 30, and 15 minutes) in case of alcoholic extract, while the percentage of reducing their vitality with the aqueous extract was 100% at 60 and 40 minutes, and at 30 and 15 minutes, the percentages were 9.67% and 19.32% compared to the control group (94.84% and 96.98%, respectively)).

Conclusion: The alcoholic and aqueous extracts of *M. sylvestris* leaves were able to kill protoscolices *in vitro*. It was also seen that the alcoholic extract showed a greater effect than the aqueous extract.

Keywords: Hydatidosis, *Echinococcus Granulosus*, Protoscolices, Extracts of *Malva Sylvestris L*

Introduction

Cystic Echinococcosis (CE) is an animal disease of importance due to its seriousness. It is prevalent in many regions of the world. It is endemic to most countries such as Peru, Brazil, Argentina, Chile, Uruguay, Iraq, Iran, Turkey, the Levant (including Syria, Jordan and Palestine), and some countries in Africa and western China.[1–3] This disease is caused by the infection of animals or humans with the protoscolices (larval stage) of the genus Echinococcus, and is thus considered among Cyclozoonotic diseases.[4,5] The life cycle of the *E. granulosus* includes carnivores as the definitive host and herbivores as the intermediate host. Humans become infected with hydatidosis by swallowing fertilised parasite eggs contaminated with water or food, as do herbivores, which causes material and economic losses in livestock.[6,7]

It was found that only dogs are infected with the adult worms of E. granulosus.[8] The infection has been found to be associated with certain types of professions such as farmers and herders as a result of close contact between dogs, sheep, and humans in such occupations. Infection in Lebanon is exacerbated in people working in leather tanning due to the use of dog faeces in tanning solutions.[9] Thus, hydatid cysts spread to all parts of the body except for hair and nails.[10]

Two cases of female patients were diagnosed with an unusual hydatidosis muscle injury in the thigh. Both of them came from an infected area. The cyst was extracted after they were given a hypertonic solution and an antiparasitic treatment.[11] Due to of the long period of time for the onset of symptoms that may reach 4 years, and as a result of pain when walking and an increase in size, the injury was diagnosed in the lateral region of the left thigh, which ended in a cutaneous fistula. Petit et al. reported another similar case.[12]

Cystic echinococcosis treatment may result in a few complications, and surgery is the best treatment option.[13] Since the flow of hydatid fluid inside the body is the main cause of secondary parasitic infection, it is recommended to neutralise the contents of the hydatid cyst before opening or removing the cyst using agents that are lethal and effective for protoscolices infections.[14,15] As for chemotherapy, mebendazole and albendazole are widely used to neutralise cyst content during and after surgery. These drugs, Ag-nitrate, cetrimide, albendazole, and mebendazole may cause adverse side effects such as leukaemia, liver necrosis, and cholangitis.[16,5]

Many researchers have tried to find chemical components from natural sources such as plants with anti-parasitic activities. Most of the world's population (60%–80%) relies on traditional medicines to treat many diseases.[17,18] Medicinal plants are an important source of compounds that contain effective biological substances for the treatment of serious diseases all over the world.[20]

Materials and Methods

Plant: *Malva Sylvestris L.*

It is a herbaceous plant belonging to the Malvacea family. Its height ranges between 10 and 30 cm. Its leaves are round with serrated edges, palmate-veined and have a long neck.[20,21] This plant has multiple medicinal properties, including the treatment of infections of the respiratory passages and mouth, laryngitis and emphysema, and the treatment of skin eczema. It is also helpful in wound healing.[22] Its leaves are used as an antiseptic, bactericide and fungicide. It is also characterised as an anti-inflammatory for the urinary tract.[20,23]

Active Ingredients

M. Sylvestris has many effective compounds such as flavones, phenols, sugars, alkaloids, steroids, saponins, binary and triple terpenes and volatile oils.[22,24]

Plant Source

The leaves of the *M. Sylvestris* plant were obtained from the local market and were cleaned and washed. They were then dried under the shade, ground into powder and stored until use.

