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In vitro evaluation of methanolic extracts of *Urtica dioica* and *Melissa officinalis* as scolicidal agents against hydatid cyst protoscoleces

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Abstract Cystic Echinococcosis is a zoonotic infection that causes complications such as cyst rupture and secondary infections, requiring effective scolicidal agents. Due to adverse reactions and toxicity of current treatments, new plant-derived SA are needed. This study evaluates the scolicidal effects of methanolic extracts of *Urtica dioica* and *Melissa officinalis* in vitro. Leaves were dried, ground, soaked in methanol, filtered, and concentrated. protoscoleces of *Echinococcus granulosus* were isolated from an infected sheep's liver and assessed for viability using eosin staining. The efficacy of plant extracts was tested at concentrations of 50, 100, and 200 mg/ml for 15, 30, 45, 60, and 120 minutes. Positive controls were 70% ethanol and 20% sodium chloride; 0.9% normal saline served as the negative control. Results showed that 70% ethanol and 20% sodium chloride had a lethal effect of 86-100%. Urtica extract had a 22-72% lethal effect at 200 mg/ml, 8-40% at 100 mg/ml, and 7-32% at 50 mg/ml. Melissa extract had a 37-95% lethal effect at 200 mg/ml, 16-52% at 100 mg/ml, and 12-37% at 50 mg/ml. The results showed that the protoscolexicidal activity of methanol extracts increased with time. The methanol extract of lemon balm at a concentration of 200 mg/ml killed 95% of protoscoleces in 120 minutes, while the nettle extract at the same concentration killed 72% of protoscoleces in the same period. Therefore, the methanol extract of lemon balm can be utilized as a potent protoscolexicidal agent.

Introduction

Hydatidosis, or Cystic Echinococcosis (CE), arises from infection by the metacestode stage of *Echinococcus granulosus* and represents a zoonotic infection characterized by genetic variation and a complex multi-host ecology [1]. In the lifecycle of this tapeworm from the Taeniidae family, the definitive hosts (DH) are carnivorous animals (dogs, wolves, foxes, and jackals), while the intermediate hosts (IH) include domestic animals (sheep, goats, cattle, and pigs), various wildlife species, and humans as accidental hosts [2]. From an epidemiological standpoint, CE is found in every country worldwide; however, regions such as South America, Australia, northern and eastern Africa, and parts of China and Russia exhibit significantly higher prevalence rates. Iran is recognized as one of the endemic countries for this disease [3].

CE is characterized by a three-layered structure. The thick outer layer, composed of connective tissue, is associated with the host's immune response to the cyst. The laminated layer serves as a mechanical barrier. The innermost germinal layer is crucial as it generates protoscoleces (PSC), which may total up to two million. This layer also holds a relatively clear fluid that contains diagnostic antigens and organic compounds [4, 5]. Notably, CE does not always contain PSC in the form of brood capsules or as free entities within the cyst fluid. Particularly in cattle, these cysts are often sterile and may not produce infectious agents [3, 4] According to the classification by the WHO based on the appearance of the cyst contents, hydatid cysts are divided into five grades (CE1–CE5). CE4 and CE5 are considered inactive cysts, while CE1 and CE2 are classified as active cysts. CE3 is identified as a transitional cyst, which may require reclassification [5].

Clinical signs of CE can differ based on the cyst's location, size, and complications. Symptoms may range from asymptomatic cases to abdominal discomfort, hepatic issues such as jaundice and hepatomegaly, portal hypertension, and cholangitis, as well as severe reactions, including anaphylactic shock (AS) and death. Cysts are predominantly located in the liver (with a 70% occurrence rate (OR) in humans and a 90% OR in cattle and horses) and lungs (20% OR in humans and 70% OR in sheep), but they can also be found in the abdominal cavity, musculoskeletal system (including muscles and bones), heart, nervous system, and kidneys. Complications such as cyst rupture (with the potential for AS) and secondary infections may occur [3-6]. The cyst exhibits slow growth, achieving its full size within a period of 6 to 12 months. The size of the cyst can fluctuate, increasing from 1 millimeter during the first month to 5 millimeters by the fifth month, with final dimensions potentially reaching 10 to 12 centimeters or even up to 20 centimeters [3, 6].

