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Antiparasitic activity of *Astragalus brachycalyx* subsp. *brachycalyx* extract against hydatid cyst protoscoleces and its effect on induction of apoptosis: an *in vitro* and *ex vivo* study

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ABSTRACT

Introduction: This study aims to evaluate *in vitro* and *ex vivo* antiparasitic activity of *Astragalus brachycalyx* subsp. *brachycalyx*. root chloroformic extract against hydatid cyst protoscoleces and its effect on induction of apoptosis.

Methods: Various concentrations of the *A. brachycalyx* root chloroformic extract (56.25, 112.5, 225, and 450 mg/mL) were treated with hydatid cyst protoscoleces collected from the liver of infected sheep for 5-60 minutes *in vitro* and *ex vivo*. Eosin exclusion test was also utilized to measure the mortality of protoscoleces. Moreover, the extract effect was assessed on apoptosis induction in hydatid cyst protoscoleces by caspase-3 activity measurement.

Results: The mortality rate of protoscoleces in *in vitro* was 100% after being exposed to 450 and 225 mg/mL of *A. brachycalyx* extract for 20 and 30 minutes and in *ex vivo* for 30 and 60 minutes, respectively. Following 48 h treatment of protoscoleces, *A. brachycalyx* chloroformic extract at the doses of 56.25, 112.5, 225, and 450 mg/mL, dose-dependently motivated the caspase-3 enzyme ranging from 8.8% to 29.6%

Conclusion: *A. brachycalyx* root chloroformic extract had a significant protoscolicidal effect; however, extra surveys are required to assess its efficacy and safety as a promising protoscolicidal agent in clinical settings.

Implication for health policy/practice/research/medical education:
This study revealed that *Astragalus brachycalyx* subsp. *brachycalyx* root chloroformic extract had a significant protoscolicidal effect *in vitro* and *ex vivo*. This result may help in providing new drugs. However, extra surveys are required to assess its efficacy and safety as a promising protoscolicidal agents in clinical settings.

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Introduction

Hydatidosis is a dangerous and global zoonotic disease among humans and animals caused by the larval stage of *Echinococcus granulosus* (1,2). Dogs and candies are the ultimate hosts of this parasite (2). Domestic animals such as sheep, cows, goats, and humans are considered as intermediate hosts of this parasite (3). Adult worms in the small intestine of carnivores produce eggs containing six-hooked embryos that are transmitted to the environment by dog feces (4). The larval stage (hydatid cyst, metacestode) develops in various organs of intermediate hosts, such as the liver, lungs, brain, following the ingestion of these eggs (5). There may be no clinical symptoms at the onset of this disease; however, after a while, clinical symptoms may appear due to the growth of cysts depending on their size and location (6). Benzimidazoles are typically applied to treat hydatidosis, the most important of which is albendazole. It is regarded as the definitive treatment for large and active cysts (6,7).

Protoscolicidals such as formalin, silver nitrate, and hypertonic saline could be injected into the cyst in order to reduce the potential risk during surgery (8). However, these substances have side effects such as fibrosis, biliary stricture, and acute and diffuse hepatic necrosis (10,11).

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Attempts have been made to find a novel protoscolicidal agent with minimal side effects and high impact. Plants are among the natural elements that have long been considered by humans due to their economic values, medicinal properties, diversity, and availability (12). Nowadays, the consumption of medicinal herbs to treat diseases is growing, and evaluating the features of herbal compounds and demonstrating the action mechanisms of these combinations in the treatment of numerous diseases have been considered (12).

In recent years, several *in vitro* and *in vivo* studies have been conducted on the protoscolicidal effects of some plants (e.g., *Lepidium sativum, Eucalyptus globulus, Nigella sativa, Artemisia aucheri, Zataria multiflora, Allium sativum,* and *Quercus infectoria*) and their products; however, the results of these studies, due to their different efficacy and the lack of precise mechanisms are not very reliable. Hence, more complete and accurate studies seem necessary (13).

Astragalus is one of the most prevalent herbs belonging to the Fabaceae family, with more than 2000 species (14). It has been proven that *Astragalus* spp. plants are widely applied in traditional medicine to treat numerous disorders, such as gastrointestinal problems, allergies, anorexia, diabetes, and infectious diseases (14,15). *Astragalus* spp. plants have exhibited many therapeutic applications, such as anti-cancer, antioxidant, neuroprotective, hepatoprotective, and antimicrobial activities (15). *Astragalus brachycalyx* subsp. *brachycalyx*. is a perennial plant growing in arid areas with 0.5-1-meter high, which is native in middle east countries such as Iran, Iraq, Turkey (15,16). To the best of our knowledge, there is no report investigating the antiparasitic effects of *A. brachycalyx*; hence, the current study was intended to evaluate the antiparasitic effects of *A. brachycalyx* root chloroformic extract on hydatid cyst protoscoleces *in vitro* and *ex vivo* due to its medicinal, biological, and antimicrobial properties.

