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Chemical composition, efficacy and safety of *Pistacia vera* (var. Fandoghi) to inactivate protoscoleces during hydatid cyst surgery

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ABSTRACT

At present, various scolicidal agents have been used for inactivation of protoscoleces during hydatid cyst surgery, however, they are associated with serious adverse side effects including sclerosing colangitis (biliary tract fibrosis), liver necrosis and methaemoglobinaemia. This investigation was designed to evaluate the chemical composition and *in vitro* scolicidal effects of *Pistacia vera* (var. Fandoghi) essential oil against protoscoleces of hydatid cysts and also its toxicity in mice model. The components of the *P. vera* essential oil were identified by gas chromatography/mass spectroscopy (GC/MS) analysis. Protoscoleces were aseptically aspirated from sheep livers having hydatid cysts. Various concentrations of the essential oil (25–200 μl/mL) were used for 5–30 min. Viability of protoscoleces was confirmed using eosin exclusion test (0.1% eosin staining). In addition, forty male NIH mice were used to determine the acute and sub-acute toxicity of *P. vera* essential oil for 2 and 14 days, respectively. The main components of *P. vera* essential oil were limonene (26.21%), α-pinene (18.07%), α-thujene (9.31%) and α-terpinolene (9.28%). Findings of the present study demonstrated that the *P. vera* essential oil at the concentrations of 100 and 200 μl/mL killed 100% protoscoleces after 10 and 5 min of exposure, respectively. The LD_{50} values of intraperitoneal injection of the *P. vera* essential oil was 2.69 ml/kg body weight, and the maximum nonfatal doses were 1.94 ml/kg body weight. No significant difference ($P > 0.05$) was observed in the clinical chemistry and hematological parameters following oral administrations of *P. vera* essential oil at the doses 0.1, 0.2, and 0.4 ml/kg for 14 days. The obtained findings demonstrated new chemical composition and promising scolicidal activity of the *P. vera* with no significant toxicity which might be used as a natural scolicidal agent in hydatid cyst surgery.

1. Introduction

Cystic echinococcosis, (CE, Hydatidosis), caused by the larval stage of the dog tapeworm *Echinococcus granulosus*, remains a major public health problem on several continents and is reemerging in several countries [41]. Until the 1980s, surgery was the only option for treatment of hydatid cysts. Chemotherapy with benzimidazole compounds and, later, treatment by PAIR (cyst puncture, aspiration, injection of chemicals, and re-aspiration) were subsequently introduced. However, these treatments have increasingly supplemented or even replaced surgery [5]. First-line chemotherapy of CE with albendazole and mebendazole is a challenge due to having problems of hepatotoxicity, severe leucopenia, thrombocytopenia and alopecia [19]. During hydatid surgery there is a high risk of intraoperative spillage of the cyst contents (protoscoleces) and subsequent recurrence of hydatid cysts and secondary infection, which is observed in nearly 10% of the postoperative cases [12]. The use of effective scolicidal agents is necessary to reduce the risk of recurrence [4,28]. Recently, it has been proven that current scolicidal agents such as hypertonic saline, silver-nitrate, cetrimide, and ethanol are associated with some serious adverse effects including sclerosing collangitis [15,32]. For this reasons, enormous efforts have been made to reach new scolicidal agents especially from natural resources with low side effects and more efficacies for hydatid cyst surgery.

Historically, due to having fewer side effects, high availability, and high efficacy, natural products and their compounds have been used as a valuable resource of traditional remedy [7,33].

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The genus *Pistacia* belongs to the family Anacardiaceae. Among 15 known species of pistachios, only 3 species grow in Iran, including *P. vera*, *P. Khinjuk* and *P. atlantica* [6]. *P. vera* is the only species of the genus cultivated commercially, and the rest of the species are mostly used as rootstocks for *P. vera* [29]. Different parts of *P. vera* including resin, leave, fruit, and aerial parts have been traditionally used as analgesic, carminative, astringent, stomachic, aphrodisiac, antitussive, diuretic, and expectorant [6]. Reviews have also reported *P. vera* as having various biological activities such as anti-inflammatory, antinociceptive, antiatherogenic, and anti-diabetic effects [11,14,16]. Furthermore, antibacterial, antiviral, antifungal, and antiparasitic activities of this plant against several pathogenic strains have previously been demonstrated [13,26,27,31,30]. Previous studies have shown that main constituents of *P. vera* essential oil are hydrocarbon and oxygenated monoterpens such as *α-pinene* [39]. However, some factors such as plant species and part, sex of cultivars, geographical origin, harvesting time, and climatic conditions could affect on the chemical composition and functional activity of the plants [3,36]. The present study was designed to investigate the chemical composition of *P. vera* essential oil, scolicidal activity against protoscoleces of *E. granulosus* and its toxicity in the mice model.

