

Protoscolicidal Effects and Acute Toxicity of Essential Oil and Methanolic Extract of *Cuminum cyminum* Seeds

Amir Keyhani, Amir Tavakoli Kareshk, Razieh Tavakoli Oliaei, Hossein Mahmoudvand

ABSTRACT

Surgical treatments were the gold standard for treatment of cystic echinococcosis (CE) until the last 30 years. Existing scolicidal agents such as hypertonic saline and silver-nitrate which have been used for inactivation of protoscoleces during surgery are associated with some serious adverse effects. The present study was designed to investigate *in vitro* scolicidal activity of *Cuminum cyminum* L. against protoscoleces of hydatid cysts and its acute toxicity in mice model. Protoscoleces were aseptically aspirated from sheep livers having hydatid cysts. Various concentrations of the essential oil (12.5- 100 µl/mL) and methanolic extract (12.5-100 mg/mL) were used for 5 to 60 min. Viability of protoscoleces was confirmed using eosin exclusion test (0.1% eosin staining). Moreover, thirty-two male NMRI mice were used to determine the acute toxicity of *C. cyminum*.

C. cyminum methanolic extract at the concentrations of 100 and 50 mg/ml killed 100% protoscoleces after 10 and 20 minutes exposure. Moreover, all protoscoleces were killed upon 10 and 20 minutes of exposure to 50 and 25 µl/mL *C. cyminum* essential oil, respectively. The essential oil demonstrated no mortality up to the dose of 1 ml/kg, but 25% mortality at dose of 2ml/kg was occurred. The methanolic extract showed no mortality up to the dose of 4 g/kg. The obtained results indicated the potent scolicidal activity of *C. cyminum* with no significant toxicity which might be used as a natural scolicidal agent in hydatid cyst surgery. However, further investigation will be needed to confirm these results by checking in a clinical setting as a new scolicidal agent.

Keywords: Cumin; scolicidal effects; cystic echinococcosis; hydatid cyst; toxicity

Amir Keyhani, Amir Tavakoli Kareshk, Razieh Tavakoli Oliaei
Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran.

Hossein Mahmoudvand
Razi Herbal Medicine Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran.

Corresponding Author:
Hossein Mahmoudvand
e-mail: dmahmodvand@gmail.com

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Introduction

Hydatid disease or cystic echinococcosis (CE) is between the main neglected parasitic infections around the world which typically caused by the metacestodes form of the *Echinococcus granulosus* [1]. CE can grow slowly for several years up to the onset of clinical manifestations or as an accidental finding on some radiography examinations such as ultrasound (US) scan that demanded for not related reasons [2]. Clinical symptoms can indicate that the cyst is already problematical, e.g., cysts rupture, secondarily infections, or leaking and producing anaphylactic shock [2]. Since last decades the gold standard treatment for hydatidosis was surgery; whereas chemotherapy with albendazol, mebendazole and also PAIR (cyst puncture, aspiration, injection of chemicals and re-aspiration) were then expanded which have progressively further supplemented or surprisingly replaced surgery [2]. When surgery is doing the use of influential scolicidal agents to avoid reappearance and secondary infection of hydatid cyst is compulsory. [3]. Recent investigations demonstrated

or have shown that accessible scolical drugs because of having severe complications such as sclerosing cholangitis should be used with caution [4]. This indicated that discovery of a novel scolical agent with low complications and higher performance is one of the most important concerns of surgeons.

Of years ago until now, herbs and spices have been applied for prevention and treatment of a broad spectrum of illnesses such as infectious ones [5, 6]. *Cuminum cyminum* L. (Apiaceae family) as an aromatic herb broadly demonstrated some medicinal features such as antidiarrhoeal, anti-diabetic, antioxidant, anti-epileptic, anti-stress, hepatoprotective and renoprotective, as well as anti-bacterial, and anti-fungal in modern medicine [8]. To the best of our knowledge, there have been no studies made on scolical effects of *C. cyminum*. Thus, this study was aimed to assess protoscolical activity of *C. cyminum* against protoscoleces of hydatid cysts and also its acute toxicity in mice.

Materials and methods

Plant collection

Seeds of *C. cyminum* were collected from the rural region of Bam district, Kerman province, Iran in May 2014. The plant materials 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 1357-1).

Preparing of methanolic extract

Air dried seeds (100 g) were extracted by percolation method with 80% methanol successively for 72 h. in room temperature. The extract was passed through filter paper (Whatman No.3, Sigma, Germany) to remove plant debris. Finally, the extract was concentrated in vacuum at 50°C using a rotary evaporator (Heidolph, Germany) and stored at -20°C, until testing [9].

