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In Vitro Protoscolicidal Activity of Pomegranate (*Punica Granatum*) Rind and Barberry (*Berberis Vulgaris*) Alcoholic Extracts against Hydatid Cysts Caused by *Echinococcus granulosus*

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ABSTRACT

Background and objectives: Echinococcosis is a global cosmopolitan zoonotic disease and a major veterinary and public health issue. In humans, echinococcosis usually develops following close contact with infected dogs or ingestion of the parasite eggs. Until now, no effective vaccine has been commercially developed, and treatment is only focused on controlling hydatidosis. This study was conducted to evaluate the protoscolicidal activity of alcoholic extracts of pomegranate rind and barberry.

Methods: The alcoholic extracts of pomegranate rind and barberry were prepared by mixing 330 g of powdered plants with 1,000 ml of 70% ethanol. A concentrate of viable protoscolices (PCSs) was obtained from hydatid cysts found in the lungs and liver of sheep. Next, PCSs were treated with four different concentrations (5, 10, 20, 30, and mg/ml) of each extract for 10, 20, 30, and 60 minutes. The eosin exclusion test was performed to assess viability of the PCSs.

Results: The mortality rate caused by treatment with the extracts ranged between 25% and 100%. Complete inactivation of PCSs was achieved after 60 minutes of exposure to 15 mg/ml of the pomegranate rind extract and 30 mg/ml of the barberry extract.

Conclusion: Given their favorable anti-PCSs activity, combination of conventional synthetic albendazole with the alcoholic extracts of pomegranate rind and barberry might induce higher anti-PCS activity with lower side effects. It is recommended to evaluate the anti-PCSs activities of the pomegranate rind and barberry alcoholic extracts in vivo and ex vivo.

Keywords: Echinococcus granulosus, pomegranate fruit rind, Berberis.

INTRODUCTION

Echinococcosis, also known as hydatidosis, is a parasitic infection of farm and wild life animals, including herbivorous and omnivorous mammals. The frequency of this global veterinary and public health concern varies depending on factors including climate, agriculture, development, and education (1). The disease is caused by the larval stage of a well-known dwarf tape-worm of dogs, known as *Echinococcus*. Hydatidosis is characterized by formation of bladder-like, fluid-filled, and space-occupying cysts of variable sizes, following the growth of oncospheres in the internal organs of intermediate hosts. Farm and feral ruminants act as intermediate hosts, while canines are known as definitive hosts. When infected, the adult worms in the definitive hosts release their eggs into the environment. The intermediate hosts, if come into close contact with feces of infected canines, may ingest the eggs. Similarly, accidental ingestion of the worm eggs by humans can result in development of typical hydatid cysts, mainly in visceral organs such as the liver and lungs. The subsequent need for hospitalization as well as medical and surgical treatment costs impose a great financial burden, especially in the Eastern Mediterranean (e.g. Iran, Turkey, Iraq, and Tunisia) (2), with an estimated annual cost of 194 million US dollars (3).

There are four taxonomically valid species within the genus *Echinococcus* including *Echinococcus granulosus* (cystic hydatidosis), *Echinococcus multilocularis* (multivesicular hydatidosis), *Echinococcus vogeli* (polycystic hydatidosis), and *Echinococcus oligarthrus* (4). On the other hand, within the genus *Echinococcus* and based on the genetic, biological, and morphological properties, 11 distinct genotypes (strains) have been identified across the world (4). These genotypes demonstrate dissimilar socio-economical significances and geographical dispersion patterns (5) with seven of them (G1, G2, G3, G5, G6, G7, and G9) also implicated in zoonotic cases (4).

While open surgical intervention remains the principle modality of hydatidosis therapy worldwide, it may be life-threatening for the patients. Accidental spillage of the hydatid fluid containing live protoscolices (PCSs) during the operation might result in anaphylactic shock or formation of secondary cysts and recurrence of the disease. Similarly, with popularity of the more recent puncture-aspiration-injection-reaspiration procedure (6), complete inactivation of all PCSs remains a challenge, as there is no ideal protoscolicide agent available. While benzimidazole is currently the only licensed synthetic protoscolicide accessible in the market, effective agents with less side effects are highly demanded. Until now, a broad range of protoscolicidal substances have been introduced (7, 8). With the emergence of resistance against modern chemical drugs in some parasites, the effectiveness of plant extracts as harmless parasiticidal agents is coming to attention. This so-called herbal or ethnomedical medicine is most popular in Africa, Asia, and Latin America. In herbal medicine, a great deal of plant species are used for disease treatment based on the old traditions (9).