2. Materials and methods

2.1. Collection of plant materials

The plant materials (branch) investigated in this work were collected from rural regions of Kerman province, south east of Iran, from May to September 2013. They were identified by a botanist of the Botany Department of Shahid Bahonar University, Kerman, Iran. A voucher specimen of the plant materials was deposited at the Herbarium of Department of Pharmacognosy of School of Pharmacy, Kerman University of Medical Science, Iran (KF 1136). The collection of plant materials in this study was carried out on private land, and no specific permissions were required for this location.

2.2. Isolation of essential oil

One hundred grams of powdered plant materials were subjected to hydro-distillation for 3 h using an all-glass Clevenger-type apparatus. The essential oil obtained was dried over anhydrous sodium sulfate, and stored in darkness at 4 °C in airtight glass vials closed under nitrogen gas until testing [38].

2.3. Gas chromatography/mass spectrometry (GC/MS) analysis of essential oil

2.3.1. GC analysis

GC analysis was carried out by a Hewlett-Packard 6890 with a HP-5MS column (30m × 0.25 mm, film thickness 0.25 mm). The column temperature was maintained at 55 °C for 3 min and programmed to 180 °C at a rate of 5 °C per min, and kept constant at 220 °C for 5 min. Injector and interface temperatures were 220 °C and 290 °C, respectively. The flow rate of Helium as carrier gas was (1 ml/min C.F). The percentages were calculated by electronic integration of FID peak areas without the use of response factors correction. Linear retention indices for all components were determined by coinjection of the samples with a solution containing homologous series of C8–C22 *n*-alkanes.

2.3.2. GC/MS analysis

GC/MS analysis was performed using a Thermoquest-Finnigan gas chromatograph equipped with fused silica capillary DB-5 column (30m × 0.25 mm, film thickness 0.25 mm) coupled with a TRACE mass (Manchester, UK). Helium was used as carrier gas with ionization voltage of 70 eV. Ion source and interface temperatures were 220 °C and 290 °C, respectively. Mass range was from 40 to 400 μ. Oven temperature program was the same given above for the GC.

2.3.3. Identification of the essential oil components

The components of the essential oil were identified by comparison of their relative retention time and mass spectra with those of standards Wiley 2001 library data of the GC/MS system or with those of reported in the literature data [2].

2.4. Collection of protoscoleces

The protoscoleces of *E. granulosus* were collected from livers of naturally infected sheep slaughtered at Kerman abattoir, and transferred to the Parasitology Laboratory at the Department of Parasitology and Mycology, Kerman University of Medical Sciences. The hydatid fluid aspirated by a 50 ml syringe and aseptically transferred into a flask was left for 30 min for protoscoleces to settle down. The supernatant was discarded and the protoscoleces were washed two times with PBS (pH 7.2) solution. The number of protoscoleces per ml was adjusted as 2×10^3 protoscoleces in 0.9% NaCl solution with at least 90% viability rate. The viability of the protoscoleces was confirmed by their flame cell motility and impermeability to 0.1% eosin solution under a light microscope.