Materials and Methods

Preparing and approving *Astragalus brachycalyx*

Herbarium specimens from *A. brachycalyx* roots were first collected from different regions of Lorestan Province in May 2021. After approving the species by a botanist, a specimen was kept in Razi Herbal Medicines Research Center, Khorramabad, Iran (No. LUMS-26354).

Specimen preparation and extraction

The roots were thoroughly washed and dried in the laboratory. Then, they were properly ground and stored in the refrigerator until use. The extraction process was performed using the maceration procedure. In this method, the ground powder was added to 70% methanol, and then, a homogeneous mixture was provided by a glass stirring rod. After extraction, the alcoholic fraction was separated from the extract and the chloroformic extract remained, which was kept at -20°C to be used for other experiments after being dried by the rotary evaporator (17,18).

Primary phytochemical analysis

The primary phytochemical analysis of the *A. brachycalyx* root chloroformic extract was accomplished to check the presence of tannins, saponins, alkaloids, flavonoids, glycosides, etc, based on the previous investigation (17).

Preparing of protoscoleces

Livers of sheep infected with hydatid cysts were collected from the Khorramabad abattoir and transferred to the Department of Parasitology, Razi Herbal Medicines Research Center. The outer surface of the cyst was first disinfected and sterilized with 70% alcohol. Then, hydatid fluid was removed. Afterward, the hydatid fluid was centrifuged and the supernatant was washed 3 times with sterile normal saline and transferred to sterile tubes (19).

Viability

At this stage, 0.1% eosin solution (1 g of eosin powder in 1000 mL of distilled water) was used to calculate the number of viable protoscoleces. During staining, dead protoscoleces absorbed the eosin stain and turned red. However, viable protoscoleces did not absorb the stain and remained colorless. The viability of protoscoleces was observed by flame cell vibrations and body contractions (20).

Evaluating protoscolicidal effect *in vitro*

First, 56.25, 112.5, 225, and 450 mg/mL concentrations of *A. brachycalyx* extract were applied to a suspension of protoscoleces (1×10^3 per mL) and their viabilities were calculated after 5, 10, 20, 30, and 60 minutes. These concentrations were selected based on initial experiments. Sterile normal saline + Tween 20 and 20% silver nitrate were simultaneously used as negative and positive controls, respectively. All these steps were performed on 48-well plates in triplicate (21)

Evaluating protoscolicidal effect *ex vivo*

For this purpose, naturally infected sheep livers were used. Initially, more than 50% of the hydatid fluid was aspirated to obtain protoscoleces, and the viability rate of which was confirmed by the 1% eosin staining. Then, 3 hydatid cysts were considered at extract concentrations of 56.25, 112.5, 225, and 450 mg/mL. The entire inner surfaces of the cysts were filled by injecting the extract. A small amount of the cyst fluid was removed after 5, 10, 20, 30, and 60 minutes and combined with 0.1% eosin. A drop of protoscoleces was placed on the slide, to which a drop of 1% eosin solution was added and observed under a microscope after 1 minute (22).

Effect of induction of apoptosis

At this stage, the effects of plant extract on the enzymatic activity of caspase-3 and subsequent induction of programmed cell death (apoptosis) in hydatid cyst protoscolices were studied. In summary, after 48 hours of protoscolices treatment with different concentrations of the extract, the mixture was centrifuged at 700 rpm for 5 minutes at 4°C. In the next step, the remaining precipitate containing the protoscoleces was lysed and centrifuged again at high speed (18 000 rpm) for 12 minutes. Lastly, the mixture supernatant (85 µL) was added the buffer (85 µL) and caspase-3 (pNA-DEVD-Ac, Sigma-Aldrich, USA) substrate (10 µL); the combination was again kept warm for 120 minutes at 37°C. The absorption of the tested combination was evaluated at 410 nm by an ELISA reader (23).

Statistical analysis

The data were analyzed using SPSS 25.0. After calculating the central tendency and dispersion, the Shapiro-Wilk and one-way analysis of variance (ANOVA) tests were employed. The significance level was considered to be $P < 0.05$.

Results

Phytochemical analysis

By phytochemical examination, the presence of saponins, flavonoids, terpenoids, and glycosides was confirmed in *A. brachycalyx* chloroformic extract (Table 1).