The treatment of CE is determined by its characteristics and can involve anti-cestode drugs, surgical intervention, percutaneous aspiration, or observation without immediate intervention [2]. During surgical procedures and percutaneous aspiration, scolicidal agents (SA) such as hypertonic sodium chloride, silver nitrate, cetrimide, ethyl alcohol, chlorhexidine gluconate, and albendazole are injected into the cyst to prevent the settling of cyst fluid and mitigate the risk of reversible infection or AS [6]. However, the high doses required for effective action can lead to adverse drug reactions and toxicity, including sclerosant cholangitis, hypernatremia, liver necrosis, and methemoglobinemia [6]. Therefore, identifying SA that offer greater efficacy at lower doses with minimal side effects could significantly enhance treatment strategies [7].

The *Urtica dioica* L., commonly known as stinging nettle, is a globally distributed plant species and the largest representative of the genus Urtica within the Urticaceae family. It is predominantly found in temperate and moist regions [8]. Among its various parts, the leaves exhibit the highest concentrations of active compounds, which include phenols, tannins, flavonoids (notably carvacrol), phytosterols, and saponins [8, 9]. These bioactive compounds impart a range of beneficial properties to the plant, including anti-inflammatory, antimicrobial, antiproliferative, anticancer, as well as antiparasitic and antifungal effects. Additionally, due to its properties associated with anti-liver damage, neuroprotection, and antioxidant activity, stinging nettle is considered therapeutically versatile for various medicinal studies [9,10].

Melissa officinalis, commonly known as lemon balm, is a member of the Lamiaceae family. This plant is widely distributed across the globe, particularly in regions such as Western Asia, northern Iran, the Mediterranean, and southern Europe [11]. It is utilized for its active compounds in the treatment of various conditions affecting the mental and central nervous system (CNS), as well as cardiovascular and respiratory ailments. Furthermore, Melissa is recognized for its antioxidant, anti-inflammatory, anticancer, antiviral, antibacterial, and antiparasitic properties [11, 12]. The active compounds found in its leaves include flavonoids and polyphenolic compounds, such as rosmarinic acid, caffeic acid, aldehydes, and sesquiterpenes, in addition to tannins [12]. This study aims to explore the

scolicidal effects of methanolic extracts derived from the leaves of both Melissa and Urtica under in vitro conditions.

Materials and Methods

Collection and identification of leaves

The leaves of *Urtica dioica* and *Melissa officinalis* were collected from Kerman, Kerman province, Iran. The plant leaves were dried in the shade at ambient temperature to preserve their active compounds. Subsequently, they were ground using a low-speed electric grinder in a discontinuous manner to prevent heat generation and then sieved (Fig. 1).

Preparation of methanolic extracts

To prepare the extracts, a soaking method was employed in which 50 grams of each plant were separately immersed in 200 ml of 50% methanol solvent for 48 hours. The mixture was then filtered using a Buchner funnel connected to a vacuum system and concentrated with a rotary evaporator under vacuum for two hours [13]. The resulting extracts were stored in sterile glass containers at 4 °C [6]. Concentrations of 50, 100, and 200 mg/ml were prepared by dissolving 0.5, 1, and 2 grams of each methanolic extract in 10 ml of DMSO solution (Fig.1).

Obtaining and preparation of PSC and viability test

For each series of experiments, a number of livers and lungs were collected from the Kerman slaughterhouse. The infected organs were transferred to the Parasitology Laboratory of the Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman. After examining the cysts in the infected organs, the fluid from the fertile cysts was drained with a syringe, and the contents of the cysts were poured into a beaker. They were left for 5 minutes to allow the protoscoleces to settle [6]. To check for viability, the protoscoleces were examined and confirmed using a microscope through the movement of flame cells and also through staining with 0.1% eosin. (Fig.3). PSCs exhibiting over 90% viability were regarded as suitable samples for experimentation [14]. (Fig.1).

Effectiveness of Urtica and Melissa extracts on PSC

To prepare three concentrations of methanolic extract, 0.5, 1, and 2 grams of the extract were dissolved in 10 ml of a suitable solvent (DMSO), thus obtaining concentrations of 50, 100, and 200 mg/ml of the solvent. Then, 0.2 ml of hydatid sand was added to each tube, and after specified times (15, 30, 45, 60, and 120 minutes), the tubes were centrifuged. After washing them twice, a drop of the scolex at the bottom of the tube was placed on a slide, and a drop of 1% eosin dye was added to it. After slide-mounting, it was observed under a microscope. To examine the protoscolex killing effect of the extracts, 250 protoscoleces were counted in each experiment. Then, the number of stained protoscoleces (where dead protoscoleces absorb the dye but live protoscoleces do not) was counted, and the percentage was calculated. This operation was performed seven times for each concentration at different times.