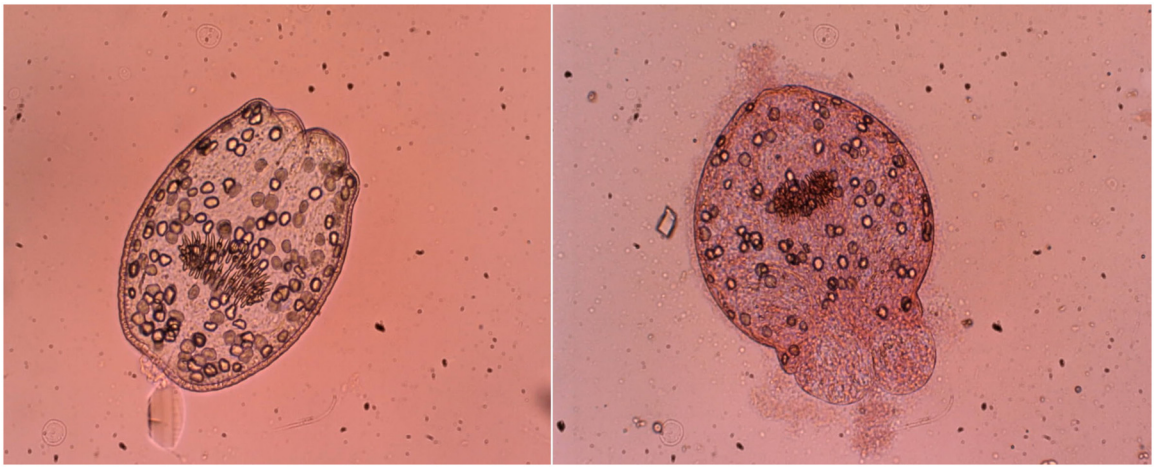

Fig. 1. Live (left) and death (right) protoscoleces of hydatid cysts after exposure with 0.1% eosin.

2.5. Effect on protoscoleces

To determine the scolicidal effects of *P. vera* essential oil against protoscoleces of *E. granulosus,* various concentrations of the oil were used for 5, 10, 20 and 30 min. Initially, 0.5 ml of the protoscoleces (2×10^3/mL) solution was placed in test tubes. Then 0.5 mL of different concentrations of the essential oil (25, 50, 100, and 200 μl/mL) was added to each test tube. The contents of the tubes were gently mixed and then incubated at 37 °C for 5, 10, 20 and 30 min. At the end of each incubation time the upper phase was carefully removed so as not to interrupt the protoscoleces. Fifty μL of 0.1% eosin stain (Sigma-Aldrich, St. Louis, MO, USA) was then added to the remaining settled protoscoleces and mixed gently. The upper portion of the solution was discarded after 10 min of incubation. The remaining pellet of protoscoleces was then smeared on a glass slide, covered with a coverslip and examined under a light microscope. The percentages of dead protoscoleces were determined by counting 300 protoscoleces [21]. In this study, normal saline containing Tween 20 and 20% hypertonic saline were used as negative and positive control, respectively.

2.6. Viability test

Eosin exclusion test was used to determine the viability of protoscoleces [35]. Eosin solution with a concentration of 0.1% (1 g of eosin powder in 1000 ml distilled water) was used. After exposure to the stain, alive protoscoleces remained colorless and showed characteristic muscular movements and flame cell activity, whereas dead protoscoleces absorbed eosin and colored red (Fig. 1).

2.7. Toxicity effects

2.7.1. Animals

Forty male NIH mice (6–8 weeks old) were obtained from the Animal Breeding Stock Facility of Razi Institute of Iran (Karaj, Iran). Animals were housed in a colony room with a 12:12 h light/dark cycle at 21 ± 2° C and were handled according to standard protocols for the use of laboratory animals. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Kerman University of Medical Science (Permit Number: 91/27, 2013).

2.7.2. Acute toxicity test

To determine the acute toxicity, various doses of *P. vera* essential oil (0.5, 1, 2, and 3 ml/kg) were injected as intraperitoneally into groups of five mice. The number of deaths was counted at 48 h after treatment. LD_{50} values were determined by the Probit test in SPSS software [16].

2.7.3. Sub-acute toxicity

Twenty mice were randomly divided into four groups with 5 mice per group. The first group (control) was administrated normal saline orally, and the second to fourth groups were orally administrated *P. vera* essential oil at the doses of 0.1, 0.2, and 0.4 ml/kg, respectively, for 14 consecutive days.

