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ORIGINAL ARTICLE

In vitro Protoscolicidal Effects of *Cinnamomum zeylanicum* Essential Oil and Its Toxicity in Mice

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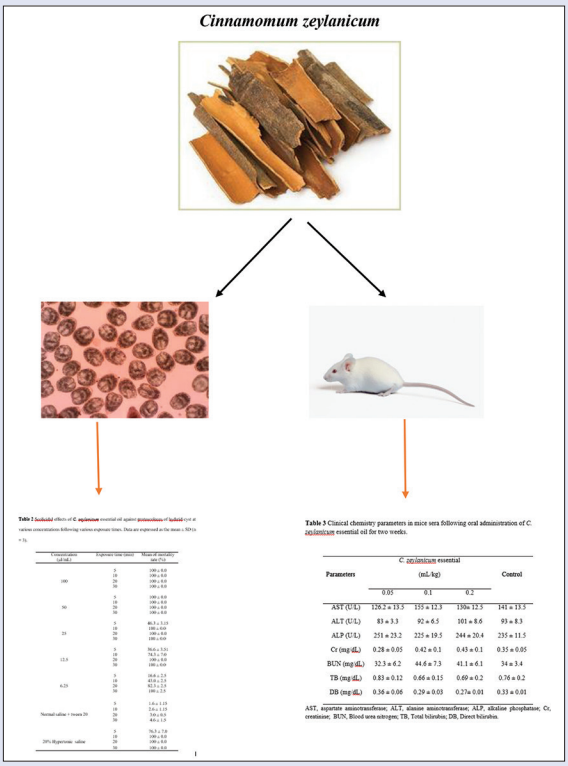
ABSTRACT

Background: This study investigates the scolicidal effects of *Cinnamomum zeylanicum* essential oil against the protoscoleces of hydatid cysts and its toxicity in the mice model. **Materials and Methods:** Gas chromatography/mass spectroscopy analyses were used to identify the constituents of essential oil. Protoscoleces were treated with different concentrations of the essential oil (6.25–100 µL/mL) in each test tube for 5–30 min. The viability of protoscoleces was confirmed using eosin exclusion test (0.1% eosin staining). Forty-eight male NMRI mice were also used to determine the toxicity of *C. zeylanicum* essential oil (0.5–4 mL/kg). **Results:** The main components were found to be cinnamaldehyde (91.8%), ρ metoxicinamate (1.57%), and α pinene (1.25%). Findings indicate that *C. zeylanicum* essential oil with the concentrations of 100 and 50 µL/mL killed 100% of protoscoleces after 5 min of exposure. Also, the lower concentrations of *C. zeylanicum* essential oil motivated a late protoscolicidal effect. The LD_{50} value of intraperitoneal injection of *C. zeylanicum* essential oil was 2.07 mL/kg body weight after 48 h, and the maximum nonfatal dose was 1.52 mL/kg body weight. The results also showed that there was no significant toxicity following oral administration of *C. zeylanicum* essential oil for 2 weeks. **Conclusion:** The results exhibited the favorable scolicidal activity of *C. zeylanicum*, which could be applied as a natural scolicidal agent in hydatid cyst surgery.

Key words: Cystic echinococcosis, gas chromatography/mass spectroscopy, hydatid cyst, protoscoleces

SUMMARY

- We evaluated the efficacy of *Cinnamomum zeylanicum* essential oil against hydatid cyst protoscoleces
- The viability of protoscoleces was confirmed using eosin exclusion test (0.1% eosin staining)
- Forty-eight male NMRI mice were also used to determine the toxicity of *C. zeylanicum* essential oil
- C. zeylanicum with potent scolicidal activity could be applied as a natural scolicidal agent in surgery.



Abbreviations used: GC/MS: Gas chromatography/mass spectrometry analysis; CE: Cystic echinococcosis; LD50: Lethal dose 50%; I.p: Intraperitoneally.