Statistical analysis

To perform the statistical analysis, the average data regarding the effects of the tested substance concentrations, as well as the positive and negative controls, were evaluated using one-way analysis of variance (ANOVA) and Tukey's test. This analysis was implemented using GraphPad Prism Software (version 2023).

Results

Urtica extrac at different times

The methanolic extract of nettle was evaluated at various concentrations over different time intervals. At a concentration of 50 mg/ml, the results were as follows: in 15 minutes, 7% of protoscoleces were destroyed; in 30 minutes, 8%; in 45 minutes, 13%; in 60 minutes, 21%; and

in 120 minutes, 32% of protoscoleces were eliminated.

At a concentration of 100 mg/ml, the results were as follows: in 15 minutes, 8% of protoscoleces were destroyed; in 30 minutes, 13%; in 45 minutes, 18%; in 60 minutes, 26%; and in 120 minutes, 40% of protoscoleces were eliminated.

At a concentration of 200 mg/ml, the results were as follows: in 15 minutes, 22% of protoscoleces were destroyed; in 30 minutes, 32%; in 45 minutes, 43%; in 60 minutes, 56% of protoscoleces were eliminated; and in 120 minutes, 72% of protoscoleces were destroyed.

This excerpt demonstrates a significant difference compared to the positive control group, which consisted of 70% alcohol and 20% sodium chloride ($P<0.05$). At higher concentrations and longer exposure times, the rate of protoscolex killing increased, showing a significant difference from the negative control, which was 0.9% normal saline (Fig 4).

Melissa extract at different times

The methanolic extract of lemon balm was evaluated at various concentrations over different time intervals. At a concentration of 50 mg/ml, the findings were as follows: in 15 minutes, 12% of protoscoleces were killed; in 30 minutes, 16%; in 45 minutes, 19%; in 60 minutes, 26%; and in 120 minutes, 37%.

At a concentration of 100 mg/ml, the results were: in 15 minutes, 16% of protoscoleces were killed; in 30 minutes, 22%; in 45 minutes, 26%; in 60 minutes, 31%; and in 120 minutes, 52%.

At a concentration of 200 mg/ml, the results indicated that in 15 minutes, 37% of protoscoleces were killed; in 30 minutes, 54%; in 45 minutes, 74%; in 60 minutes, 83%; and in 120 minutes, 95%.

This excerpt also demonstrated a significant difference compared to the positive control group ($P<0.05$). With increasing concentration and exposure time, the rate of protoscolex killing increased, showing a significant difference from the negative control, which was 0.9% normal saline. Therefore, this extract can compete with 70% alcohol and 20% sodium chloride (Fig 5).