2.7.4. Determination of clinical chemistry and hematological parameters

Following the experimental period, animals were fasted overnight and anaesthetized. According to the guidelines of the Kerman University of Medical Science (Kerman, Iran) for the care and use of laboratory animals, we used Ketamine (100 mg/kg) and Xylazine (10 mg/kg) combination for anesthesia which in it some alpha-2 adrenoreceptor agonists (i.e., Xylazine, Medetomidine) do have analgesic properties and other analgesics like opioids were not used. Moreover, due to the no signs of pain were observed in the process of the study with the anesthetic treatment utilized no specific separate analgesics were used. Sodium pentobarbital (70 mg/kg, i.p.) was used as euthanasia agent and then the abdomen was opened, and blood samples were collected from the heart. Due to compliance with all standards of sterilization, surgeries were per-formed using instruments sterilized by autoclave and under laminar flow hood. For hematological studies, total blood was collected into tubes containing ethylenediamine tetraacetic acid (EDTA) anticoagulant, and hematological parameters, including hemoglobin, hematocrit, white blood cell counts, red blood cell counts, and platelet counts were measured. To measure clinical chemistry parameters in serum, blood was collected into tubes containing no anticoagulant, allowed to clot, and serum was separated by centrifugation at 2000*g* for 20 min. The assays of aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), creatinine (Cr), blood urea nitrogen (BUN), and bilirubin (direct and total), were performed using Roche diagnostics kits (Mannheim, Germany).

2.8. Statistical analysis

In the present study, all the tests were performed in triplicate. Data analysis was carried out by using SPSS statistical package (version 17.0) (SPSS Inc., Chicago, IL, USA). Differences between test and control groups were analyzed by *t*-test. In addition, $P < 0.05$ was considered statistically significant.

3. Results

3.1. GC/MS analysis of essential oil

Table 1 shows the results obtained by GC/MS analysis of *P. vera* essential oil. Twenty-five compounds were identified,

Table 1
Essential oil composition of *P. vera* identified by GC/M.

Components	KI[a]	% Composition
Tricyclene	956	1.24
α-Thujene	973	**9.31**
α-Pinene	987	**18.07**
Camphene	993	4.41
Sabinene	1017	0.6
β- Pinene	1026	3.06
β- Myrcene	1031	1.87
α-Terpinene	1035	1.57
Δ-Carene	1060	2.84
Benzene, 1-methyl	1072	0.68
Limonene	1097	**26.21**
γ-Terpinene	1101	2.15
α-Terpinolene	1179	**9.28**
1,8-menthadien-4-ol	1184	1.14
3-Cyclohexen	1196	1.28
α-Terpineol	1199	0.65
Bicyclo[2.2.1]heptan-2-ol	1234	7.5
Thujopsene	1288	0.38
β-Himachalene	1297	0.68
Cembrene	1354	0.9
b-Caryophyllene	1428	2.06
Bis(2-ethylhexyl) phthalate	1448	0.56
1,13-Tetradecadiene	1498	0.64
delta.-Cadinene	1581	0.6
Cyclohexadecane	1587	0.25
Total		97.93

[a] Kovats index on non-polar DB-5 ms column in reference to *n*-alkanes.

representing 97.93% of the total oil. The main components were limonene (26.21%), α-pinene (18.07%), α-thujene (9.31%) and α-terpinolene (9.28%).

3.2. Scolicidal effects

Fig. 2 shows scolicidal activity of various concentrations of the *P. vera* essential oil (25–200 μl/mL) for 5, 10, 20 and 30 min against protoscoleces of hydatid cysts. *P. vera* essential oil in all concentrations exhibited significant scolicidal effects in comparison with the control group ($P < 0.05$). Results demonstrated that essential oil at the concentration of 200 μl/mL after 5 min of exposure killed 100% protoscoleces. Similarly, all of the protoscoleces were killed after 10 min of exposure to the concentration of 100 μl/mL of essential oil. In contrast, essential oil at the concentration 50 μl/mL killed 16.6, 69.3, 96, and 100% of the protoscoleces, respectively and at the concentration 25 μl/mL killed 7.3, 39.3, 65.6 and 100% of the protoscoleces after 5, 10, 20 and 30 min exposure, respectively. Findings also showed that by increasing the exposure time with *P. vera* essential oil in all concentrations the mortality rate was significantly increased ($P < 0.05$). The mortality rate of protoscoleces in the negative controls was 4.3% after 30 min incubation; while, 20% hypertonic saline as positive control resulting in 100% mortality of protoscoleces after 10 min of exposure.

3.3. Acute toxicity effects

Acute toxicity effects of *P. vera* essential oil were evaluated on male NIH mice. The LD_{50} values of intraperitoneal injection of the *P. vera* essential oil was 2.69 ml/kg body weight, and the maximum nonfatal doses were 1.94 ml/kg body weight.