According to the World Health Organization, albendazole is recommended for treatment of cystic and alveolar echinococcosis. This drug is also the only safe option for serious cases with multiple cysts, cysts in the brain, and immunocompromised individuals. However, albendazole is not always efficient and might cause liver toxicity and other side effects. Overcoming such side effects has been made possible through synergistic combination of drugs. So far, a number of herbal compounds have been examined in vitro and in vivo against *Echinococcus*, with only few satisfying enough to be clinically used (10).

Pomegranate (*Punica granatum L.*) is a fruit-bearing shrub or small tree native to Asia. This is a broadly cultivated tree along the Silk Road, with a long history of use in traditional medicine (11). Various phytochemicals, such as alkaloids, tannins, and volatile oils have been found in different parts of pomegranate, which exhibit a wide spectrum of bioactivities, including antiparasitic, antimicrobial, and antioxidant properties (11) as well as protective effects against ultraviolet radiation (12). *Berberis vulgaris*, a member of the Berberidaceae family, is indigenously found in semi-tropical regions where it is chiefly used as food. Pharmacologically, *B. vulgaris* has been proven to show antimicrobial, antileishmanial, and antimalarial properties. Berberine and berbamine are the two important alkaloids present in *B. vulgaris* that

exhibit anti-inflammatory and parasiticidal effects (13).

This study comparatively assess efficacy of pomegranate peel and barberry alcoholic extracts against hydatid cysts.

MATERIALS AND METHODS

Preparation of PCS concentrates

In August 2020, two livers and two lungs from sheep bearing typical hydatid cysts (Figure 1) were collected freshly from the Meisam Abattoir (Tehran, Iran) and transported on ice to the parasitology laboratory of the AJA Army University of Medical Sciences (Tehran, Iran) on the same day. Next, the liver and lung samples were thoroughly inspected for any evident pathological changes, such as caseation and/or calcification. The fluid-filled cysts representing a tender texture were selected and gently washed. In order to extract PCSs, the liquid content of cysts was aspirated aseptically using sterile plastic syringes (20 ml) and pooled in a glass measuring cylinder. The suspension was left to settle at room temperature for 30 minutes, and then the supernatant was removed. The PCSs concentrate was washed with phosphate buffer saline (pH 7.2) and centrifuged twice at 2,000 rpm for 2 minutes. For unification of the procedure, the nominal concentration of PCSs was determined using a hemocytometer and adjusted with 0.9% NaCl solution to obtain 5×10^3 PCSs with >95% viability (14).

Viability of the PCSs was examined by using the eosin staining procedure, also known as the eosin exclusion test (15). In brief, 10 µl of eosin stock solution 0.1% (w/v) were added to 10 µl of the PCSs concentrate on a glass slide (16). After 15 minutes, the droplet was covered with a glass slip and examined under a light microscope at 100× magnification. The dead-to-viable ratio (e.g. numbers) among 300 PCSs was calculated (15).

Live PCSs are not permeable towards eosin, demonstrating the typical muscular movements and the flame cell motion, while dead PCSs absorb the red stain and display the distinctive reddish color (17).

Peels of *P. granatum* and fruits of *B. vulgaris* were acquired from a private and well-reputed herbal remedies store in Tehran. Authentication of the plant samples was conducted by a botanist (one of the co-authors) at the AJA University. To obtain the alcoholic extracts, 330 g of each plant were weighted and grinded to obtain a fine powder. In a glass beaker, 1,000 ml of 70% ethanol were added to the powder and thoroughly stirred for 5 minutes. The mixture was left to settle in a dark place, at room temperature, for 72 hours. Then, the mixture was passed through a sterilized Whatman filter paper. Using a rotary evaporator, the filtrate was subjected to evaporation until a dark-brown, semi-dried, and soft paste was obtained (Figure 1).