Evaluating protoscolicidal effect *in vitro*

The results presented in Table 2 show that *A. brachycalyx* chloroformic extract significantly reduced the viability of hydatid cyst protoscoleces at the different concentrations of 56.25, 112.5, 225, and 450 mg/mL (P <0.001). The mortality rate of hydatid cyst protoscoleces was 100% after being exposed to 450 and 225 mg/mL of *A. brachycalyx* extract for 20 and 30 minutes, respectively. The extract concentration of 56.25 mg/mL showed the lowest protoscolicidal property, so 84.3% of the protoscoleces were killed after 60 minutes of exposure. The mortality rates of protoscoleces were 4.1% and 100% in negative and positive controls after 60 and 20 minutes, respectively.

Evaluating protoscolicidal effect *ex vivo*

The results presented in Table 3 show *A. brachycalyx* root chloroformic extract significantly reduced the viability of hydatid cyst protoscoleces at the different concentrations of 56.25, 112.5, 225, and 450 mg/mL ex vivo ($P < 0.001$). The mortality rate of hydatid cyst protoscoleces was 100% after being exposed to 450 and 225 mg/mL of *A. brachycalyx* root chloroformic extract for 30 and 60 minutes, respectively. The results indicated this extract required more time *ex vivo* than *in vitro* to have the same protoscolicidal effect. The mortality rates of protoscoleces

Table 1. Phytochemistry of the *Astragalus brachycalyx* root chloroformic extract

Phytochemical test	Results
Alkaloid	+
Flavonoid	+
Saponin	+
Tannin	-
Polyphenol	+
Quinone	+
Steroid	+
Terpenoid	-

Note: (+) showed a positive result from *A. brachycalyx root chloroformic extract*.

Table 2. Effect of different concentrations of *Astragalus brachycalyx* root chloroformic extract on hydatid cyst protoscoleces at different times (5-60 min) *in vitro*, analyzed by one-way ANOVA

Concentration (mg/mL)	Time (min)	Mortality rate %	*P* value
450	5	41.3 ± 3.15	<0.001*
	10	73.3 ± 3.51	
	20	100.0 ± 0.0	
	30	100.0 ± 0.0	
	60	100.0 ± 0.0	
225	5	31.3 ± 2.51	<0.001*
	10	49.6 ± 2.51	
	20	91.3 ± 4.05	
	30	100.0 ± 0.0	
	60	100.0 ± 0.0	
112.5	5	18.3 ± 1.51	<0.001*
	10	34.3 ± 2.51	
	20	58.6 ± 3.15	
	30	89.3 ± 5.12	
	60	100.0 ± 0.0	
56.25	5	6.6 ± 0.51	<0.001*
	10	14.6 ± 1.51	
	20	31.3 ± 2.51	
	30	47.6 ± 3.05	
	60	84.3 ± 4.51	
Normal saline	5	0.0 ± 0.0	-
	10	0.0 ± 0.0	
	20	1.5 ± 0.3	
	30	3.3 ± 0.15	
	60	4.6 ± 0.51	
Silver nitrate	5	44.3 ± 2.51	<0.001*
	10	82.1 ± 3.15	
	20	100.0 ± 0.0	
	30	100.0 ± 0.0	
	60	100.0 ± 0.0	

* Significant difference compared to the control group (normal saline). The results are presented as mean ± SD (n=3).

Table 3. Effect of different concentrations of *Astragalus brachycalyx* chloroformic extract on hydatid cyst protoscoleces at different times (5-60 min) *ex vivo*, analyzed by one-way ANOVA

Concentration (mg/mL)	Time (min)	Mortality rate %	*P* value
450	5	33.4 ± 3.15	<0.001*
	10	59.3 ± 2.51	
	20	90.3 ± 4.15	
	30	100.0 ± 0.0	
	60	100.0 ± 0.0	
225	5	22.3 ± 2.51	<0.001*
	10	31.6 ± 2.51	
	20	56.3 ± 2.05	
	30	82.3 ± 4.15	
	60	100.0 ± 0.0	
112.5	5	8.6 ± 0.32	<0.001*
	10	13.6 ± 1.51	
	20	27.6 ± 2.15	
	30	49.6 ± 2.51	
	60	83.2 ± 4.15	
56.25	5	3.3 ± 0.51	<0.001*
	10	9.3 ± 1.51	
	20	16.9 ± 1.51	
	30	33.1 ± 3.05	
	60	56.6 ± 4.51	
Normal saline	5	0.0 ± 0.0	-
	10	0.0 ± 0.0	
	20	0.0 ± 0.0	
	30	1.3 ± 0.15	
	60	2.3 ± 0.51	
Silver nitrate	5	40.1 ± 2.51	<0.001*
	10	63.5 ± 3.15	
	20	100.0 ± 0.0	
	30	100.0 ± 0.0	
	60	100.0 ± 0.0	

* Significant difference compared to the control group (normal saline). The results are presented as mean ± SD (n=3).

were 2.3% and 100% in negative and positive controls after 60 and 20 minutes, respectively.