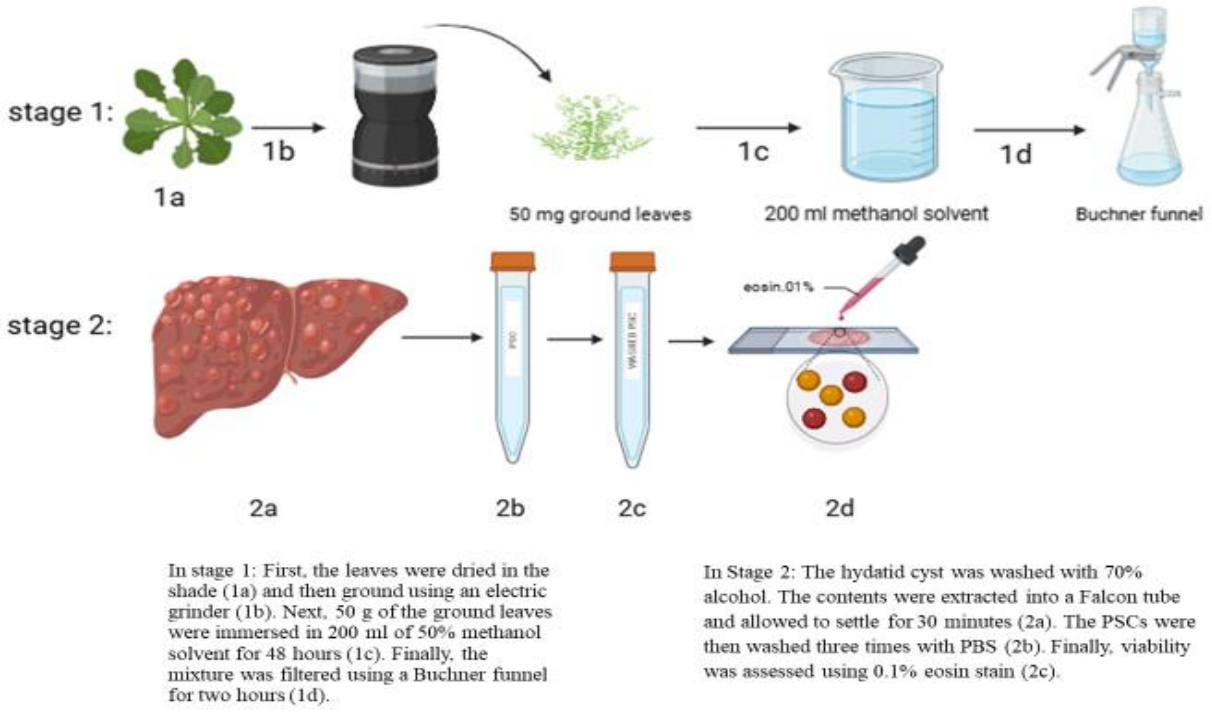

Figure 1. Collection of leaves, preparation of methanolic extracts of Urtica and Melissa, obtaining and preparation of PSC and viability test.

