



## *In vitro* lethal effects of various extracts of *Nigella sativa* seed on hydatid cyst protoscoleces

Hossein Mahmoudvand<sup>1, 2</sup>, Arash Asadi<sup>3</sup>, Majid Fasihi Harandi<sup>2</sup>, Fariba Sharififar<sup>4</sup>, Sareh Jahanbakhsh<sup>1, 2</sup>, Ebrahimm Saedi Dezaki<sup>2\*</sup>

<sup>1</sup> Research Center for Tropical and Infectious Diseases, Kerman University of Medical Science, Kerman, Iran

<sup>2</sup> Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran

<sup>3</sup> Department of Medical Parasitology and Mycology, Kerman University of Medical Sciences Kerman, Iran

<sup>4</sup> Department of Pharmacognosy, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran

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### ABSTRACT

**Objective(s):** There are several scolicidal agents for inactivation of hydatid cyst protoscoleces during surgery, but most of them are associated with adverse side effects such as sclerosing cholangitis and liver necrosis. The present study was aimed to evaluate scolicidal effects of various extracts of *Nigella sativa* seeds against protoscoleces of hydatid cyst in an *in vitro* model.

**Materials and Methods:** Protoscoleces were aseptically aspirated from naturally infected livers of sheep and goats. Various concentrations of the different extracts of *N. sativa* (5 to 50 mg/ml) were used for 5 to 60 min. Viability of protoscoleces was confirmed by 0.1% eosin staining.

**Results:** The findings exhibited that methanolic extract at the concentration of 50 mg/ml after 10 min of incubation, and aqueous extract at the concentration of 50 mg/ml after 30 min of incubation can kill 100% of protoscoleces. In addition, all of experiments revealed dose-dependent and also time-dependent scolicidal effect of various extracts of *N. sativa* on the protoscoleces of hydatid cyst.

**Conclusion:** The results of the present study demonstrated that *N. sativa* may be a natural source for the production of new scolicidal agent for use in hydatid cyst surgery. However, further studies will be required to evaluate scolicidal effects of *N. sativa* in the *in vivo* model.

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## Introduction

Hydatidosis or cystic echinococcosis (CE) is a chronic zoonosis affecting humans as well as domestic animals caused by the larval stage of a cosmopolitan parasitic cestode *Echinococcus granulosus*. It has been identified as a global public health and economic problem particularly in developing regions including Iran, which causes serious morbidity and death if left untreated (1). The dog is the definitive host, in which adult tapeworms attached to the intestinal epithelium undergo sexual reproduction, leading to the development of eggs. Intermediate hosts such as humans as well as domestic livestock including cattle, sheep, camels, pigs become infected following ingestion of eggs through direct contact with dog or indirectly through food, water or soil contaminated with eggs. The released embryos penetrate the intestinal wall and via the portal system enter mainly into the liver (50-70%), lungs (20-30%), or any other organs where the hydatid cysts grow up (2). At present, surgical removal is still the ideal and preferred treatment for

CE in many parts of the world, including Iran (3). In addition, chemotherapy with benzimidazoles (albendazole, mebendazole) and PAIR (puncture, aspiration, injection, re-aspiration) are also recommended as alternative treatments to surgery, especially for the patients who do not suffer from complicated cases of CE (3, 4). To reduce the risk of intraoperative spillage of the cyst contents (scolices) during surgery and subsequently recurrence of CE and secondary infection, which is observed in nearly 10% of the postoperative cases, the use of effective scolicidal agents are necessary (4, 5). Existing scolicidal agents including hypertonic saline, silver nitrate, cetrimide, and ethanol, which commonly used as scolicidal agents, are associated with adverse effects such as sclerosing colangitis, liver necrosis and methaemoglobinaemia (6-8). For these reasons, the development of new scolicidal agents especially from natural products due to having fewer side effects, low cost, high availability and higher efficacy is an urgent need for surgeons (9).