Preparation of Plant Extracts

Aqueous Extract

The powder of leaves of *M. Sylvestris* (40 g) was taken in a beaker and 400 ml of distilled water was added to it. The mixture was stirred by a magnetic stirrer for 24 hours. Then the mixture was filtered through four pieces of medical gauze and poured into the centrifuge tubes at a speed of 3000 rpm for 10 minutes. After this, the mixture was put in Petri dishes and placed in an oven at 40 °C to dry. Finally, the extract was scraped, collected in clean glass vials, and kept in the refrigerator for further use.[25]

Preparation of Alcoholic Extract

The powder of leaves of *M. Sylvestris* (50 g) was taken in the Soxlet apparatus. 500 ml of ethyl alcohol (70%) was added to it and was left undisturbed. The solvent was evaporated by rotary evaporation. The mixture was finally placed in clean Petri dishes. The dry mass was transferred to an incubator and kept for 24 hours at 50 °C. It was weighed and kept in the refrigerator in sterile and dark-coloured containers until use.[26,27]

Source of Hydatid Cyst

The hydatid cysts were obtained from the sheep of the butchery in Al-Mawsil City, Iraq and were transferred to the laboratories.

Collecting the Protoscolices

The hydatid cysts were sterilised twice with ethyl alcohol (70%), and the cyst fluid was removed with a sterile syringe. The cyst was washed internally with PBS solution, (pH 7.2), supplemented with the antibiotic penicillin (IU20000) and Streptomycin (1 g/L), placed in test tubes, and then centrifuged at 3000 rpm for 10 minutes. The collected protoscolices were examined under a microscope.[28]

Evaluating the Vitality of the Protoscolices

The vitality of protoscolices was evaluated by monitoring the motility of protoscolices and staining with 0.1% aqueous eosin solution. The stain is not absorbed by the living protoscolices, while the dead ones absorb it.[29,30] The percentage of alive protoscolices in the sample was calculated by dividing the number of alive protoscolices

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in the sample by the total number of the calculated protoscolices x 100. The protoscolices whose vitality exceeded 95% were utilised in the next phase.[2]

Method of Studying the Effect of Aqueous and Alcoholic Extracts of *M. Sylvestris* Plant on the Vitality of the Protoscolices *In Vivo*

The mice were divided into three groups in order to demonstrate the effect of aqueous and alcoholic extracts on the growth and development of protoscolices *in vivo*. Each group consisted of five mice (6–8 weeks old), and after determining the lethal dose (LD_{50}) for both aqueous and alcoholic extracts, which amounted to 2.5 mg/ml, and 4 mg/ml, respectively, they were injected into these groups with the protoscolices into the peritoneum cavity at a rate of 2000 protoscolices/ml. The first groups of rats were injected with the protoscolices treated with the alcoholic extract of *M. Sylvestris* at a concentration of 2.5 mg/ml.

The second group of rats was injected with the protoscolices treated with the aqueous extract of *M. Sylvestris* at a concentration of 4 mg/ml.

The third group of rats that were injected with phosphate buffer saline (PBS) and protoscolices which were not treated with alcoholic or aqueous extracts of *M. Sylvestris,* were counted as positive control.

Mice Anatomy

The mice were dissected four months after the injection, and the number of hydatid cysts, if present, were counted. Their weights and diameters were measured, and the sites of infection were determined.

Results

Effect of Aqueous and Alcoholic Extracts of *M. Sylvestris* on the Vitality of *In Vitro* Protoscolices

The results of this study showed a clear effect of the aqueous and alcoholic extracts of *M. Sylvestris* leaves on the vitality of protoscolices as compared to the control group. As shown in Table 1, there were significant differences between the concentrations and exposure periods at the probability level of $p < 0.05$. According to the Duncan test, the concentration of 60 mg/ml of alcoholic extract showed the highest reduction in the vitality of the protoscolices, which caused the killing of all the protoscolices (100%) at all exposure times (60, 45, 30, and 15 minutes). The percentage of reduced vitality by the aqueous extract (60 mg/ml) was 100% in 60 and 45 minutes, while in 30 and 15 minutes, the percentages of viable protoscolices were 9.67% and 19.32%, as compared to the control group values (94.84% and 96.98%, respectively).