3.4. Sub-acute toxicity

Based on the obtained findings of LD_{50}, doses of 0.1, 0.2, and 0.4, ml/kg essential oil were chosen to determine sub-acute toxicity of *P. vera*. No death was observed in doses of 0.1, 0.2, and 0.4 ml/kg after two weeks administration. Tables 2 and 3 showed the findings of the clinical chemistry and hematological parameters following oral administration of *P. vera* essential oil for two weeks. There was no significant difference ($P > 0.05$) between oral administrations of *P. vera* essential oil at the employed doses 0.1, 0.2, and 0.4 ml/kg in comparison with control group.

Table 2
Clinical chemistry parameters in mice sera.

Parameters	*P. vera* essential oil (mL/kg)			Control
	0.1	0.2	0.4	
AST (U/L)	136.2 ± 17.6	129 ± 12.3	146 ± 13.5	124 ± 19.5
ALT (U/L)	86 ± 5.3	79 ± 6.6	93 ± 5.6	80 ± 5.3
ALP (U/L)	295 ± 22.3	266 ± 19.5	299 ± 21.2	280 ± 16.5
Cr (mg/dL)	0.38 ± 0.05	0.5.1 ± 0.1	0.58 ± 0.1	0.4 ± 0.05
BUN (mg/dL)	39 ± 6.6	47 ± 6	51 ± 6.1	40 ± 2.4
TB (mg/dL)	0.68 ± 0.1	0.9 ± 0.11	0.83 ± 0.2	0.8 ± 0.1
DB (mg/dL)	0.26 ± 0.05	0.24 ± 0.05	0.32 ± 0.01	0.2 ± 0.015

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; Cr, creatinine; BUN, Blood urea nitrogen; TB, Total bilirubin; DB, Direct bilirubin.

Table 3
Hematology parameters in whole blood.

Parameters	*P. vera* essential oil (mL/kg)			Control
	0.1	0.2	0.4	
RBC ($\times 10^6/\mu L$)	5.12 ± 0.2	6.2 ± 0.5	5.42 ± 0.24	5.4 ± 0.23
HGB (g/dL)	12.4 ± 0.8	13.2 ± 0.5	11.8 ± 0.5	10.9 ± 0.64
Hct (%)	32.5 ± 3.6	37.5 ± 4	35.3 ± 1.3	34.6 ± 1.38
WBC ($\times 10^3/\mu L$)	3.2 ± 0.25	2.4 ± 0.11	3.0 ± 0.25	2.6 ± 0.2
PLT ($\times 10^3/\mu L$)	376 ± 34.3	420 ± 42.3	389 ± 26.6	411 ± 47

RBC, red blood cell; HGB, hemoglobin; Hct, hematocrit; WBC, white blood cell; PLT, platelet.

4. Discussion

This investigation was aimed to evaluate the chemical composition of *P. vera* essential oil, its scolicidal activity against protoscoleces of hydatid cysts and also acute toxicity of *P. vera* essential oil in mice model. At present, an ideal scolicidal agent to reduce the risk of protoscoleces spillage during hydatid cyst surgery is described by its potency at lower concentrations, high efficacy in a shorter time of exposure, stability in the presence of cystic fluid, scolicidal ability inside a cyst, lower toxicity, higher