\*Corresponding author: Ebrahimm Saedi Dezaki. Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran. Tel: +98-341-3224616; Fax: +98-341-3221676; email: saedi\_e@yahoo.com

*Nigella sativa* Linn. (Ranunculaceae), black seed commonly grows in the Southern Europe, North Africa, Middle East and Western Asia. *N. sativa* called "*Siah Daneh*" in Persian, has long been traditionally used as a natural medicine for treatment of many acute, as well as, chronic conditions include hypertension, diabetes, cough, bronchitis, headache, eczema, fever and dizziness in worldwide (10). Reviews have reported *N. sativa* as having antioxidant and neuroprotective effects in addition to many other therapeutic activities such as antitumor, immunopotential, anti-inflammatory, antiasthmatic and antimicrobial properties (10, 11). Moreover, studies have revealed antibacterial, antifungal, antiviral and antiparasitic effects of *N. sativa* and its derivatives (12-16). To our knowledge, there is no study on the effect of *N. sativa* on protoscoleces of hydatid cyst in an *in vitro* model. Therefore, this study was aimed to evaluate scolicidal effects of various extracts of *N. sativa* against protoscoleces of *E. granulosus* in an *in vitro* model.

## Materials and Methods

### Collection and identifying the plant materials

The seeds of *N. sativa* as a source of the active ingredients of this plant were collected from rural regions of Bam in September 2012, Kerman province, Iran. The plant was identified by a botanist of the Botany Department of Shahid Bahonar University, Kerman, Iran. Voucher specimen (KF575) has been deposited in the Herbarium of Department of Pharmacognosy, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.

### Preparation of various extracts

The dried seeds (100 g) of *N. sativa* were grinded and extracted by percolation method with methanol and water successively for 72 hr in room temperature. The extracts were passed through filter paper (Whatman No.3, Sigma, Germany) to remove plant debris. The extracts were finally concentrated in vacuum at 50°C using a rotary evaporator (Heidolph, Germany) and stored at -20°C, until use.

### Drug dilutions

To prepare the dilutions of the various extracts of *N. sativa*, 1 g of various extracts dissolved in 9.9 ml of normal saline. In addition, to enhance the dispersal of the extracts in normal saline, 0.1 ml of dimethyl sulphoxide (DMSO) was added to the test tube and serial dilution was subsequently made to obtain extracts at 5, 10, 25 and 50 mg/ml. The selection of dilutions of the various extracts of *N. sativa* was based on initial experiments, which showed that DMSO below 1.5% had no effect on the growth of protoscoleces. In this study, the concentration of DMSO in all of the various dilutions was 1.5% and below.

### Collection of protoscolices

The protoscoleces of *E. granulosus* were collected

from livers of naturally infected sheep and goats slaughtered at Kerman abattoir, Southeast of Iran. Infected livers transferred to the Parasitology Laboratory of Kerman University of Medical Sciences, Kerman, Iran. The fluid of hydatid cysts aspirated by a 50 ml syringe and aseptically transferred into the glass cylinders. After 30 min, the supernatant was discarded and the settled protoscoleces were collected and washed three times with PBS (pH 7.2). The concentration of protoscoleces was adjusted as  $2 \times 10^3$  protoscoleces in 0.9% NaCl solution with at least 90% viability rate. The viability of the protoscolices was confirmed by their flame cell motility and impermeability to 0.1% eosin solution under a light microscope.