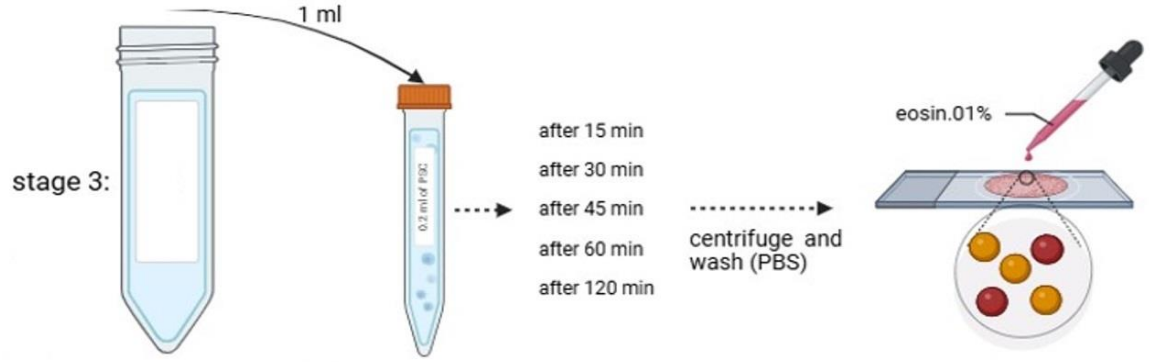

Figure 2. Effectiveness of Urtica and Melissa extracts on PSC

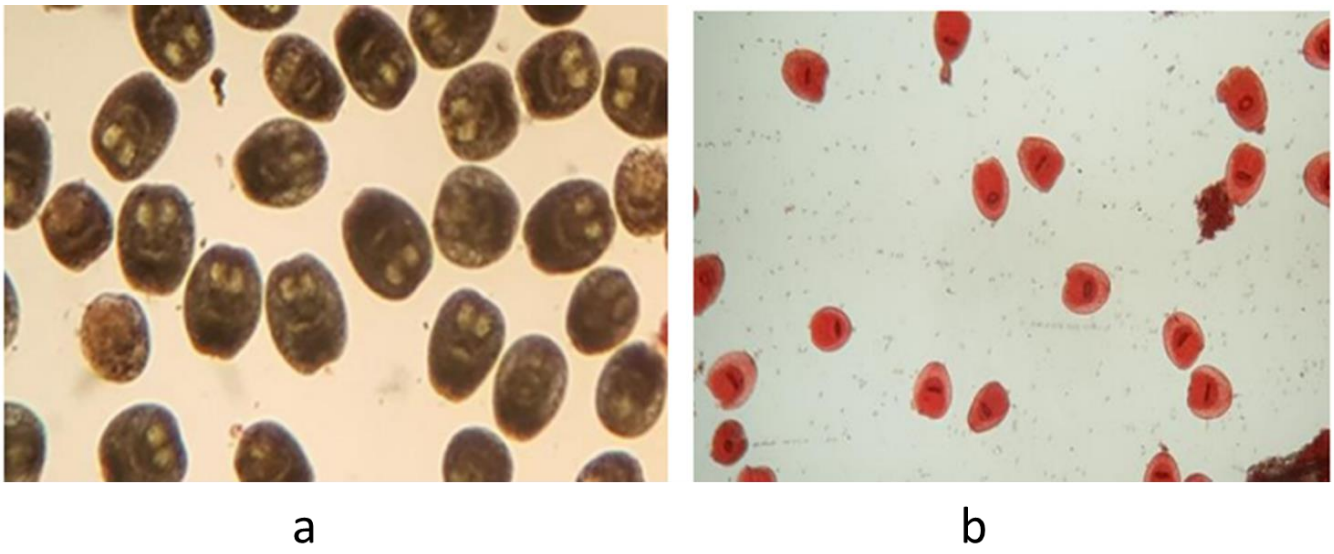

Figure 3. The viability test demonstrated that the protoscolices (PSCs) stained with 0.1% eosin could be distinguished as follows: dead PSCs appeared red (b), while live PSCs remained colorless (a).

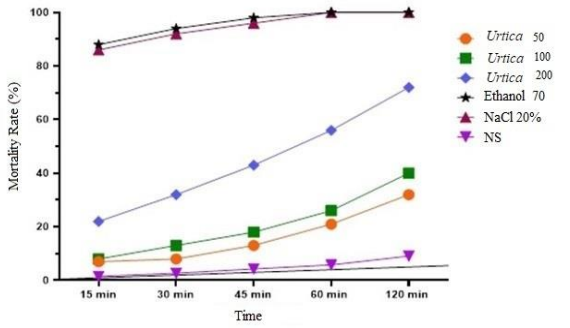

Figure 4. The relationship between the mortality rate of CE protoscoleces and various concentrations of Urtica extract is compared to a negative control and two positive controls. Each data point represents the average percentage of dead PSC derived from seven replicate experiments.

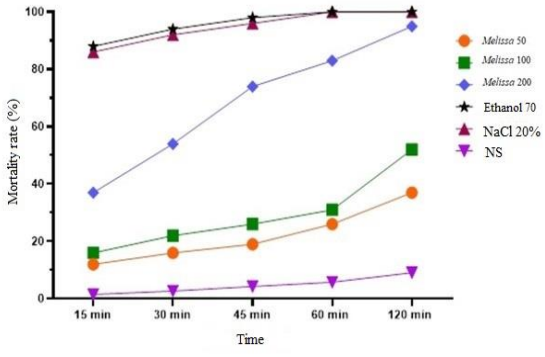

Figure 5. The relationship between the mortality rate of CE protoscoleces and various concentrations of Melissa extract is compared to a negative control and two positive controls. Each data point represents the average percentage of dead PSC derived from seven replicate experiments.

Discussion

The results of our study demonstrate the scolicidal effects of *Urtica dioica* and *Melissa officinalis*, highlighting the potential of natural plant extracts as scolicidal agents against PSC of hydatid cysts. This area of research has garnered significant attention in recent years due to the low cost, easy accessibility, and minimal adverse reactions associated with these natural compounds [15, 16].

The study indicates that both *Urtica dioica* and *Melissa officinalis*, which contain flavonoids, are known for their scolicidal properties. These flavonoids make the plants suitable candidates for therapeutic use, offering a promising alternative to conventional treatments. Many researchers have investigated the protoscolexicidal effect of various substances and plants.

