Original Article

Phytochemical Screening, Protoscolicidal Activity and Mechanisms of Action of *Taraxacum Officinale* Extract against Hydatid Cyst Protoscoleces

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ABSTRACT

Cystic echinococcosis (CE) is a parasitic disease resulting from the presence of the larval stage of Echinococcus granulosus, a species of tapeworm. The surgical treatment of CE is typically indicated when the cysts are of considerable size and located in the heart or brain. A variety of chemical agents are employed during surgical procedures to mitigate complications, including hypertonic saline solution, cetrimide-C, and silver nitrate. The plant known as Taraxacum officinale has been employed for its medicinal properties since the 10th century, reflecting a longstanding tradition of therapeutic application. The objective of this study was to conduct a phytochemical screening, evaluate protoscolicidal activity, and investigate the mechanisms of action of T. officinale ethanolic extract (TOE) against hydatid cyst protoscoleces (PSCs). The metabolites of T. officinale were extracted using ethanol, and qualitative phytochemical analyses were conducted to detect the presence of total steroid glycosides, flavonoids, saponins, anthraquinones, sterols, and terpenoids. A range of TOE concentrations (50-800 mg/mL) were prepared for the treatment of PSCs. The eosin exclusion experiment was conducted to assess the viability of the protoscoleces. PSCs were treated with TOE, and the Caspase 3-like activity assay kit was employed to quantify the degree of apoptosis induction. The TOE demonstrated the greatest efficacy at a concentration of 800 mg/ml, resulting in the complete eradication of PSCs within 60 minutes. The activity of the apoptotic enzyme caspase-3 was observed to be in the range of 11.4 to 35.7%. Scanning electron microscope (SEM) analysis of PSCs treated with TOE for 60 minutes revealed deformities in the tegument and rostellum. The study offers valuable insights into the scolicidal properties of TOE. In light of the findings, it can be posited that TOE exerts a considerable lethal impact when employed against the protoscoleces of hydatid cysts.

Keywords: Medicinal Plant, *Echinococcosis Granulosus*, Caspase 3-like, Protoscoleces.

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1. Introduction

Cystic echinococcosis (CE) is a parasitic disease resulting from the presence of the larval stage of Echinococcus granulosus, a species of tapeworm. This zoonotic disease has the potential to affect both animals, particularly herbivores, and humans. A number of countries around the globe have expressed concern about the economic and health risks associated with echinococcosis among humans and animals. Humans, who are not the primary hosts, may contract the infection through ingestion of parasite eggs expelled by canines, consumption of food and water contaminated with the eggs, or contact with infected canines. The majority of cysts develop in the liver (40-80%) and lungs (10-30%), with a smaller percentage affecting other bodily tissues. The treatment of small and inactive cysts typically involves the administration of chemotherapy using benzimidazole derivatives (albendazole and mebendazole), whereas the treatment of active and large cysts is surgical in nature. In most cases, CE is treated with surgical intervention, particularly when the cysts are large and located in the heart or brain. A variety of chemical agents are employed during surgical procedures to mitigate these complications, including hypertonic saline solution, cetrimide, and silver nitrate (3). In the postoperative period, it has been observed that 10% of patients will develop new cysts due to the spillage of hydatid fluid. Therefore, secondary hydatidosis may occur following surgery and may even result in death. The adverse effects associated with chemical drugs and the emergence of drug resistance in parasites, particularly hydatid cysts, have prompted a growing interest in the utilization of plant-based compounds and their metabolites [4]. It is therefore imperative that scolicidal agents which are both effective and safe in the inactivation or killing of protoscoleces (PSCs) be employed during surgery for cystic echinococcosis (CE). The World Health Organization (WHO) has long recognized the potential of medicinal plants as a source of treatment for a wide range of illnesses and conditions. The plant known as Taraxacum officinale has been employed for its medicinal properties since the 10th century, reflecting a longstanding tradition of therapeutic application. This versatile plant contains a plethora of bioactive constituents, including polysaccharides, flavonoids, terpenes, pigments, phytosterols, coumarins, organic acids, and carbohydrates. The plant's potent nature renders it a valuable component of traditional medicine, with its therapeutic potential attributed to the diverse range of bioactive constituents present within it. Furthermore, special components with potential bioactivity have been identified in TOE, including chlorogenic acid, taraxasterol, sesquiterpene lactones, CRA, and taraxerol. These components possess a number of properties, including choleretic, anti-rheumatic, antioxidative, and anti-inflammatory properties, as evidenced by studies (6, 7). The metabolites present in plants play a pivotal role in regulating the apoptosis pathway in E. granulosus PSCs, ultimately leading to their demise. Caspase-3 plays a pivotal role in the programmed cell death pathway. The objective of this study was to conduct a phytochemical screening, assess protoscolicidal activity, and investigate the mechanisms of action of T. officinalis extract against hydatid cyst PSCs.