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INTRODUCTION

Cystic echinococcosis (CE) is a zoonotic parasitic disease which is caused by the larval form of *Echinococcus granulosus* tapeworm.[1] CE has a worldwide distribution but is mostly prevalent in the rural areas, in which it is transmitted in a cycle between dogs (definitive host), as domestic livestock, and humans, as the intermediate host.[2,3] CE may develop in humans after the accidental ingestion of tapeworm

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eggs excreted with the feces of an infected dog. The eggs hatch in the intestine of the herbivore, penetrate into the intestinal wall, and reach the livers (50%–70%), lungs (20%–30%), or any other organs through the portal system, in which they develop to a hydatid cyst.[4,5] At present, surgical intervention (conventional or laparoscopic approaches) is the ideal treatment for CE. In addition, cyst puncture, aspiration, injection of chemicals, and reaspiration, chemotherapy with benzimidazole compounds, and watching and waiting for inactive, clinically silent cysts are the substitute treatments to surgery, particularly for the patients who cannot tolerate surgery.[6,7] Through the surgery for dropping threat of intraoperative outflow of protoscoleces (cyst contents) and followed by the repetition of CE and secondary infection, it is necessary to apply effective protoscolicidal agents.[8,9] At this time, the accessible protoscolicidal agents including hypertonic saline and silver nitrate are connected with some serious adverse effects such as biliary tract fibrosis and liver necrosis.[10-12] Therefore, the expansion of new helpful scolicidal agents, mainly from natural resources with little side effects and higher efficiency, is extremely important for surgeons.

Reviews have reported antimicrobial activity of plants and derivative essential oils, extracts, and other phytoconstituents.[13-15] Essential oils are an important supply of new antimicrobial agents as a result of their two imperative properties: further protection to people and environment in addition to low risk of the appearance of microbial resistance, given that they have components which possibly will indicate a range of mechanisms of antimicrobial effects.[16,17] Cinnamomum zeylanicum Blume (Lauraceae) has long been used as a spice and flavoring agent in different cultures around the world for several centuries. True cinnamon or *C. zeylanicum* is the inner bark of a small evergreen tree, which is considered a medication for respiratory, digestive, and gynecological disorders.[18,19] A range of pharmacological functions in antitumor, anti-inflammatory, antioxidant, antimicrobial, and antidiabetic terms has been attributed to this plant.[20-22] Previous investigations have found cinnamaldehyde, camphene, linalool, α-phelendrene, α-terpinene, and limonene as the main constituents of *C. zeylanicum* essential oil.[21] However, some factors such as location and seasonal variations could affect the chemical composition and antimicrobial activity of this essential oil.[23] This study analyzes the chemical composition of the essential oil obtained from *C. zeylanicum* bark, its scolicidal effects on protoscoleces of *E. granulosus*, and its toxicity in mice.

MATERIALS AND METHODS

Plant material

C. zeylanicum bark was collected from rural regions of Jiroft district (Kerman, Iran) in June 2014. The plant materials were identified by Dr. Mandegari, a botanist in Herbal Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran. Voucher specimen was deposited at Herbarium of Pharmacognosy Department of Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.

Extraction/isolation of essential oil

Air-dried bark (200 g) was subjected to hydrodistillation for 3 h by an all-glass Clevenger-type apparatus. The obtained essential oil was dried on anhydrous sodium sulfate and stored in darkness at 4°C in airtight glass vials closed under nitrogen gas until testing.[24]

Drug dilutions

The essential oil (0.1 mL) was dissolved in 0.97 mL of normal saline. In addition, to increase the dispersal of the essential oil, 0.03 mL of Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) was added to the test tube. Obtained solution was adequately mixed using a magnetic stirrer. Then, serial dilution was made to access the essential oil at concentrations of 100, 50, 25, 12.5, and 6.25 μL/mL. The selection of the essential oil dilutions was according to pilot experiments, which also indicated that normal saline plus Tween 20 had no effect on the growth of protoscoleces.