Effect of apoptosis induction

We studied the induction of apoptosis in protoscoleces treated with *A. brachycalyx* chloroformic extract by the enzymatic activity of the caspase-3. Followed by 48 hours treatment of protoscoleces, *A. brachycalyx* chloroformic extract at the doses of 56.25, 112.5, 225, and 450 mg/mL dose-dependently motivated the caspase-3 enzyme by ranging from 8.8% to 29.6% (Figure 1).

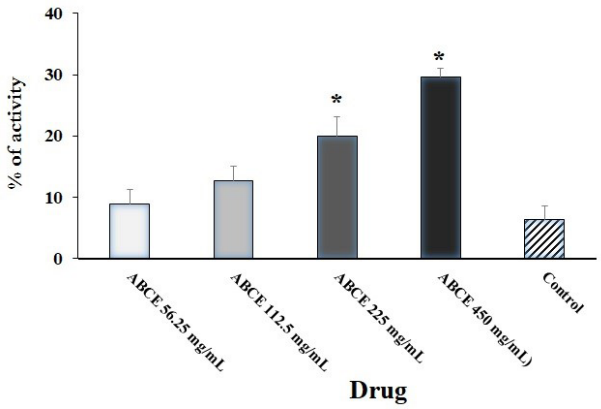

Figure 1. The percent of caspase-3 activity of protoscoleces treated with various concentrations of *Astragalus brachycalyx* subsp. *Brachycalyx* chloroformic extract (ABCE) compared with the control groups. Results are displayed as mean ± SD (n = 3). *P < 0.01 displays significant difference with the control group.

Discussion

Researchers have recently paid special attention to applying medicinal plants to treat various diseases (13). Widespread availability, cost-effectiveness, low side effects, high effectiveness, and antimicrobial and antioxidant properties are among the remarkable reasons for using medicinal plants (24). Nowadays, medication and surgery are used to treat hydatidosis; however, surgery is currently the only main treatment for hydatid cysts (6-8). This method is quite risky and has side effects; it may even lead to death when the cyst is removed (9,10). Currently, surgeons use protoscolicidals such as 20% saline solution, 3% hydrogen peroxide, and betadine to prevent these risks during hydatid cyst surgery. However, these agents may have complications such as biliary fibrosis or hepatic necrosis (8,9).

Here, the obtained *in vitro* results demonstrated that the mortality rate of protoscoleces was 100% after being exposed to 450 and 225 mg/mL of *A. brachycalyx* extract for 20 and 30 minutes, respectively. However, the *ex vivo* results indicated that the mortality rate of protoscoleces was 100% after being exposed to 450 and 225 mg/mL of *A. brachycalyx* root chloroformic extract for 30 and 60 minutes, respectively. Based on the stranded criteria, a suitable protoscolicidal agent must meet some ideals, e.g., low dosage of this agent should have high efficacy in a short time and minimal systemic adverse effects, minimal toxicity, easy to access, and affordable (25).

So far, protoscolicidal activities of a wide range of herbal extracts, e.g., *Nigella sativa, Berberis vulgaris, Allium sativum, Bunium persicum Pistacia* spp., and *Curcuma* spp., have been investigated (26,27). For example, Amiri et al have investigated the protoscolicidal effects of the methanolic extract of *Myrtus communis* and *Tripleurospermum disciforme* at concentrations of 25, 50, and 100 mg/mL against hydatid cyst protoscoleces. By eosin exclusion test, they found that the potent protoscolicidal activity of *M. communis* was observed at 100 and 50 mg/mL concentrations after 10 and 20 minutes incubation. However, *T. disciforme* had no significant protoscolicidal effect even at high concentration (28). In a study conducted by Almalk et al, the results showed that *Zingiber officinale*

ethanolic extract has the highest protoscolicidal effect with 100% mortality at concentrations of 30 and 50 mg/mL after 20 and 10 minutes, respectively. The highest scolicidal activity of *Curcuma longa* ethanolic extract was 93.2% at a concentration of 50 mg/mL followed by 30 minutes incubation (29). Methanol extract of *Olea europaea* L. has shown 100% in vitro mortality in hydatid cyst protoscoleces at concentrations of 300 and 150 mg/mL after 10 and 20 minutes of exposure, respectively. However, their *ex vivo* results revealed that *O. europaea* extract displayed 100% mortality in hydatid cyst protoscoleces at concentrations of 300 and 150 mg/mL after 12 and 25 minutes of injection of extract into the hydatid cyst, respectively (30). The difference in the effectiveness of different plant extracts on hydatid protoscoleces can be attributed to the part of the plant that was used, the extraction method, the exposure time of the protoscoleces, and the test method.