2. Materials and Methods

2.1. Plant Preparation

The plant T. officinalis was procured from a market specializing in herbal medicines in Khorramabad, Lorestan, Iran. The phytochemistry experts at the Razi Herbal Medicines Research Center in Khorramabad, Iran, were identified by a botanist and a voucher sample was subsequently deposited in the herbarium (No. 1399.221). The flowers were subsequently subjected to an air-drying process, following a double cleansing with tap and distilled water. Subsequently, the material was pulverized using a laboratory electric grinder.

2.2. Preparation of Ethanolic Extract of TOE

A powder of T. officinalis flowers was prepared by drying and then mixed with ethanol (70%, 100 mL) and allowed to stand for 24 hours at room temperature. Subsequently, the ethanolic extracts were filtered through a Whatman filter paper No. 1 (Sigma-Aldrich, Germany). The filterate was subsequently subjected to rotary evaporation under vacuum conditions (Heidolph, VE-11). The dried ethanolic extract was subsequently transferred to a refrigerator set at 4°C for further analysis.

2.3. Qualitative Analysis of Phytochemicals

The bioactive metabolites present in the ethanolic extract of TOE were identified through the application of the following qualitative analytical techniques.

2.4. Total Steroidal Glycoside Assay

The experiments present two approaches for the detection of steroidal glycosides in TOE, based on the observation of color changes following the addition of specific reagents. This methodology has been previously described in detail in references (10, 11).

2.5. Total Flavonoid Assay

The presence of flavonoids in the TOE was confirmed through the introduction of a methanol solution containing aluminum nitrate (10% w/v) and lead acetate (0.1% w/v). The specimen exhibited a yellow coloration, indicative of the presence of flavonoids (12).

2.6. Total Saponin Assay

Following the addition of 5 mg of TOE to distilled water, the solution was subjected to vigorous shaking. Following vigorous shaking, a foam layer was observed over the solution, confirming the presence of saponins (11).

2.7. Anthraquinones Detection Assay

In a conical flask, 0.6 grams of TOE powder were added to 10 milliliters of benzene. Subsequently, the specimen was filtered through a Whatman filter paper after a 10-minute interval. Subsequently, 10 ml of an ammonia solution (10%) was added to the filtrate and vigorously shaken for 30 seconds to detect the presence of anthraquinones. The presence of anthraquinones can be identified by the

emergence of pink, violet, or red colors in the reaction mixture.

2.8. Salkowski's Test

A solution of 0.1g of TOE in 2 ml of 98% H_2SO_4 was prepared in a glass tube. The steroidal aglycone component of the glycoside was identified by the observation of a reddish-brown coloration (14).

2.9. Detection of Terpenoids

A total of 2 ml of chloroform was added to 5 ml of TOE. Subsequently, the reaction sample was boiled in 3 ml of concentrated sulfuric acid. The appearance of a gray coloration in the solution is indicative of the presence of terpenoids (25).