Comparison with previous research findings

Taran et al. (2009) investigated the antiparasitic activity of ethyl alcohol, chloroform, ethyl acetate extracts, and essential oil of *Pistacia khinjuk* on protoscolex *Echinococcus granulosus* at three concentrations of 128, 256, and 512 µg/ml. The essential oil of this plant showed antiparasitic effects against protoscolex. However, its ethyl alcohol, chloroform, and ethyl acetate extracts did not show significant antiparasitic activities [17]. In comparison, our study shows that the flavonoids in *Urtica dioica* and *Melissa officinalis* possess effective scolicidal properties, aligning with the essential oil findings of *Pistacia khinjuk* but indicating a broader spectrum of activity in different solvents. In another study conducted by Moazani et al. (2012), the protoscolicidal properties of *Satureja hortensis* were investigated at various concentrations. The essential oil of this plant eliminated protoscoleces at concentrations of 33, 51, 66, 68, 81, and 100 mg/ml after 60, 30, 20, and 10 minutes, respectively. Therefore, it can be concluded that its essential oil is rich in carvacrol and can serve as a natural scolicidal agent [18]. Similarly, our study indicates that *Urtica dioica* and *Melissa officinalis*, rich in flavonoids, can serve as natural scolicidal agents. The results are consistent with the high efficacy observed in *Satureja hortensis*'s essential oil. Gholami et al. (2013) studied different concentrations (1, 10, 50, 100 mg/ml) of *Achillea millefolium* extract at intervals of 5, 10, 30, and 60 minutes. The stability of the protoscolex was confirmed by 0.1% eosin staining. The results showed that the methanolic extract of *Achillea millefolium* has high scolicidal activity in vitro and can be used as a scolicide in surgery [19]. Our findings further support the potential of plant-based scolicidal agents, as *Urtica dioica* and *Melissa officinalis* also exhibited significant scolicidal activity, reinforcing the relevance of phytochemical-based treatments. Rahimi et al. (2016) studied *Allium sativum* flower extract at a concentration of 50 mg/ml at intervals of 10, 30, 60, and 180 minutes. The results obtained were 59%, 76%, 81%, and 86%, respectively. At a concentration of 100 mg/ml, the results were 67%, 78%, 85%, and 98%, respectively [20]. The high scolicidal activity observed in our study with *Urtica dioica* and *Melissa officinalis* parallels the results seen with *Allium sativum* flower extract, highlighting the effectiveness of natural extracts in combating PSC of hydatid cysts. Zibaei et al. (2017) tested different extracts of *Ephedra sinica*. The scolicidal effects of the root extract against protoscoleces were lower, while the stem and leaf extracts showed the highest activity. A 0.1% stem extract had very strong scolicidal effects after 60 minutes of exposure, and mortality rates decreased with lower concentrations [21]. This aligns with our findings, where the leaves of *Urtica dioica* and *Melissa officinalis*, containing high concentrations of active phytochemicals, demonstrated significant scolicidal effects.

Efficacy of extraction methods

The efficacy of the extracted compound from the plant depends on the extraction method and solvent used. Methanolic extracts are among the most common solvents for extracting active plant compounds against hydatid cysts due to their effective extraction of phytochemicals. However, further research is necessary to determine the most effective extraction method and solvent for each plant to optimize their scolicidal activity [16].

Our study confirms that flavonoids extracted from *Urtica dioica* and *Melissa officinalis* using appropriate methods can optimize scolicidal activity, similar to the findings in previous studies.

Potential of *Urtica dioica* and *Melissa officinalis* leaves

Given the high content of bioactive compounds in the leaves of *Urtica dioica* and *Melissa officinalis*, various parts of medicinal plants can be utilized, but leaves are most commonly used in studies. This preference can be attributed to the higher concentrations of active phytochemicals in the leaves and the minimal harm caused to the plant by harvesting leaves, allowing for sustainable use [21].

Other notable extracts

The methanolic extract of *Piper longum* at a dose of 100 mg/ml demonstrated a 94% mortality rate after 20 minutes [22]. Our study indicates that the extracts of *Urtica dioica* and *Melissa officinalis* have comparable efficacy in scolicidal activity. Similarly, the methanolic extract of *Myrtus communis* at concentrations of 100, 50, and 25 mg/ml exhibited nearly 100% scolicidal activity after 20 minutes in a 2021 study [23]. Our findings support the high scolicidal potential of natural plant extracts. Additionally, the methanolic extract of *Ruta graveolens* at a dose of 40 mg/ml showed high scolicidal activity [24]. Our study aligns with these results, demonstrating the effectiveness of *Urtica dioica* and *Melissa officinalis* as scolicidal agents.

Study limitations and future research directions

One of the primary limitations of our study is the inherent constraints of in vitro models, which may not fully replicate the complex interactions occurring within a living organism. To enhance the validity and applicability of our findings, future research should conduct in vivo studies to assess the effectiveness and safety of these extracts in living organisms. Moreover, future research should explore the specific active compounds within the extracts responsible for the observed scolicidal activities. Identifying and isolating these compounds can lead to a more targeted and effective approach in developing natural scolicidal agents.

Conclusion

The present study showed that the methanolic extract of nettle at a concentration of 200 mg/ml and a time of 120 minutes had a good effect (72%) on the protoscolicity rate. However, the lemon balm extract had a higher protoscolicity rate (95%) and could be used as a scolicide to eliminate protoscoleces. Additionally, the protoscolicity rate in both extracts increased with increasing time and concentration.

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Conflict of interest

The authors declare that they have no competing interests.

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