### Scolicidal effects of *N. sativa*

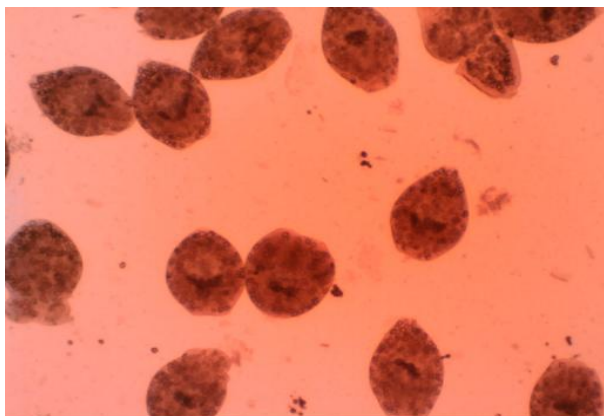
To determine the scolicidal activity of various extracts of *N. sativa* upon the protoscoleces of hydatid cysts, various concentrations of the extracts were used for 10, 20, 30 and 60 min. Initially, 0.5 ml of the protoscoleces ( $2 \times 10^3$ /ml) solution was placed in test tubes. In the next step, 0.5 ml of various concentrations of the extracts was added to each test tube, separately. The contents of the tubes were slowly mixed and then incubated at 37°C for 10, 20, 30 and 60 min. At the end of each incubation time, the upper phase was carefully removed so as not to interrupt the protoscoleces. Fifty  $\mu$ l 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 cover glass and examined under a light microscope. The percentages of dead protoscoleces were determined by counting 200 protoscoleces. Furthermore, normal saline and 20% hypertonic saline were used as negative and positive control, respectively.

### Viability test

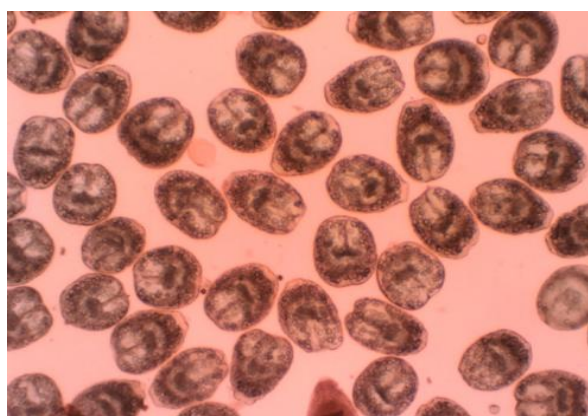
For evaluation of the viability of protoscoleces, we used eosin exclusion test. In this test, eosin solution with a concentration of 0.1% (1 g of eosin powder in 1000 ml distilled water) was used. A few minutes after exposure to the eosin stain, dead protoscolices absorbed eosin and colored red (Figure 1), whereas, alive protoscolices remained colorless and showed characteristic muscular movements and flame cell activity (Figure 2) (17).

### Statistical analysis

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



**Figure 1.** Dead protoscolece of hydatid cysts after exposure to *N.sativa* with 0.1% eosin



**Figure 2.** Live protoscolece of hydatid cysts after exposure with 0.1% eosin

**Results**

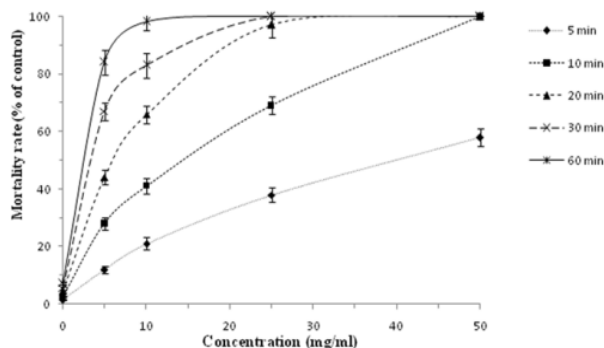
**Scolicidal effects upon protoscoleces**