2.10. Protoscoleces and Their Viability Evaluation

PSCs were obtained from the livers of sheep that had been naturally infected and subsequently slaughtered at the Khorramabad abattoir in Iran. The fluid contents of the cysts were transferred to glass tubes for the purpose of harvesting the PSCs. Subsequently, the contents of the glass tubes were allowed to settle at the end of the tube. The liquid above the solid was then removed, and the PSCs that had settled were rinsed three times with sterile normal saline (16).

2.11. Viability Test

The viability of PSCs was evaluated through the use of the eosin exclusion assay. The test was conducted using a 0.1% eosin solution, prepared by dissolving 1 g of eosin powder in 1,000 ml of distilled water (Sigma-Aldrich, Germany). When subjected to eosin staining, deceased PSCs absorb the dye and display a red coloration (Figure 1), while living PSCs display normal flame cell activity and muscular movement (16).

2.12. Protoscoleces Treatment

In test tubes, 0.5 ml of protocol cells (2×10^3/ml) were prepared in accordance with the prescribed methodology. Subsequently, 0.5 mL of TOE was introduced at varying concentrations (50-800 mg/ml) to each tube. Following gentle mixing of the tubes, they were incubated at 37°C for a period of 10–60 minutes. The upper phase of each sample was removed while the PSCs remained. PSCs that had settled at the bottom of the dish were stained with a low concentration (0.1%) of eosin in order to assess the impact of TOE treatment on their mobility and permeability. Furthermore, normal saline (NS) and hypertonic saline (HS) were employed as negative and positive controls, respectively. Subsequently, the glass slides were examined under light microscopy to ascertain whether any alterations in the appearance of the PSCs had occurred.

2.13. Effect of TOE on Protoscoleces Ultrastructure

The impact of TOE on the ultrastructure of PSCs was investigated through a series of procedures. Initially, the PSCs were treated with TOE at a concentration of 800 mg/mL. Following this, the cells were fixed in 2.5% glutaraldehyde for four hours at room temperature. Subsequently, the cells were washed with PBS, dehydrated with increasing concentrations of alcohol, and then dried with a critical point dryer for 45 minutes. The processed PSCs were mounted on a metal stub, coated with gold using a scanning electron microscope (SEM) E5100 device, and analyzed with an SEM 5410LV at 15-25 kV.

2.14. Induction of Caspase-3-like of Protoscoleces by TOE

The caspase-3-like activity of TOE-treated PSCs was evaluated in accordance with the manufacturer's protocol to assess the induction of apoptosis in these cells. In the course of the experiment, caspase-3 activity released a substrate that was bound to pNA, which could then be measured using a spectrophotometer. The activation of caspase-3 in PSCs was evaluated 48 hours following treatment with TOE at concentrations of 5, 10, 20, 40, and 80 mg/mL. Subsequently, the TOE-treated PSCs were subjected to centrifugation at 4°C and 600 rpm for a period of 5 minutes. To lyse the PSCs, they were subjected to a probe sonicator for a period of five minutes, after which the lysate was subjected to centrifugation at 20,000 rpm for a period of ten minutes. A mixture of 5 µl of the supernatant was combined with 85 µl of PBS buffer. Subsequently, the sample was incubated at 37°C for two hours with 10 liters of caspase-3 (pNA-DEVD-Ac) substrate. To quantify the colorimetric absorption of the sample at 405 nm, an ELISA reader was employed.

2.14. Statistical Analysis

The data analysis was conducted using the Statistical Package for the Social Sciences (SPSS) software, version 20. To ascertain the existence of differences among the groups, a one-way analysis of variance (ANOVA) was conducted with a predetermined significance level of $P<0.05$.

3. Results

3.1. Phytochemical Characterization of *T. officinalis* Extract

The preliminary phytochemical studies, as presented in Table 1, confirmed the presence of saponins, flavonoids, terpenoids, anthraquinones, steroidal aglycones, and glycosides in TOE.