Evaluation of the in vitro protoscolicidal activity of P. granatum and B. vulgaris

Four different concentrations (5, 10, 15, and 30 mg/ml) of the alcoholic extracts were prepared by dilution with normal saline (18). Moreover, four treatment interval times (10, 20, 30, and 60 minutes) were considered. For evaluation of the protoscolicidal activity of the extracts, 0.5 ml of each concentration was added to a glass test tube and later mixed with 0.5 ml of the PCSs concentrate. The cocktail was mixed thoroughly and incubated at 37 °C for the mentioned time intervals. Subsequently, the supernatant was aspirated and replaced with 0.5 ml of eosin stain (0.1% w/v) and subjects to gentle stirring. The relative percentages of dead-to-viable PCSs were determined microscopically using a hemocytometer slide. The number of dead and viable PCSs was counted in 10 randomly chosen fields. To ensure accuracy of the findings, all experiments were conducted five times, and the average scores of all five readings were used for analysis. Isotonic saline was used as the control.

Data were entered into the MSTAT-C for processing and analysis. P-values ≤ 0.05 were considered as statistically significant.

RESULTS

The mortality rate (e.g. scolicidal activity) of PCSs following treatment with the *P. granatum* and *B. vulgaris* alcoholic extracts at different concentrations and intervals was calculated and recorded (Table 1 and Figure 2). Exposure to either of the two extracts at all tested concentrations resulted in significant PCS inactivation compared with the control group ($p<0.001$). Furthermore, increasing the exposure time and concentration of the extracts significantly increased the mean scolicidal activity ($p<0.01$) (Table 1). At concentration of 5 mg/ml, the *B. vulgaris* alcoholic extract had higher scolicidal activity (78.3% at 60 min) compared with that of the

P. granatum peel extract (Table 1, Figure 2). An almost similar level of scolicidal activity was noted after 60 minutes of treatment with both extracts at concentration of 10 mg/ml, which eliminated 70-80% of PCSs (Table 1 and Figure 2). When assessing exposure to both extracts at concentration of 15 mg/ml for 30 minutes, the *P. granatum* peel extract displayed a stronger scolicidal activity against the PCSs compared with the *B. vulgaris* extract; this activity increased to 100% after 60 minutes of treatment (Table 1 and Figure 2). A significant rise in the scolicidal activity of both extracts was observed after increasing exposure time from 30 to 60 minutes (p<0.05). Quite interestingly, at concentration of 30 mg/ml, the *B. vulgaris* alcoholic extract exhibited the highest scolicidal activity in as short as only 5 minutes post-exposure, where 98.3% of the PCSs were inactivated. Furthermore, the complete inactivation (100%) of PCSs was achieved 30 minutes post-exposure (Table 1 and Figure 2).

Overall, at lower concentrations and longer exposure times, the *P. granatum* peel extract showed better efficacy, while at higher concentrations and shorter exposure times *B. vulgaris* performed better.

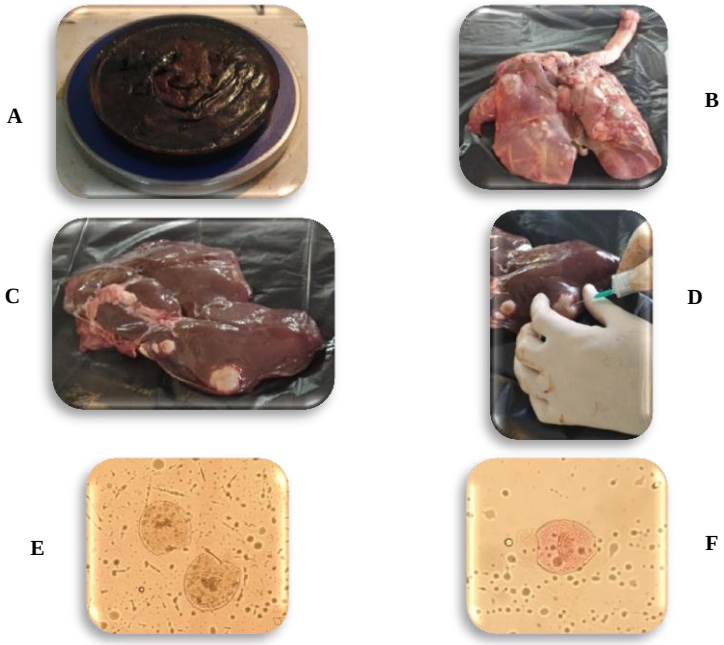

Figure 1- Overview of laboratory works in the present study. A: The dark-brown semi-dried and soft paste; B: Sheep lungs bearing hydatid cysts; C: Sheep liver bearing hydatid cysts; D: Aspiration of cyst fluid; E: Viable protoscolex after exposure to the eosin solution; F: Dead protoscolex after exposure to the eosin solution