The lowest percentage of reducing the vitality of these protoscolices was at a concentration of 15 mg/ml at 15 minutes for both aqueous and alcoholic extracts (89.33% and 91.00% respectively, while those of the control group were 92.86% and 95.23%, respectively).

As for the concentration of 30 mg/ml of the aqueous extract, it reduced the vitality percentage of the protoscolices at 60, 45, 30, and 15 minutes to 43.00%, 61.33%, 76.00%, and 86.33%, respectively; all these values differed significantly with each other. In the alcoholic extract, the percentage of vitality decreased to 40.67%, 53.67%, 68.00%, and 80.67% at 60, 45, 30, and 15 minutes, respectively. The corresponding values for the control group were 93.50% and 96.45%, respectively.

The 45 mg/ml concentration of the aqueous extract reduced the percentage of vitality at 60 and 45 minutes to 7.33% and 10.67%, respectively, which differed significantly from each other. It decreased to 26.33% and 34.34% at 30 and 15 minutes respectively, while the corresponding value for the control group reduced to 94.89%. As for the alcoholic extract, it reduced the vitality percentage to 9.00%, 16.00, 34.67%, and 63.00% at 60, 45, 30, and 15 minutes, respectively, while the corresponding value for the control group was 96.71%.

The average values of vitality percentages for concentrations 60 mg/ml, 45 mg/ml, 30 mg/ml, and 15 mg/ml were 0.00, 30.67, 60.75, and 73.41 respectively for alcoholic extract. In its effect, the vitality decreased to 26.59%, 33.25%, 46.34%, and 58.67% at 60, 45, 30, and 15 minutes respectively, as compared with the control group in which the vitality was found to be 95.07%.

Table 1.Effect of Aqueous and Alcoholic Extracts of Leaves of *M. sylvestris* Plant on Protoscolices (of Sheep Origin) *In Vitro*

Extract	Concentration (mg/ml)	Control Group Vitality % (Min. 0)	Viable Protoscolices (%) at Specific Time (Minutes)				Mean Viable Protoscolices (%)
			15	30	45	60	
Alcoholic extract	15	95.23	91.00 j	82.67 i	63.33 g	56.67 f	73.41 D
	30	93.50	80.67i	68.00 h	53.67 f	40.67 e	60.75 C
	45	96.71	63.00 g	34.67 d	16.00 c	9.00 b	30.67 B
	60	94.84	0.00 a	0.00 a	0.00 a	0.00 a	0.00 A

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Aqueous extract	Mean	95.07	58.67 D	46.34 C	33.25 b	26.59 A	-
	15	92.86	89.33 m	87.33 lm	80.67 k	72.00 i	82.33 D
	30	96.45	86.33 l	76.00 j	61.33 h	43.00 g	66.67 C
	45	94.89	34.34 e	26.33 e	10.67 c	7.33 b	19.67 B
	60	96.98	19.32 d	9.67 c	0.00 a	0.00 a	7.25 A
	Mean	95.295	57.33 D	49.83 C	38.17 B	30.58 A	-

Duncan's test was used; $p < 0.05$ was considered significant. Values followed by different letters are significant. Similar letters mean there are no significant differences. Different letters mean there are significant differences.

Effect of Aqueous and Alcoholic Extracts of *M. Sylvestris* on the Vitality of In Vivo Protoscolices Observed After Four Months

After conducting the above-mentioned experiments related to the effect of alcoholic and aqueous extracts of *M. Sylvestris* on the vitality of protoscolices in the laboratory and observing their vitality rates, protoscolices treated with the extracts were injected into the peritoneum of laboratory mice to investigate the effect of these substances on them *in vivo*. Four months later, the mice were dissected to investigate the presence and growth of secondary hydatid cysts. These cysts were clearly visible in laboratory mice injected with untreated Pprotoscolices (control group) (Figure 1) and those treated with the extracts (Figures 2 and 3).