As shown in Figures 3 and 4, various extracts of *N. sativa* at different concentrations following different exposure times had potent scolicidal effects against protoscoleces of hydatid cysts. The findings revealed that methanolic extract of *N. sativa* in comparison with the aqueous extract had strong scolicidal effects on protoscoleces of hydatid cysts. While, the mortality rate of protoscoleces in the negative control group was 7.1% after 60 min of exposure, and 100% scolicidal effect was observed with methanolic extract at the concentrations of 50 and 25 mg/ml after 10 and 30 min of incubation, respectively. The scolicidal effect of the methanolic extract of *N. sativa* at concentration of 10 mg/ml was 21.3, 41.6, 66.3, 83.6 and 98.3% after 5, 10, 20, 30 and 60 min of incubation, respectively. These values for the concentration of 5 mg/ml were 12, 28.3, 44.6, 67.3 and 86% respectively. In contrast, aqueous extract at the concentration of 50 and 25 mg/ml killed 100 and 96% of protoscoleces after 30 and 60 min of incubation, respectively. The scolicidal activity of the aqueous extract of *N. sativa* at concentration of 10 mg/ml was 10.3, 24, 37.6, 52.6 and 68.3% after 5, 10, 20, 30 and 60 min of

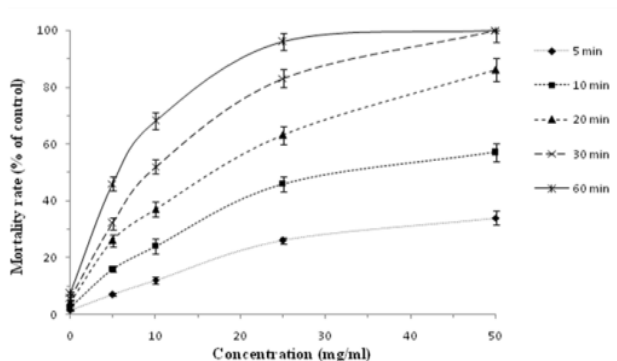
incubation, respectively. The above values for the concentration of 5 mg/ml were only 5.3, 16.6, 27.3, 32.3 and 46.6%, respectively. The scolicidal power of 20% hypertonic saline as the positive control was 100% after 10 min of application. Therefore, the scolicidal activity of methanolic and aqueous extract at the concentrations of 50 mg/ml after 10 and 30 min of incubation respectively was similar to the scolicidal activity of positive control. The scolicidal effect of various concentrations of the *N. sativa* extracts particularly methanolic extract was extremely significant ( $P<0.05$ ) compared to the control group (normal saline) at all exposure times. In the present study, all of experiments revealed dose-dependent and also time-dependent scolicidal effect of various extracts of *N. sativa* on the protoscoleces of hydatid cyst.

**Discussion**

The results of this study demonstrated that the various extracts of *N. sativa* seed, especially methanolic extract possess potent scolicidal activity and might be used as a natural scolicidal agent in CE surgery. Although, in the various studies the scolicidal effects of hypertonic saline (18), silver nitrate (19), cetrimide (20), ethyl alcohol (95%)



**Figure 3.** *In vitro* lethal effects of methanolic extract of *Nigella sativa* against protoscoleces of hydatid cyst at the various concentrations following various exposure times



**Figure 4.** *In vitro* lethal effects of aqueous extract of *Nigella sativa* against protoscoleces of hydatid cyst at the various concentrations following various exposure times