Isolation of essential oil

One hundred grams of crushed seeds were subjected to hydro-distillation for 3h using an all-glass cleverger-type apparatus. The essential oil obtained was dried over anhydrous sodium sulfate, and stored in darkness at 4°C in airtight glass vials closed until testing [10].

Drug dilution

For the preparation of dilutions of the *C. cyminum* essential oil, 0.1 mL of the essential oil was dissolved in 0.97 mL of normal saline. In addition, to enhance the dispersal of the essential oil in normal saline, 0.03 mL of Tween 20 (Sigma-Aldrich, St Louis, MO, USA) was added to the test tube. Serial dilutions were carried out to obtain the essential oil at the concentrations of 12.5 to 100µl/mL. To prepare different concentrations of methanolic extract, 100 mg of the methanolic extract was dissolved in 1 mL normal saline and then serial dilution were performed to obtain the methanolic extract at the concentrations of 50, 25, 12.5 mg/mL. The choice of doses was based on preliminary experiments, which also indicated that normal saline plus Tween 20 did not cause effect on the growth of parasite.

Collection of protoscoleces

Hydatid cysts protoscoleces were collected from the livers of naturally infected sheep and goats slaughtered at Kerman abattoir, southeastern Iran. The hydatid fluid aspirated by a 50 mL syringe and aseptically transferred into a flask was left to set 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.

Effects on protoscolices

To find out protoscolical effects of *C. cyminum* essential oil, 0.5 mL of the protoscoleces (1×10^3 /mL) solution was placed in test tubes. Then 0.5 mL of various concentrations of the essential oil and methanolic extract was added to each test tube, separately. The contents of the tubes were quietly mixed and then incubated at 37°C for 5, 10, 20, and 30 min. then the superior part was cautiously detached so as not to break off the protoscoleces.

Viability test

To do this, 50 µL of 0.1% (1 g of eosin powder in 1,000 ml distilled water) eosin stain (Sigma-Aldrich, St Louis, MO, USA) was added to the residual settled protoscoleces and mixed softly. The superior part of the solution was throwing away after 10 min of incubation. After this time, protoscoleces was smeared on a glass slide, covered with a cover glass and tested under a light microscope. The live

protoscoleces kept colorless and represented muscular motions and flame cell action, at the same time the dead protoscoleces captivated eosin and colored red (Fig 1). The percentages of viability of protoscoleces were determined by counting 300 protoscoleces. Moreover, normal saline and hypertonic saline 20% was applied as negative and positive control, respectively [11].

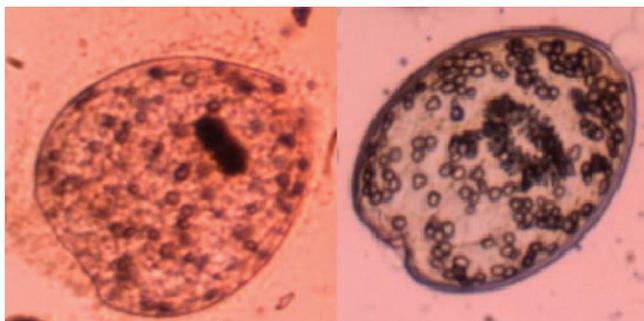


Figure 1. Death (left) and live (right) protoscoleces of hydatid cysts after exposure with 0.1% eosin.

Animals

Thirty-two male NMRI 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 $21 \pm 2^\circ\text{C}$ and were handled according to standard protocols for the use of laboratory animals. The experimental procedures carried out in this study complied with the guidelines of the Kerman University of Medical Science (Kerman, Iran) for the care and use of laboratory animals (Permit no. 2016/279).

Toxicity effects

To determine the acute toxicity, various doses of *C. cyminum* essential oil (0.5-4 ml/kg) and methanolic extract (0.5-4 g/kg) were injected as intraperitoneally into groups of four mice and the mortality and morbidity was recorded after 24 h [12].

Statistical analysis

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). To determine the acute toxicity, LD50 values were determined by the Probit test in SPSS software. In evaluation of protoscolicidal effects of *C. cyminum*, the difference between various concentrations was

evaluated by ANOVA which followed by *t*- test and *p* values less than 0.05 were considered significant.

Results

Protoscolicidal effects

Table 1 shows scolical effects of *C. cyminum* methanolic extract at the various concentrations following different exposure times. *C. cyminum* methanolic extract at the concentrations of 100 and 50 mg/ml killed all over of protoscoleces after 10 and 20 minutes exposure, respectively. Likewise, lower concentrations of methanolic extract forced a postponed scolical activity. Table 2 shows 100% protoscoleces were killed upon 10 and 20 minutes of exposure to 50 and 25 $\mu\text{L}/\text{mL}$ *C. cyminum* essential oil, respectively. Obtained results also confirmed that lower concentrations of *C. cyminum* essential oil had a belated scolical effects. The mortality rate of protoscoleces in the negative and positive control was 4.3 and 100% after 30 and 10 min exposure, respectively. These findings demonstrated that essential oil and methanolic extract of *C. cyminum* at all of concentrations had considerable ($p < 0.05$) scolical effects in comparison with the control group.