Table 2 shows the groups of mice injected with protoscolices treated with aqueous and alcoholic extracts at respective concentrations of 4 mg/ml and 2.5 mg/ml, and a reduction ratio of 92.5% and 98.5%, respectively. In the case of alcoholic extract, the average number of developing secondary hydatid cysts in the liver was 0.9, the average diameter of the cysts was 0.5 mm, and their average weight was 2.3 mg. This group did not show any developing secondary hydatid cysts in the spleen. In the control group, the average number of secondary cysts developing in the liver was 6.0, their average diameter was 3.67 mm, and their average weight was 35.15 mg. As for the effect of the aqueous extract on its discolouration and development, its reduction rate was 92.5%. The average number of secondary hydatid cysts developing in the liver in this group was 0.4, with an average diameter of 1.76 mm and an average weight of 3.1 mg, while the average number of secondary hydatid cysts developing in the spleen was 0.87, with an average diameter of 0.66 mm and an average weight of 3.67 mg.

Table 2.Details of Developing Hydatid Cysts in the Mice in the Control, Aqueous Extract, and Alcoholic Extract Groups Four Months after Injecting Them with Protoscolices and Treating Them with Extracts of *M. sylvestris Leaves*

Method	Transactions	No. of Repeat Mice	Hydatid Cysts in the Liver			Reduction Percentage	Hydatid Cysts in the Spleen			Reduction Percentage	General Reduction Percentage
			No. (average)	Diameter (average) (mm)	Weight (average) (mg)		No. (average)	Diameter (average) (mm)	Weight (average) (mg)		
Control	C^+	5	6.0	3.67	35.15	0.0	0.60	1.78	15.28	0.0	0.0
Alcoholic Extract	2.5 mg/ml	5	0.9	0.50	2.30	97.0	0.00	0.00	0.00	100.0	98.5
Aqueous Extract	4 mg/ml	5	0.4	1.76	3.10	94.0	0.87	0.66	3.67	91.0	92.5

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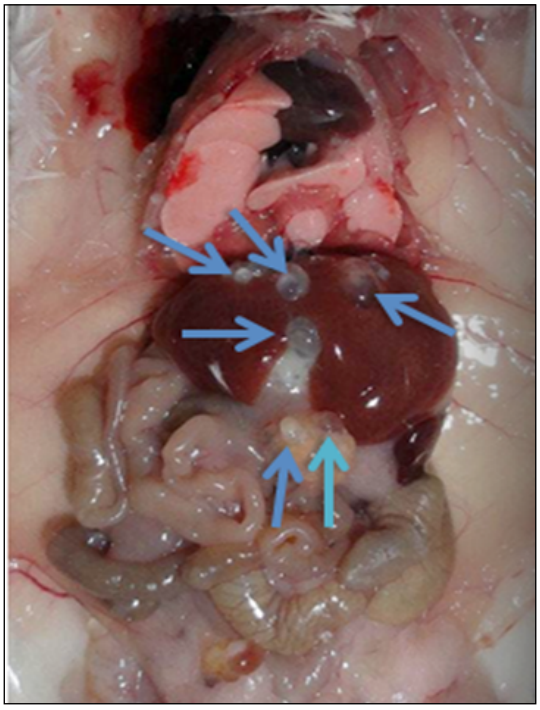