Gas chromatography/mass spectrometry analysis

Gas chromatography analysis

Gas chromatography (GC) analysis was carried out by a Shimadzu QP5050 with a HP-5MS column (30 m × 0.25 mm, film thickness 0.25 mm). The column temperature was retained at 60°C for 3 min and programmed to 180°C at a rate of 5°C/min and maintained constant at 275°C for 5 min. Injector and interface temperatures were 230 and 280°C, respectively. The flow rate of helium as carrier gas was (0.9 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-C20 *n*-alkanes.

Gas chromatography/mass spectrometry analysis

GC/mass spectrometry (MS) analysis was carried out by a Thermoquest-Finnigan gas chromatograph equipped with fused silica capillary DB-5 column (30 m × 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 230 and 280°C, respectively. Mass range was from 40 to 300 U. Oven temperature program was the same given above for the GC.

Identification of the essential oil components

The constituents were identified using 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 showed in the literature data.[25]

Collection of protoscoleces

The protoscoleces of hydatid cysts were collected from the livers of naturally infected sheep and goats slaughtered at Kerman abattoir, Southeast of Iran and transferred to the Parasitology Laboratory at the Department of Parasitology and Mycology, Kerman University of Medical Sciences (Kerman, Iran). All hydatid cyst fluid aseptically aspirated by a 50 mL syringe and transferred into a glass tube was left for 30 min for protoscoleces to settle down. After throwaway the supernatant, the protoscoleces were washed two times with PBS (pH 7.2) solution. Finally, the number of protoscoleces per mL was adjusted as 2×10^3 protoscoleces in 0.9% NaCl solution with at least 90% viability rate.[26]

Effect on protoscoleces

To evaluate scolicidal effects, various concentrations of the essential oil were evaluated during 5, 10, 20, and 30 min. Initially, 0.5 mL of the protoscoleces (2×10^3/mL) solution was poured in each test tube. Then,

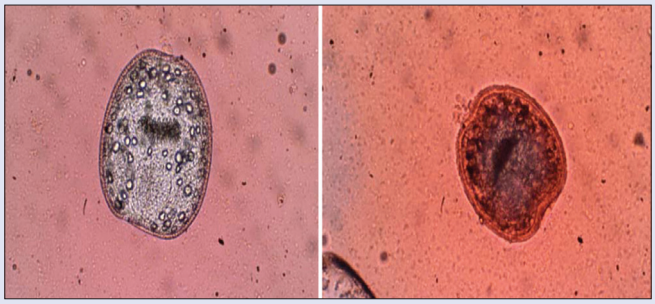

Figure 1: Live (left) and death (right) protoscoleces of hydatid cysts after exposure with 0.1% eosin

0.5 mL of different concentrations of the essential oil was added to test tubes. The tubes were mixed gently and then incubated at 37°C for 5, 10, 20, and 30 min. After incubation, 50 µL of 0.1% eosin stain was added to the remaining settled protoscoleces and mixed gently again. After 10 min of incubation, the upper portion of the solution was discarded. The remaining pellet of protoscoleces was then smeared on a glass slide and examined under a light microscope. The mortality rate of the protoscoleces was determined by counting 300 protoscoleces.[27] Moreover, normal saline plus Tween 20 and 20% hypertonic saline were used as negative and positive control, respectively.

Viability test

The viability was confirmed by flame cell motility and impermeability of the protoscoleces to 0.1% eosin (Sigma-Aldrich, St. Louis, MO, USA) solution under a light microscope (Smyth & Barrett, 1980). After exposure, live protoscoleces remained colorless and exhibited characteristic muscular movements and flame cell activity, whereas dead protoscoleces absorbed stain and colored red [Figure 1].[28]

Toxicity

Animals

Forty-eight male NMRI mice (6–8 weeks old) were purchased from the Animal Breeding Stock Facility of Razi Institute of Iran (Karaj, Iran). Mice were kept in a colony room with a 12:12 h light/dark cycle at 21°C ± 2°C and were handled according to standard protocols for the use of laboratory animals. The experimental procedures performed in the present study were in line with the guidelines of the Kerman University of Medical Science (Kerman, Iran) for the care and use of laboratory animals (Permit no. 92/279).