Acute toxicity

Acute toxicity effects of *C. cyminum* were determined on male NMRI mice. The essential oil demonstrated no mortality up to the dose of 1 ml/kg, but 25% mortality at dose of 2ml/kg was occurred. The methanolic extract showed no mortality up to the dose of 4 g/kg.

Discussion

From long time ago, antimicrobial property of remedial herbs has been logically revealed in experiments with essential oils, extracts and phytoconstituents [13]. Nowadays, essential oils as one of the most important herb formatives have effective antimicrobial activities because of owing two special features: their natural basis generally means appropriate safety to human and environment; and also because of low threat for rising of microbial resistance as a result of possessing components with a range of mechanisms of antimicrobial effects [6]. As reported by the World Health Organization (WHO), an perfect scolical drug qualified by its power at lesser concentrations, high efficiency in a brief time of contact, permanence in the

Table 1. Protoscolicidal effects of *C. cyminum* methanolic extract against protoscoleces of hydatid cyst at various concentrations following various exposure times. Data are expressed as the mean \pm SD (n = 3).

Concentration of <i>C. cyminum</i> metabolic extract (mg/mL)	Exposure time (min)	Mean of mortality rate (%)
100	10	100 \pm 0.0
	20	100 \pm 0.0
	30	100 \pm 0.0
	60	100 \pm 0.0
50	10	83.5 \pm 2.12
	20	100 \pm 0.0
	30	100 \pm 0.0
	60	100 \pm 0.0
25	10	37.5 \pm 3.53
	20	72.5 \pm 3.53
	30	100 \pm 0.0
	60	100 \pm 0.0
12.5	10	12.5 \pm 3.53
	20	39.0 \pm 5.65
	30	68.0 \pm 5.65
	60	100 \pm 0.0
Normal saline	10	1.6 \pm 0.5
	20	2.5 \pm 0.5
	30	3.3 \pm 0.5
	60	5.3 \pm 0.5
20% Hypertonic saline	10	100 \pm 0.0
	20	100 \pm 0.0
	30	100 \pm 0.0
	60	100 \pm 0.0

attendance of cystic fluid, scolical skill indoor a cyst, minor toxicity, high ease of use, and capability for quick preparation [1]. To date, scolical activity of a range of chemical and natural products including hypertonic saline, silver nitrate and mannitol, selenium nanoparticles, honey and some plant extracts have been proven [14-19]. However, they are accompanying with some adverse side effects for example biliary tract fibrosis and their usefulness is contentious. Our results revealed that *C. cyminum* essential oil at the concentrations of 50 and 25 μ L/mL and methanolic extract at the concentrations of 100 and 50 mg/mL killed 100% protoscoleces after 10 and 20 minutes exposure respectively. Therefore it could be concluded that scolical activity of *C. cyminum* particularly its essential oil is comparable with the existing scolical agents.

Based on the previous study, in the initially phytochemical analysis of *C. cyminum* extract there are some compounds such as terpenoids, phenols, flavonoids, and fatty acids [8]. Recently, Oroojalian *et al* [20] have recognized nineteen compounds in the essential oil of *C. cyminum* L. from Mashhad, Iran, whereas the main components were cuminaldehyde (30.2%), p-cymene (14.1%), γ -terpinene (12.8%), safranal (9.4%), and β -pinene (6.4%), respectively. In the other study on the chemical composition of *C. cyminum* L. from the Alborz mountain α -pinene (29.2%), limonene (21.7%), 1, 8-cineole (18.1%), linalool (10.5%), and α -terpineole (3.17%) were found as the major compounds [21]. However, some factors such as geographical source and collecting time could influence on the chemical composition and functional activity of plant essential oils [22-24].

Table 2. Protoscolicidal effects of *C. cyminum* essential oil against protoscoleces of hydatid cyst at various concentrations following various exposure times. Data are expressed as the mean \pm SD (n = 3).