Figure 1.Mouse of Control Group Extract of *M. sylvestris Leaves*

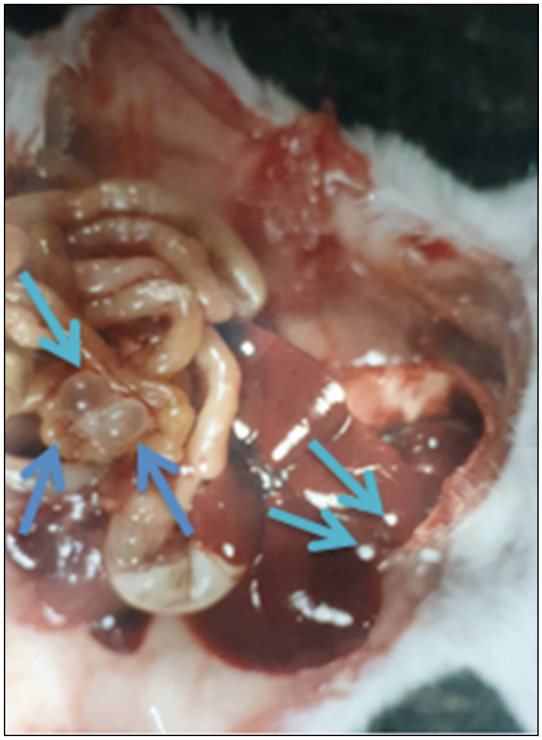

Figure 2.Mouse Treated with Aqueous

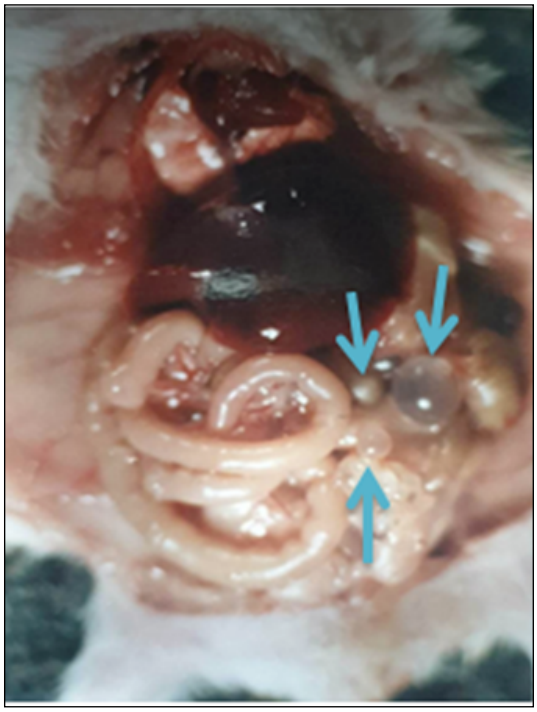

Figure 3.Mouse Treated with Alcoholic Extract of *M. sylvestris Leaves*

Discussion

Due to the great role that medicinal plants have been playing in caring for human and animal health in the world with the secondary compounds of pharmacological importance and the lack of side effects, this study came to demonstrate the effect of aqueous and alcoholic extracts of the *M. Sylvestris* plant on the protoscolices of sheep origin (*in vitro* and *in vivo*).

The results of this study were similar for both alcoholic and aqueous extracts of *M. Sylvestris* leaves at concentrations of 60 and 45 mg/ml, which caused 100% of all protoscolices to be killed. In a study that used the aqueous and alcoholic extract of tamarind leaves at a concentration of 100 mg/ml, the extract was observed to have caused 100% of all Pprotoscolices to be killed in a period of 4 hours, revealing the superiority of *M. Sylvestris* extracts to the extracts used in our study.[26]

Another study used the aqueous extract of *Lepidium sativum L.* plant at a concentration of 75 mg/ml at 30, 45 and 60 minutes. The extract was seen to cause a decrease in the vitality of the primary heads to 35.33%, 33.00% and 10.33% respectively.[31] The extract used in our study proved to be a better option as at a concentration of 45

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mg/ml, it led to a decrease in the vitality of primary heads to 34.67%, 16.00%, and 9.00% for the alcoholic extract, and to 26.3%, 10.67%, and 7.33% for the aqueous extract, and at the same time intervals, respectively.

The finding of the study is similar to the result of a study done by AL Qaisi et al. which used the alcoholic extract of *Ruta graveolens L.* leaves at a concentration of 40 mg/ml.[32] It killed all the Protoscolices (100%) at 75 minutes, proving to be a better extract at this time interval over the extracts of the *M. Sylvestris* plant, while the extracts of the *M. Sylvestris* plant outperformed it in the exposure period of 60 and 45 minutes.