Acute toxicity effects

To determine the acute toxicity, various doses of *C. zeylanicum* essential oil (0.5–4 mL/kg) were intraperitoneally administrated into four groups (six mice in each group). The number of deaths was counted at 48 h after treatment. LD_{50} values were determined by the Probit test in SPSS software.[29]

Determination of clinical chemistry and hematological parameters

Twenty-four mice were randomly divided into four groups with 8 mice per group. The first group (control) was administrated normal saline orally (orogastric gavage), and the second to fourth groups were orally administrated *C. zeylanicum* essential oil at the doses of 0.05, 0.1, and 0.2 ml/kg, respectively, for 14 consecutive days.

Following the experimental period, animals were fasted overnight and anesthetized. According to guidelines of the Kerman University of Medical Sciences (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 and medetomidine) do have analgesic properties and other analgesics such as opioids were not 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. In this work due to compliance with all standards of sterilization, we did not use any antibiotics. For hematological studies, total blood was collected into tubes containing ethylenediaminetetraacetic acid anticoagulant, and biochemical 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 aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine (Cr), blood urea nitrogen (BUN), and bilirubin (direct and total) were performed using Roche Diagnostics Kits (Mannheim, Germany).[30,31]

Statistical analysis

Obtained results are expressed as the mean ± standard error of mean. Data analysis was carried out using SPSS statistical package version 17.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA with Tukey's *post hoc* test was used to assess differences between experimental groups. In addition, $P < 0.05$ was considered statistically significant.[32]

RESULTS

Gas chromatography/mass spectrometry analysis of essential oil

Yellow-colored essential oil (yield 1.6% v/w) was obtained by

Table 1: Essential oil composition of *Cinnamomum zeylanicum* identified by gas chromatography/mass spectroscopy

Peak number	Compound	RT	Peak area (%)
1	α-pinene	4.81	1.25
2	Cis-ocimene	6.73	0.9
3	Camphene	7.54	0.62
4	Ocimene	8.69	0.6
5	Linalool	9.12	0.21
6	Tricyclene	10.26	0.4
7	Cinnamaldehyde	17.06	91.8
8	ρ-metoxicinamate	21.05	1.57
9	Trans-caryophyllene	22.32	0.31
10	Humulene	24.18	0.12
11	Benzenethanamine	26.47	0.42
12	Trans-cinnamyl acetate	28.91	0.61
	Total		98.81

RT: Retention time

Table 2: Scolicidal effects of *Cinnamomum zeylanicum* essential oil against protoscoleces of hydatid cyst at various concentrations following various exposure times

Concentration (µL/mL)	Exposure time (min)	Mean of mortality rate (%)
100	5	100±0.0
	10	100±0.0
	20	100±0.0
	30	100±0.0
50	5	100±0.0
	10	100±0.0
	20	100±0.0
	30	100±0.0
25	5	46.3±3.15
	10	100±0.0
	20	100±0.0
	30	100±0.0
12.5	5	36.6±3.51
	10	74.3±7.0
	20	100±0.0
	30	100±0.0
6.25	5	16.6±2.5
	10	43.0±2.5
	20	82.3±2.5
	30	100±2.5
Normal saline + Tween 20	5	1.6±1.15
	10	2.6±1.15
	20	3.0±0.5
	30	4.6±1.5
20% hypertonic saline	5	76.3±7.0
	10	100±0.0
	20	100±0.0
	30	100±0.0

Data are expressed as the mean±SD (n=3). SD: Standard deviation

hydrodistillation method and analyzed using GC/MS. Table 1 shows the obtained results by the GC/MS analysis of *C. zeylanicum* essential oil. Thirteen compounds were identified, which represented 98.93% of the total oil. The main components were cinnamaldehyde (91.8%), ρ-metoxicinamate (1.57%), and α-pinene (1.25%).