3.2. Scolicidal Studies of TOE

The scolicidal activity of TOE was examined at varying concentrations for a period of 10–60 minutes, as illustrated in Table 2. The findings indicated that TOE at a concentration of 50 mg/ml exhibited scolicidal activity of approximately 40% at its maximum efficacy. However, 100% scolicidal activity of TOE was observed at 800 mg/mL after 60 minutes of exposure. Similarly, following 30 and 20 minutes of exposure, PSCs in the negative and positive control groups exhibited mortality rates of 5.22 and 100%, respectively.

3.3. Effect of TOE on Protoscoleces Ultrastructure

Figure 2 illustrates the alteration in cyst permeability to eosin following TOE exposure for 10, 30, and 60 minutes. Following a one-hour incubation period, PSCs treated with 800 mg/ml TOE exhibited ultrastructural damage and rostellar disorganization. A number of studies have indicated that PSCs exhibit blebs as a consequence of apoptosis-mediated death. Figure 2 additionally illustrates the presence of asymmetry in the teguments and deformation of the cyst structure.

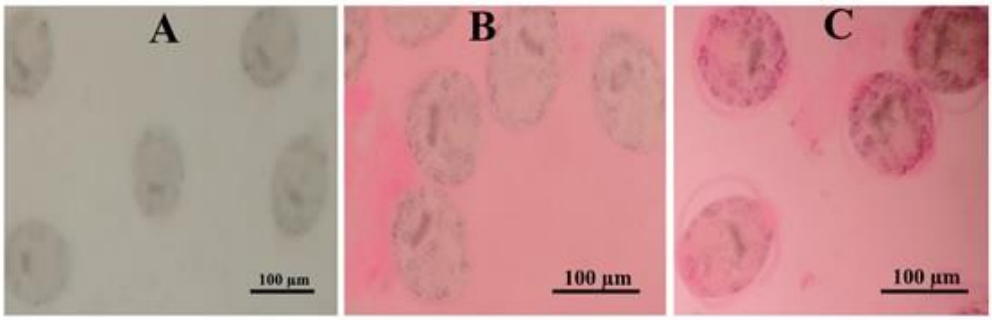

Figure 1. Light microscopy images of PSCs. A) Untreated and non-stained PSCs (Control), B) Untreated PSCs stained with eosin, and C) TOE-treated PSCs (800 mg/ml) after 60 min.

Table 1. Qualitative analysis of metabolites of *T. officinalis extract*

phytochemicals	Result	Appearance
Saponins	+	Foam layer formation
Flavonoids	+	Yellow
Terpenoids	+	Grey
Steroidal glycoside	+	Green or brown
Anthraquinones	+	Pink, Violet, or Red
Salkowskis	+	Reddish-brown

Table2. Protoscolicidal effects of *T. officinalis* extract against protoscoleces at various concentrations during the exposure times.

Treatment Dose (mg/ml)	Treatment time (min)	Mortality rate of protoscoleces (%)	Treatment Dose (mg/ml)	Treatment time (min)	Mortality rate of protoscoleces (%)
50	10 20 30 60	10.05±3.33 20.43±3.57 30.93±6.36 40±10.00	800	10 20 30 60	55.05±3.33 75.43±3.57 95.93±6.36 100±0.00
100	10 20 30 60	15.09±3.18 25±3.16 33±6.30 45±2.10	0.9% Normal saline	10 20 30 60	3.05±3.33 5.22±2.30 8.33±4.18 16.3±5.20
200	10 20 30 60	25.16±4.16 30±5.60 39±6.60 50±1.45	20% Hypertonic saline	10 20 30 60	98.05±3.33 100±0.00 100±0.00 100±0.00
400	10 20 30 60	32.16±4.16 36±4.22 45±3.12 65±9.33	-	-	-