<i>C. cyminum</i> essential oil concentration (μ l/mL)	Exposure time (min)	Mean of mortality rate (%)
50	10	100 \pm 0.0
	20	100 \pm 0.0
	30	100 \pm 0.0
	60	100 \pm 0.0
25	10	75.0 \pm 7.0
	20	100 \pm 0.0
	30	100 \pm 0.0
	60	100 \pm 0.0
12.5	10	52.6 \pm 3.53
	20	82.3 \pm 4.9
	30	100 \pm 0.0
	60	100 \pm 0.0
6.25	10	5.0 \pm 2.82
	20	8.5 \pm 2.52
	30	61.0 \pm 6.1
	60	100 \pm 0.0
Normal saline + Tween 20	10	1.6 \pm 1.15
	20	2.6 \pm 1.15
	30	3.0 \pm 0.5
	60	4.6 \pm 1.5
20% Hypertonic saline	10	100 \pm 0.0
	20	100 \pm 0.0
	30	100 \pm 0.0
	60	100 \pm 0.0

This indicated that some of these main components could be answerable for their scolical properties of this plant; while their accurate mechanisms of action are inadequately unstated [6]. However, previous investigations on antimicrobial mechanism of some terpenoids demonstrated that these components can manipulate permeability cell membranes and also arrived into the cell and disturb vital intracellular functions [25-27].

Regarding toxicity effects of *C. cyminum*, the obtained results that the essential oil had no mortality up to the dose of 2 ml/kg, but 25% mortality at dose of 4ml/kg was occurred; while, the methanolic extract showed no mortality up to the dose of 4 g/kg. In a study Haroun *et al* (2002) demonstrated that diets containing 2% *C. cyminum* fruits were not any toxic effects to rats; whereas some problems such as disorder of growth and enterohepatonephropathy were reported in the rats fed a diet containing 10% *C. cyminum* fruits [28]. However, according

to a toxicity classification, *C. cyminum* had no significant toxicity against male NMRI mice [29].

To conclude, the present study exhibited potent scolical activity of *C. cyminum* which could be used as a natural scolical agent to reduce the risk of protoscoleces spillage during hydatid cyst surgery. However, further investigation will be needed to confirm these results by checking in a clinical setting as a new scolical agent.

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Conflict of Interest

The authors declare no conflict of interest

***Cuminum cyminum* uçucu yağının ve bitkinin çekirdeklerinden hazırlanan metanollü ekstrenin akut toksisitesi ve protoskolisidal etkileri**

ÖZ

Son 30 yıla kadar, kistik ekinokokkozis (CE) tedavisinde cerrahi yaklaşım en doğru seçim olarak görülmekteydi. Hipertonik tuz çözeltisi ve gümüş nitrat çözeltisi gibi skolisidal özellik gösteren çözeltiler cerrahi işlemler esnasında kistlerin inaktivasyonu için kullanılmakla birlikte bu çözeltilerin ciddi yan etkilere de neden olduğu bilinmektedir. Bu çalışmada, *Cuminum cyminum* L.'nin hidatik kist protoskoleseslerine karşı *in vitro* skolisidal etkinliğine ek olarak oluşturulan fare modelinde akut toksisitesi de incelenmiştir. Protoskolesesler aseptik şartlarda hidatik kisti olan koyunların karaciğerlerinden aspire edilerek elde edilmiştir. Uçucu yağ (12.5-100 µl/mL) ve metanollü ekstre (12.5-100 mg/mL) farklı derişimlerde 5-60 dakika uygulanmıştır. Protoskoleseslerin canlılığı eosin eksklüzyon yöntemiyle (%0.1 eozin boyama) ile tespit edilmiştir. Buna

ek olarak, 32 erkek NMRI fare kullanılarak *C.cyminum*'un akut toksisitesi çalışılmıştır. *C.cyminum*'un metanollü ekstresi 100 ve 50 mg/ml derişimlerde uygulandığında 10 ve 20 dakika maruziyet sonrasında protoskoleseslerin %100'ünü öldürmüştür. *C.cyminum*'un uçucu yağı, 50 ve 25 µl/mL derişimlerde uygulandığında ise sırasıyla 10 ve 20 dakika maruziyet sonrasında tüm protoskoleseslerin öldüğü tespit edilmiştir. Uçucu yağ ile yapılan çalışmalarda 1 ml/kg doza kadar ölüm saptanmazken 2 ml/kg dozda ölüm oranı %25'tir. Metanollü ekstre ile çalışıldığında ise 4 g/kg doza kadar ölüm gerçekleşmemiştir. Elde edilen bulgular değerlendirildiğinde *C. cyminum*'un potansiyel skolisidal etkinliğine ek olarak ciddi bir toksiste göstermediği ve bu nedenle hidatik kist cerrahisinde bitkisel kaynaklı bir skolisidal olarak kullanılabilceği önerilmiş ancak önerininin doğrulanması için ileri klinik çalışmalara ihtiyaç duyulduğu da belirtilmiştir.

Anahtar kelimeler: Kimyon; skolisidal etki; kistik ekinokokkozis; hidatik kist; toksisite

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