Effect on protoscoleces

Table 2 shows the scolicidal effects of *C. zeylanicum* essential oil with different concentrations following various exposure times. *C. zeylanicum* essential oil with the concentrations of 100 and 50 μL/mL killed 100% of protoscoleces after 5 min of exposure. Likewise, the mean mortality rate of protoscoleces with the concentration of 25 μL/mL was 100% after 10 min of incubation. However, lower concentrations showed moderate protoscolicidal effects so that, at the concentration of 12.5 μL/mL, 36.6, 74.3, 100, and 100% of protoscoleces and, at the concentration of 6.25 μL/mL, 16.6, 43, 82.3, and 100% of protoscoleces were killed after 5, 10, 20, and 30 min of incubation, respectively. However, the mortality rate of protoscoleces in the negative and positive controls was 4.3% after 30 min and 100% after 5 min of exposure, respectively. Therefore, the obtained findings demonstrated that the essential oil of *C. zeylanicum* at all of these concentrations had more significant ($P < 0.05$) scolicidal effects than the control group.

Acute toxicity

Acute toxicity effects of *C. zeylanicum* essential oil on male NMRI mice were determined. The LD_{50} value of intraperitoneal injection of *C. zeylanicum* essential oil was 2.07 mL/kg body weight after 24 h, and the maximum nonfatal dose was 1.52 mL/kg body weight.

Clinical chemistry and hematological parameters

In the present study and according to the results of LD_{50}, the doses of 0.05, 0.1, and 0.4 ml/kg of *C. zeylanicum* essential oil were selected. The obtained findings indicated that no death was observed in doses of 0.05, 0.1, and 0.2 ml/kg after 2 weeks. Tables 3 and 4 are shown the results of the clinical chemistry and hematological parameters following oral administration of *C. zeylanicum* essential oil for 2 weeks. There was no significant difference ($P > 0.05$) between oral administrations of *C. zeylanicum* essential oil at the doses 0.05, 0.1, and 0.4 mL/kg and control.

Table 3: Clinical chemistry parameters in mice sera following oral administration of *Cinnamomum zeylanicum* essential oil for 2 weeks

Parameters	*Cinnamomum zeylanicum* essential (mL/kg)			Control
	0.05	0.1	0.2	
AST (U/L)	126.2±13.5	155±12.3	130±12.5	141±13.5
ALT (U/L)	83±3.3	92±6.5	101±8.6	93±8.3
ALP (U/L)	251±23.2	225±19.5	244±20.4	235±11.5
Cr (mg/dL)	0.28±0.05	0.42±0.1	0.43±0.1	0.35±0.05
BUN (mg/dL)	32.3±6.2	44.6±7.3	41.1±6.1	34±3.4
TB (mg/dL)	0.83±0.12	0.66±0.15	0.69±0.2	0.76±0.2
DB (mg/dL)	0.36±0.06	0.29±0.03	0.27±0.01	0.33±0.01

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; Cr: Creatinine; BUN: Blood urea nitrogen; TB: Total bilirubin; DB: Direct bilirubin

Table 4: Hematology parameters in whole blood of mice following oral administration of *Cinnamomum zeylanicum* essential oil for 2 weeks

Parameters	*Cinnamomum zeylanicum* essential (mL/kg)			Control
	0.05	0.1	0.2	
RBC (×l0^6/μL)	4.1±0.15	3.6±0.25	2.9±0.24	3.4±0.3
HGB (g/dL)	12.2±0.55	10.2±1.17	10.6±0.6	11.3±0.45
Hct (%)	34.8±3.1	30.1±1.51	32.2±1.6	32.6±2.18
WBC (×l0^3/μL)	2.6±0.15	3.3±0.18	3.1±0.25	2.9±0.2
PLT (×l0^3/μL)	216±15	197±14	208±2	185±17

RBC: Red blood cell; HGB: Hemoglobin; Hct: Hematocrit; WBC: White blood cell; PLT: Platelet

DISCUSSION

Consistent with the World Health Organization, a perfect scolicidal agent for dropping the risk of protoscoleces spillage through hydatid cyst surgery is explained by elevated effectiveness in a shorter time of contact, high influence at lower doses, higher accessibility, constancy in the attendance of cystic fluid, and lower toxicity.[1] Historically, herbs and spices as pure compounds, because of having low toxicity, low cost, high efficacy, and high availability, give boundless opportunities for new drug improvement.[13,33] Therefore, this investigation was designed to analyze the chemical composition of the essential oil obtained from *C. zeylanicum* barks, its scolicidal effects on the hydrated cyst protoscoleces, and its acute toxicity in the mice model.