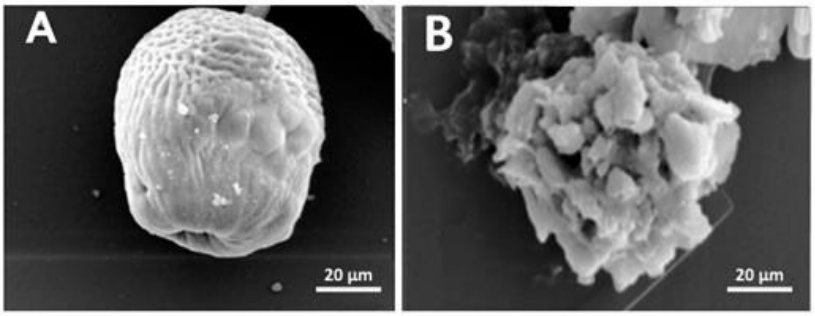

Figure 2. SEM images of PSCs. A) Untreated PSCs (normal morphology) and B) treated with 800 mg/ml of TOE expousure for 60 min.

3.4. Induction of Caspase-3-like of Protoscoleces by TOE

Given the pivotal role of caspases in apoptosis, we quantified the activity of caspases induced by TOE treatment at concentrations of 5, 10, 20, 40, and 80 mg/mL. The findings demonstrated that a dose-dependent caspase induction occurred in TOE-treated PSCs after 48 hours of treatment. As illustrated in Figure 3, PSCs treated with 5, 10, 20, 40, and 80 mg/ml of TOE exhibited activation of the caspase-3 enzyme by 3.7, 11.4, 20.5, 31.4, and 35.7%, respectively.

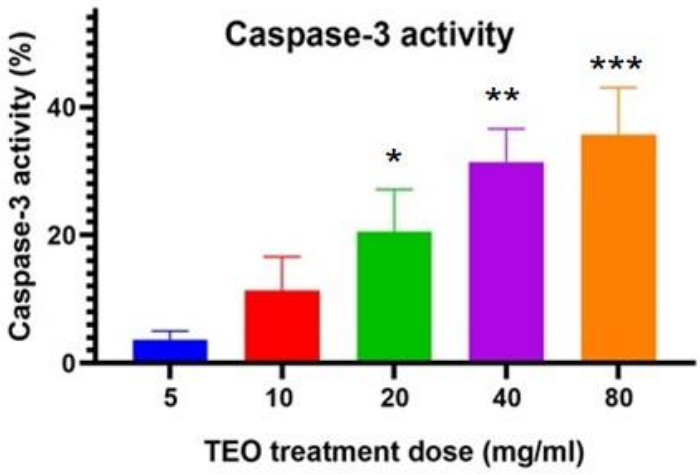

Figure 3: Caspase-3 activity in TOE-treated PSCs after 48 hours. The mean and standard deviation (Mean±SD) were calculated from the average of three replicates. *p<0.05; **p<0.01; and ***p<0.001 compared with control normal saline group.