(21), H<sub>2</sub>O<sub>2</sub> and 10% povidone iodine (22), albendazole (23), chlorhexidine gluconate (24), honey (25) and some plant extracts (26-28) have been shown. However, it has been proven that existing scolicidal agents have dangerous adverse effects and their efficacy is controversial (5). Therefore, development of new scolicidal agents with no local or systemic side effects and higher efficacy is an urgent need for surgeons for surgical success of hydatid cysts. From the past centuries, plant-derived natural products have been widely used as a valuable source of antimicrobial agents in folk medicine (29). In the recent decades, development of synthetic antimicrobial drugs caused disaffection toward natural products as an attractive resource for antimicrobial agents (30). However, emergence of some adverse effects in the use of these synthetic drugs caused shift in situation and interest in field of ethnobotanical research (31). Results of the present study revealed that the methanolic extract of *N. sativa* at the concentration of 50 mg/ml after 10 min of incubation and its aqueous extract at the concentration of 50 mg/ml after 30 min of incubation was able to kill 100% of protoscolecocytes of *E. granulosus*. These results are comparable with the scolicidal effects of some existing scolicidal agents such as 20% hypertonic saline (15 min), 20% silver nitrate (20 min), 0.5 to 1% cetrimide (10 min), H<sub>2</sub>O<sub>2</sub> 3% (15 min) and 95% ethyl alcohol (15 min). So far, in the various studies, antibacterial, antifungal and antiviral effects of *N. sativa* seeds have been demonstrated (12, 14-16). In addition, antiparasitic effects of *N. sativa* extracts to treat some parasitic infections have been investigated. Agrawal *et al*, (1979) demonstrated significant antihelminthic activity of *N. sativa* seeds against some pathogenic cestode and nematode parasites compared to those of piperazine (12). In another study, Mahmoud *et al* (2002) showed that *N. sativa* oil can significantly reduce the number of *Schistosoma mansoni* worms in the liver and can decrease the total number of ova deposited in both the liver and the intestine (13). A study conducted by Nilforoushzadeh *et al* (2010) exhibited that combination of honey and *N. sativa* extract in patients with cutaneous leishmaniasis (CL) receiving glucantime is more effective to treat and improve the clinical signs as compared to honey alone (32). Fatahi Bafghi *et al* (2013) also reported that alcoholic extract of *N. sativa* possess good efficacy against cutaneous leishmaniasis in BALB/c mice (33). In addition, Okeola *et al* (2011) showed that *N. sativa* seeds had a significant antioxidant property and might be a good phytotherapeutic agent against *Plasmodium* infection in malaria (34). However, some studies indicated that *N. sativa* extracts had no significant effect to treat some parasitic infections such as balantidiasis in equines and *Cryptosporidium parvum* infection in calves (35, 36). The exact mechanism of the antiparasitic effects of *N. sativa* is not clear and further studies are required to clarify these mechanisms. Nevertheless, Suthar *et al* (2010) demonstrated that *N.*

*sativa* oil could inhibit DNA synthesis by inhibiting histone deacetylase (HDAC) enzyme interacting with the chromosomes (37). Cisplatin (a widely used chemotherapeutic drug) is toxic to the kidney. Administration of *N. sativa* can reduced the cisplatin-toxic side effects in rats including nephrotoxicity (38). Thymoquinone (TQ), the main constituent of the volatile oil of the seeds of *N. sativa*, has significant cytoprotective properties (39). Badary *et al* (1998) administered thymoquinone in the drinking water of mice at concentrations of 0.01, 0.02, and 0.03% for 90 days with no resulting mortality or signs of toxicity. The average daily intake of the compound was approximately 30, 60, or 90 mg/kg/day. They observed no changes of toxicological significance in body and organ weights, food and water intake, or urine and feces output. Tissue GSH, plasma concentrations of TP, urea, creatinine and triglycerides, and enzyme activities of ALT, LDH, and CPK were also not affected. Histological examination revealed no gross or microscopic tissue damage. The results indicate that the acute oral toxicity of TQ in mice is of a low order and it is generally well tolerated when given subchronically at doses previously shown to have cytoprotective activity (40). Thus, according to these evidences, we can conclude that the seed extract of *N. sativa* could be considered as a safe scolicidal agent.

## Conclusion

The findings of the present study demonstrated a potent scolicidal activity of various extracts of *N. sativa* especially for its methanolic extract against protoscolecocytes of hydatid cyst, so *N. sativa* can be considered as a natural source for the production of new scolicidal agent for use in hydatid cyst surgery. However, further studies will be required to confirm these findings by checking the other derivatives of *N. sativa* such as essential oil and its active components in the *in vitro* model. Moreover, more research is needed to evaluate mode of actions and *in vivo* effects of this plant extracts.

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The authors declare that there is no conflict of interest in this study.

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