It is similar to the result of a study conducted by Jasim which used the aqueous extract of the *Petroselinum sativum* at a concentration of 75 mg/ml.[33] The results were observed at 30, 45, and 60 minutes. It caused a decrease in the vitality of the protoscolices to 16.00%, 12.33% and 100% in a row. This study outperformed it with a concentration of 45 mg/ml, and its consistency within the 60-minute period, which caused 100% of all Pprotoscolices to be killed . As for the concentration of 50 mg/ml, the reason for reducing the vitality of the Pprotoscolices to 67.28%, 33.25%, and 17.00% in succession for the same times, with the superiority of this study with a concentration of 45 mg/ml.

The present study also showed similarity with the result of Niazi et al. which used *Olea europaea* plant extract at a concentration of 300 and 150 mg/ml, and caused the killing of all primary heads in the exposure period of 10 and 20 minutes, respectively.[34] This extract proved to be better than the one used in our study in terms of exposure time, while the latter was better in terms of concentration.

Another similar study was conducted by Jasim.[35] It used the aqueous extract of apricot (*Prunus armeniaca*) seeds at a concentration of 200 mg/ml, which caused the killing of all protoscolices at all times, proving the superiority of the extracts used in our study (at concentrations of 60 and 45 mg/ml). The result differed from that of another study which used the alcoholic extract of Zataria multiflora at a concentration of 10 and 25 mg/ml.[36] It caused the killing of all protoscolices after 1 and 3 minutes of treatment, respectively, and showed its superiority over this study in concentration and duration of exposure.

As for its direct effect on the protoscolices *in vivo,* and at a concentration of 4 mg/ml, it led to a decrease in the numbers, diameters, and weights of secondary hydatid cysts in laboratory animals (albino mice). The control group showed the formation of many secondary hydatid cysts and their growth in the tissues and cavities of the body. The liver was the most affected organ. As for the spleen, no cyst had grown in it similar to the result of a study, [37] which used the alcoholic extract of the seeds of the *Nigella sativa* plant at a concentration of 10 mg/ml and revealed the reduction of the vitality to zero.

In the case of the aqueous extract, some secondary hydatid cysts appeared in the liver and spleen, and their reduction percentage was 92.5%. It is identical to the result obtained in a study [38] which used the aqueous extract of the leaves of the *Populus euphratica* at a concentration of 15 mg/ml. The results showed a decrease in the vitality to 92.33%. Thus, we find that the efficiency of the alcoholic extract is better than the aqueous extract as the reduction percentages for both were 98% and 92% respectively.

The growth of secondary hydatid cysts in the positive control mice may be due to the protoscolices that were injected. As for the decrease in the number of cysts in the treated groups, it is attributed to the effectiveness of the plant extract and its effect on the vitality of these protoscolices. Their growth and development are affected by their vitality and the immune resistance of the animal body.[39]

As for its density in the liver, it may be due to the nature of its tissue and blood vessels, which hold it back as the first filter for blood, and give it the opportunity to grow into secondary hydatid cysts.[40]

Hence, the effect of the aqueous and alcoholic extract of the leaves of the mallow plant is clear because of the active substances, flavones, present in it, which work to reduce sugars, and the lack of ATP, which is equipped for the vital activities of the parasite. Alkaloids that interact with metabolic proteins are essential for the vitality of protoscolices which leads to the destruction of the cell membrane and its proteins and lipids.[41]

Phenol, which has an effect on the enzyme acetylcholinesterase, controls the permeability and flexibility of the cell membrane. It can reduce the permeability of the membrane, leading to the passage of different taxonomic materials without control, thereby resulting in the death of the parasite.[42] These potential natural compounds such as phenolic compounds and essential oils may result in the death of these protoscolices.

Conclusion

The alcoholic and aqueous extracts of *M. Sylvestris* leaves were able to kill protoscolices in vitro and led to a reduction in the numbers, diameters, and weights of developing secondary hydatid cysts in vivo. The alcoholic extract showed a greater effect than the aqueous extract, and this may be due to the solubility of some substances in it and the aqueous extract not being soluble. More studies should be conducted in-depth, which are aimed at increasing awareness regarding the active and influencing substances on the E. granulosus parasite.

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