4. Discussion

Cystic endometriosis (CE) is typically managed with surgical intervention, particularly when the cysts are substantial in size and situated in vital organs. A variety of chemical agents, including hypertonic tonic salt solution, cetrimide-C, and silver nitrate, are employed during surgical procedures to mitigate the risk of secondary infection and anaphylactic shock. However, these agents are associated with several serious adverse effects, thus the search for a new agent with high efficacy and low toxicity represents a crucial priority for surgeons. The objective of this study was to conduct a phytochemical screening, assess the protoscolicidal activity, and elucidate the mechanisms of action of T. officinalis extract against hydatid cyst PSCs. Our findings revealed that the TOE at a concentration of 50 mg/ml exhibited scolicidal activity of approximately 40% at its maximum efficacy. Nevertheless, the TOE exhibited 100% scolicidal activity at a concentration of 800 mg/mL after a 60-minute exposure period. Similarly, following 30 and 20 minutes of exposure, PSCs in the negative and positive control groups exhibited mortality rates of 5.22 and 100%, respectively. Additionally, our findings demonstrated that TOE altered the permeability of cysts, resulting in ultrastructural damage and rostellar disorganization in PSCs. Despite the promising antimicrobial effects of T. officinale extracts against various bacteria (Gram-positive and Gram-negative) and fungi (Candida spp., Aspergillus spp. and viral pathogens (hepatitis B virus, HCV). However, the antiparasitic effects of this plant are reported in few studies. For example, Díaz et al. (2013) reported the in vitro effects of T. officinale on infective larvae and eggs of the Haemonchus contortus nematode with the 50% lethal concentration at 248 µg/mL. Atwa et al. (2002) demonstrated that treatment of mice infected with Schistosoma mansoni with a combination of T. officinale extract and praziquantel resulted in a significant reduction in the number of worms, eggs, and the size of hepatic granulomas. Additionally, the levels of IL-6 and TNF-α, two key inflammatory cytokines, were also decreased (19). In this study, we confirmed the presence of saponins, flavonoids, terpenoids, anthraquinones, steroidal aglycones, and glycosides in TOE. Prior research has indicated that the extract derived from the isolation of various components of T. officinalis contains a range of significant compounds, including flavonoids, terpenoids, anthraquinones, steroidal aglycones, and glycosides [20]. The discrepancy in the compounds present in these extracts can be attributed to several factors, including the geographical origin of the plant, the specific plant part utilized, the extraction methodology, the type of extract,

and the analytical approach employed to quantify the extract constituents [20]. A review of the literature revealed that certain compounds, including flavonoids and terpenoids, demonstrated antiparasitic activity against various parasitic strains, such as Leishmania spp., Trypanosoma spp., Plasmodium spp., and Cryptosporidium spp. [21]. It has been reported that these compounds display antimicrobial effects through a number of mechanisms, including the improvement of drug uptake or reduction of drug efflux, inhibition of nucleic acid synthesis, disruption of cytoplasmic membrane function, cell membrane damage, and disruption of energy metabolism. It can therefore be concluded that the potent protoscolicidal efficacy of this extract is attributable to the presence of these compounds. It has been documented that apoptosis serves a dual purpose in the interaction between the host and hydatid cysts, facilitating both survival and suppression mechanisms [23]. Caspase enzymes, most notably caspase-3, are indispensable for the progression of apoptosis, particularly with regard to DNA fragmentation and the structural alterations associated with cellular demise. Our findings demonstrated that, following a 48-hour TOE treatment, a dose-dependent caspase induction was observed in TOE-treated PSCs. PSCs treated with concentrations of 50, 100, 200, 400, and 800 mg/ml demonstrated activation of the caspase-3 enzyme at rates of 3.7, 11.4, 20.5, 31.4, and 35.7%, respectively. These findings suggest that TOE, through the induction of apoptosis, is capable of killing PSCs. The present research indicates that TOE has a favorable efficacy in killing PSCs of Echinococcus granulosus. Similarly, the structural and ultramorphological alterations of the membrane, along with caspase-3 induction in PSCs treated with TOE, may contribute significantly to its protoscolicidal activity. Nevertheless, further in-depth research is necessary to gain insight into the potential mechanisms of action and to substantiate these findings.

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Authors' Contribution
KC was responsible for designing the experiments, while PS, MZ, and MB conducted the experiments and gathered data. H and M oversaw, guided, and coordinated the study, with AM handling data curation and software tasks. AKK reviewed and edited the manuscript. All authors have reviewed and approved the final version of the manuscript for publication.

Ethics
The research presented in this study was conducted in accordance with the ethical standards set forth by the ethics committee at Lorestan University of Medical Sciences in Khorramabad, Iran. The committee approved the study under the ethics reference number IR.LUMS.REC.1399.221.

Conflict of Interest
There are no competing interests that require disclosure.

Data Availability
All data generated or analyzed during the course of this study has been incorporated into this published article.

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