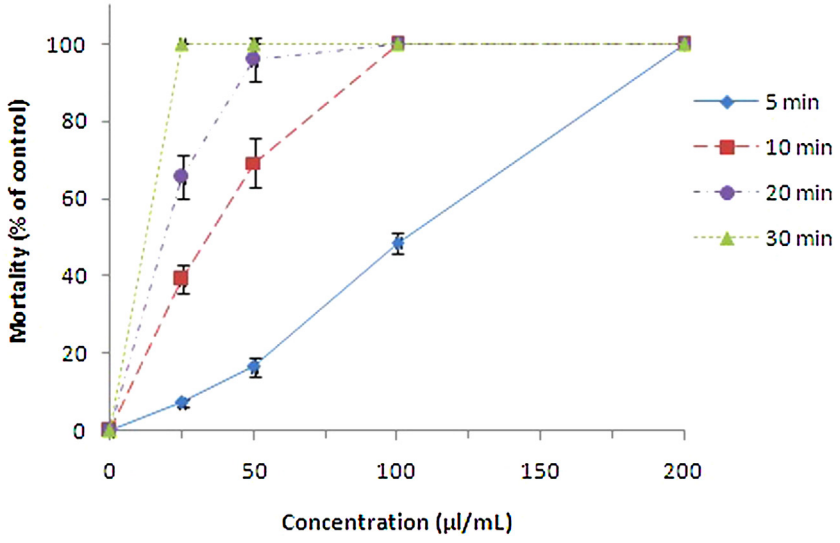

Fig. 2. Scolicidal effects of *P. vera* essential oil against protoscoleces of hydatid cyst at various concentrations following various exposure times. By increasing the exposure time with *P. vera* essential oil in all concentrations the mortality rate was significantly increased ($P < 0.001$) in comparison with control group. Data are expressed as the mean ± SD (n = 3).

availability, and ability for rapid preparation [41]. Reviews have been demonstrated the scolicidal effects of different chemical and natural products such as hypertonic saline, silver nitrate and mannitol, cetrimide, ethyl alcohol (95%), H_2O_2 and 10% povidone iodine, chlorhexidine gluconate, selenium nanoparticles, honey and some plant extracts [28,22–25,18]. However, these scolicidal agents have some limitations in use due to having serious adverse effects. The obtained results indicated that *P. vera* essential oil at concentrations of 200 and 100 μl/mL killed 100% protoscoleces after 5 and 10 min exposure, respectively. Present findings revealed that scolicidal activity of *P. vera* is comparable with the some current scolicidal agents such as 20% hypertonic saline (15 min), 20% silver nitrate (20 min), 0.5–1% cetrimide (10 min), H_2O_2 3% (15 min), and 95% ethyl alcohol (15 min). Therefore, these findings supported the idea that *P. vera* might be a natural source for the production of a new scolicidal agent for use in hydatid cyst surgery.

In the evaluation of chemical composition of *P. vera* essential oil using GC/MS, we found that the main components are limonene (26.21%), α-pinene (18.07%), α-thujene (9.31%) and α-terpinolene (9.28%). In contrast with our results, several studies have reported that α-pinene and α-terpinolene are the most components of *P. vera* essential oil. Therefore, the plant analyzed in this research was a new chemotype of *P. vera.* However, It has been proven that chemical composition of essential oils depend on the species, climate, and time of collection along with growth stage, thereby altering the biological activities studied [3].

The phytochemical screening of the *P. vera* essential oil showed the presence of terpenoids, phenols, and flavonoids in this plant [6]. So far, individual activities of these compounds have been demonstrated [8]. Furthermore, in the various studies potent antimicrobial activities of these compounds such as terpenic derivates, carvacrol, *p*-cymene, thymol, carvone, limonene, and terpinene have been proven [37,40,10,1]. Therefore, phytoconstituents in these plants could be responsible for their scolicidal activity though their exact mode of action is poorly understood. However, in the case of antimicrobial mechanism of some terpenoids compounds such as monoterpens, as the main component of *P. vera*, [34] revealed that they diffuse into pathogen and damage cell membrane structures. On the other hand, other reports suggested that the antimicrobial activity is related to ability of terpenes to affect not only permeability but also other functions of cell membranes, these compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites [9,17].

In regard to toxicity, the LD_{50} of the intraperitoneal injection of the *P. vera* essential oil was 2.69 g/kg and the maximum non-fatal dose was 1.94 g/kg. In our study, no significant difference ($P > 0.05$) was observed in the clinical chemistry and hematological parameters following oral administrations of *P. vera* essential at the doses 0.1, 0.2, and 0.4 ml/kg for 14 days. Thus, according to the toxicity classification, *P. vera* essential oil had no significant toxicity against male NIH mice [20]. In line with these results, Orhan et al. [30] showed that *P. vera* extract did not exert any cytotoxic effect on mammalian L6 cells even at very high concentrations. Therefore, it can be suggested that the *P. vera* derivatives are safe for mammalian cells.

5. Conclusion

The findings of the present study demonstrated new chemical composition and promising scolicidal activity of the *P. vera* with no significant toxicity which might be used as a natural scolicidal agent in hydatid cyst surgery. However, further studies will be needed to confirm these results by checking the essential oil in a clinical setting as a new scolicidal agent.

Conflict of interest

The authors declare no conflict of interest.

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