Table 1- Protoscolicidal activity of pomegranate rind and barberry alcoholic extracts at various concentrations and exposure times

Alcoholic extract	Concentration (mg/ml)	Mortality rate (%) after 10 minutes	Mortality rate (%) after 20 minutes	Mortality rate (%) after 30 minutes	Mortality rate (%) after 60 minutes
Pomegranate rind	5	25.16 ± 0.58	32.34 ± 0.76	39.9 ± 1.11	49.06 ± 1.54
	10	48.56 ± 1.00	55 ± 0.70	67.38 ± 0.78	71.6 ± 1.01
	15	56.46 ± 1.00	78.1 ± 0.73	91 ± 0.89	100 ± 0
	30	89.22 ± 0.74	93.24 ± 0.74	99.6 ± 0.48	100 ± 0
Isotonic saline (Control)	-	3.5 ± 0.44	4.14 ± 0.37	4.5 ± 0.44	4.5 ± 0.44
Barberry	5	37.84 ± 1.60	50.32 ± 0.68	57.58 ± 0.72	78.3 ± 0.40
	10	43.24 ± 0.67	49.6 ± 0.58	60.16 ±0.80	80.8 ± 0.50
	15	65.22 ± 0.50	80.1 ± 0.73	85.3 ± 0.50	89.62 ± 0.37
	30	98.3 ± 0.50	99 ± 0.89	100 ± 0	100 ± 0
Isotonic saline (Control)	-	4.92 ± 0.38	5.9 ± 0.37	6.3 ± 0.24	6.76 ± 0.38

Data are presented as mean ± standard deviation.

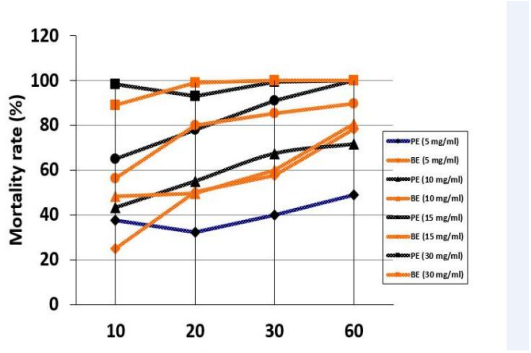

Figure 2-Protoscolicidal activity of the pomegranate rind extract (PE) and barberry alcoholic extract (BE) at various exposure times

DISCUSSION

The current OIE-World Organization for Animal Health regulations do not classify hydatidosis as a notifyable disease, although it remains a serious endemic disease of ruminants in the Mediteraniean region. Not surprisingly, hydatidosis in farm animals has been reported from different parts of Iran, with subsequent human cases of cystic and alveolar forms of the disease in which extra-hepatopulmonary cysts are still being reported (19, 20). Given the emergence of albendazole-resistant cystic echinococcosis cases, co-application of albendazole with synergistic herbal drugs might be an alternative and effective strategy. Previously, the synergistic effect of *Zataria multiflora* (21), aqueous extract of *Trametes robiniophila* (22), and alkaloids from *Sophora moorcroftiana* seeds (23) on albendazole against cystic echinococcosis has been demonstrated. Recently, Labsi et al. reported the anti-cyst-formation and liver-integrity-enhancing effects of a combinational treatment consisting of albendazole and pomegranate rind extract against cystic echinococcosis in a murine model (24). Moreover, the pomegranate rind extract has been shown to exert hepatoprotective properties, making this herbal product an important therapeutic agent for the treatment of fibrosis and oxidative damage (25).

Given the presence of the dog/sheep strain (G1) of *Eccinococus* in Iran (2), it is essential to conduct protoscolocidal activity studies on this strain. In the present study, treatment with 15 mg/ml alcoholic extract of pomegranate rind and 30 mg/ml barberry for 30 and 60 minutes fully-inactivated viable PCSs. Considering the in vitro nature of this study, ex vivo and in vivo reassessment of the findings on sheep and other farm ruminants seems necessary. Conducting dose-ranging studies will help to establish optimal working dose with minimal side effects.

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DECLARATIONS

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Ethics approvals and consent to participate

This research has been approved by the Scientific and Ethics Committee of the Iranian AJA University of Medical Sciences.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding publication of this article

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