Several investigations have reported the antimicrobial effects of *Astragalus* spp. against various pathogenic bacteria (e.g., *Escherichia coli, Pseudomonas aeruginosa*, *Salmonella typhimurium,* and *Klebsiella pneumoniae* as gram-negative bacteria and *Staphylococcus aureus* and *Enterococcus faecalis* as gram-positive bacteria), fungi (e.g., *Aspergillus flavor*, *Aspergillus niger*, *Aspergillus fumigatus,* and *Candida albicans*), viruses (e.g., influenza virus), and parasitic strains (e.g., *Toxoplasma gondii* and *Eimeria papillata*) (31-33). However, to the best of our knowledge, there is no investigation on anti-parasitic effects of *A. brachycalyx*.

According to the previous investigations the phenolic and flavonoid compounds are the most frequent phytochemicals of *Astragalus* spp., while several subclasses of flavonoids, e.g., flavones, flavonols, flavanones, flavanonols, chalcones, aurones, isoflavones, isoflavones, and pterocarpans have been extracted from the genus *Astragalus* (34,35). In connection with antimicrobial effects of flavonoid phytochemicals, previous reports exhibited that these phytochemicals displayed their antimicrobial action through distracting the function of cytoplasmic membrane, inhibiting nucleic acid synthesis, destructing cell-membrane, triggering apoptosis, disturbing metabolism of energy, stopping the biofilm formation, altering the membrane permeability, and decreasing the pathogenicity (36,37). Moreover, previous studies have showed that polyphenols compounds, e.g., flavanols, flavonols and phenolic acids display their antimicrobial action by hindering the virulence factors, interfering cytoplasmic membrane, preventing the formation of biofilm creation, destructing cell membrane, hanging-up DNA synthesis, and synergistic effects with synthetic drugs, etc. (38,39). Therefore, it may be proposed that the relevant protoscolicidal effects of *A. brachycalyx* root chloroformic extract are linked to the existence of phenolic and flavonoid phytochemicals in these herbs.

Induction of apoptosis or programmed cell death is one of the strategic molecular mechanisms of the tested antimicrobial agents, which is provoked by external factors and eventually leads to the cell's self-destruction (40). Hence, apoptosis makes available a perfect way to kill the *E. granulosus* protoscoleces. Caspases, especially caspase-3, are well-known as the key mediators of apoptosis (41). Here, we measured the caspase-3 like activity of protoscoleces incubated with *A. brachycalyx* root chloroformic extract through the colorimetric protease method. The results showed that following 48 hours treatment of protoscoleces, *A. brachycalyx* chloroformic extract, at 56.25, 112.5, 225, and 450 mg/mL, dose-dependently motivated the caspase-3 enzyme by ranging from 8.8% to 29.6%. These findings confirmed that the induction of apoptosis is one of the possible protoscolicidal mechanisms of *A. brachycalyx* root chloroformic extract against *E. granulosus* protoscoleces.

Conclusion

The results revealed that *A. brachycalyx* root chloroformic extract had a good protoscolicidal effect *in vitro* and *ex vivo*; however, extra surveys are required to assess its efficacy and safety as a promising protoscolicidal agent in clinical settings. Although the probable protoscolicidal mechanisms of *A. brachycalyx* root chloroformic extract are not evidently assumed, however, our results showed that the stimulation of apoptosis through caspase-3 enzyme activation could be considered as one of the key mechanisms. Examination of precise protoscolicidal mechanisms of this plant, detailed analysis of the effective compounds of the plant, study of its *in vivo* and *in vitro* protoscolicidal effects, and cytotoxicity and histological toxicity studies of its compounds are important aspects of this plant. The authors hope to consider them in the near future and conduct additional studies.

Authors' contributions

JYG, AKK, MS, and HM reviewed and contributed to data collection and preparation of the manuscript. The first draft was prepared by HM, and JGY. All authors read the final version and confirmed it for publication.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication etc) have been completely observed by the authors. This project was approved by the ethics committee of Lorestan University of Medical Sciences with the ethics ID IR.LUMS.REC.1400.203.

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