The obtained findings demonstrated that *C. zeylanicum* at concentrations of 100 and 50 μL/mL completely killed hydatid cyst protoscoleces after 5 min of incubation, whereas lower concentrations of *C. zeylanicum* essential oil motivated deferred protoscolicidal effects. It was also found that the scolicidal activity of *C. zeylanicum* was analogous with the current scolicidal agents such as 20% hypertonic saline, 20% silver nitrate, 0.5%–1% cetrimide, H_2O_2 3% (15 min), and 95% ethyl alcohol with potent scolicidal effects between 10 and 20 min.

The common scolicidal agents had serious side effects such as sclerosing cholangitis (biliary tract fibrosis), liver necrosis, and methemoglobinemia.[10,11] For this reason, the present results supported the idea that *C. zeylanicum* could be a natural origin for producing a new protoscolicidal agent which can be applied in CE surgery. Previously, Sharififar *et al*.[34] reported the presence of high amounts of tannin and alkaloid as well as lack of saponins in the phytochemical screening of *C. zeylanicum* barks. Individual activities of these compounds were proven by Cowan.[35] It was found that the main components were cinnamaldehyde (91.8%), ρ-metoxicinamate (1.57%), and α-pinene (1.25%).

In line with the present results, Saleem *et al*. reported cinnamaldehyde (49.15%), limonene (15.10%), and α-pinene (1.25%) as the main components of *C. zeylanicum* bark essential oil extracted by hydrodistillation method.[19] In several studies, antimicrobial activities of cinnamaldehyde as the main component of *C. zeylanicum* against some pathogenic strains have been shown.[36] Therefore, phytoconstituents in this plant could be answerable for their scolicidal effects although their accurate manner of action is inadequately understood. However, some researchers have shown its effects on the energy creation of microorganisms.[37] The possible inhibition mechanisms of energy generation are the inhibition of glucose uptake or utilization of glucose and effects on membrane permeability.[37] Moreover, in the case of the antimicrobial mechanism of some monoterpene hydrocarbons such as limonene and α-pinene, Sikkema *et al*. indicated that they were dim into pathogen and hurt cell membrane structures.[38] In addition, other studies demonstrated that the antimicrobial activity of these compounds is related to their capability to affect not only permeability but also other functions of cell wall; these compounds might cross the cell membranes and thus penetrate into the interior of the cell and interact with the critical intracellular sites.[39,40] Previous investigations on laboratory animals have demonstrated that liver and kidney is the main object limb of drug toxicity.[41,42] Injure to the structural integrity of the liver is evaluated by increased serum levels of enzymes such as ALT, AST, ALP, and bilirubin. On the other hand, the Cr blood test and BUN are

used to evaluate kidney function. Acute and subacute toxicity is the first steps of evaluation toxicity effects all drugs. In these methods, tested drugs used in both routes of oral and intraperitoneal administration. In this study, we have used manifold concentrations in comparison with concentrations applied for its protoscolicidal scolicidal effects.

With regard to the toxicity of *C. zeylanicum* essential oil, it was found that the essential oil showed no mortality up to the dose of 2 mL/kg. However, 25% of mortality occurred at the dose of 4 mL/kg. No significant differences ($P > 0.05$) in the clinical chemistry and hematological parameters following oral administrations of *C. zeylanicum* essential oil for 14 days were detected. According to the toxicity classification, *C. zeylanicum* essential oil induced no significant toxicity among male NMRI mice.[43]

CONCLUSION

The present study demonstrated the scolicidal activity of *C. zeylanicum* which could be used as a natural scolicidal agent to reduce the risk of spillage protoscoleces during CE surgery. However, further investigation is required to confirm these findings through studying the essential oil in a clinical setting as a new scolicidal agent.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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