

In vitro evaluation of *Nigella sativa* and *Punica granatum* effect on protoscolices of hydatid cysts

Avaliação *in vitro* do efeito de *Nigella sativa* e *Punica granatum* em protoescolices de cistos hidáticos

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Abstract

Cystic echinococcosis (CE) are commonly found in the liver and lungs of affected hosts. The treatment approach is usually surgical, or giving drugs in conjunction before surgery to kill protoscolices, to avoid anaphylactic shock from leakage of hydatid fluid into the peritoneum and to decrease opportunities for recurrences. The present study was to evaluate the *in vitro* scolical efficacy of hydroalcoholic extract of *Punica granatum* peel and *Nigella sativa*, on the protoscolices of CE that collected from the lungs of infected camels. Different concentrations of extracts with different exposure times were used and a viability assay was applied to measure the scolical effect. *N. sativa* showed its highest scolical efficacy at 100 mg/mL and 10 mg/mL concentrations after 30 and 60 min. *P. granatum* peel extract showed its maximum scolical efficacy at 100 mg/mL concentration after 120 min. All experiments of the current study revealed that the extracts of both *N. sativa* and *P. granatum* had a scolical effects on the protoscolices of camel hydatid cysts. It could be concluded that *N. sativa* extract is more potent than *P. granatum* peel extract regarding scolical effect, but the efficacies of both extracts were of moderate significant correlation to exposure time and concentrations.

Keywords: Hydatid cyst, *Echinococcus granulosus*, *Nigella sativa*, *Punica granatum*, protoscolices.

Resumo

Os cistos hidáticos (equinocose cística, CE) são comumente encontrados no fígado e nos pulmões dos hospedeiros afetados. A abordagem do tratamento geralmente é cirúrgica, e algumas drogas são administradas em conjunto antes da cirurgia para eliminar protoescolices e evitar choque anafilático devido ao vazamento de fluido hidático no peritônio e diminuir as oportunidades de recorrência. O presente estudo avaliou a eficácia *in vitro* do extrato hidroalcoólico de casca de *Punica granatum* e *Nigella sativa*, sobre os protoescolices de cistos hidáticos, que foram coletados dos pulmões de camelos infectados. Concentrações dos extratos com diferentes tempos de exposição foram utilizadas e um ensaio de viabilidade foi aplicado para medir o efeito escolical. A *N. sativa* apresentou sua maior eficácia escolical nas concentrações de 100 mg/mL e 10 mg/mL após 30 e 60 min. O extrato de casca de *P. granatum* mostrou sua máxima eficácia escolical na concentração de 100 mg/mL após 120 min. Todos os experimentos do presente estudo revelaram que os extratos de *N. sativa* e *P. granatum* tiveram efeito escolical dependente da dose e do tempo de exposição dos protoescolices dos cistos hidáticos de camelo. Pode-se concluir que o extrato de *N. sativa* é mais potente que o extrato de casca de *P. granatum* em relação ao efeito escolical, mas a eficácia de ambos os extratos foi de correlação significativa moderada com o tempo de exposição e as concentrações.

Palavras-chave: Cistos hidáticos, *Echinococcus granulosus*, *Nigella sativa*, *Punica granatum*, protoescolices.

Introduction

Hydatidosis is caused by ingestion of eggs of *Echinococcus* spp. It is considered to be one of the major zoonotic diseases affects both humans and herbivorous animals. Four different

Echinococcus species; *E. granulosus*, *E. multilocularis*, *E. vogeli*, and *E. oligarthrus* are well known to cause human and animal Echinococcosis (Hydatidosis) with different forms known as cystic echinococcosis (CE), alveolar echinococcosis, polycystic echinococcosis and unicystic echinococcosis; respectively. Other *Echinococcus* species seem to be rare in people or domesticated animals, but may affect wildlife. Cystic echinococcosis, the

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most common form of the disease in people and domesticated animals, is caused by *E. granulosus sensu lato* (*E. granulosus s. l.*) (THOMPSON, 2017).

CE is found mainly in the liver and lungs, and sometimes in the kidneys, spleen, bones, brain and other organs (AMMANN & ECKERT, 1996). Commonly, surgical removal and chemotherapy are the basic means of the treatment. However, this treatment approach may give rise to hydatid fluid leakage into the abdominal cavity, which leads to the possibility of occurrences of intraperitoneal cysts and anaphylactic shock. Recently, several plant extracts, such as garlic and olive leaf extracts have been evaluated as scolicidal agents instead of using chemotherapy in conjunction with surgery (MOAZENI & NAZER, 2010; SADJJADI et al., 2008; ZIBAEI et al., 2012).

Nigella sativa seeds have wide therapeutic effects and have been reported to have significant effects against many helminth infections. Mostly, the therapeutic benefits of *N. sativa* are due to its richness in Thymoquinone (TQ), which is a major active constituent. In studies focusing on antiparasitic effects (ISSA, 2003), it has been found that different extracts of *N. sativa*, as well as TQ, have a potent anthelmintic action against *Fasciola gigantica* (ULLAH et al., 2017) and *Schistosoma mansoni* (SHENAWY et al., 2008). The results from in-vitro tests on *N. sativa* seeds against miracidia, cercariae and adult worms have shown that they have strong effects against all stages of the parasite and an inhibitory effect on egg-laying among adult female worms. The antiparasitic activity of *N. sativa* seeds was high against cestodes in children (AKHTAR & RIFFAT, 1991).

N. sativa oil has anthelmintic effect in the rats infected with *Trichinella spiralis* infection and increased the production of antibodies generated during life cycle of this parasite (ABU EL EZZ, 2005). Recently, some studies have proven potent effect of *N. sativa* extracts on the protoscolices of hydatid cysts (MAHMOUDVAND et al., 2014a,b).

Punica granatum L. commonly known as pomegranate, is a fruit-bearing deciduous shrub or small tree that is native to Asia and belongs to the family Punicaceae (QNAIS et al., 2007). Different parts of the plant, such as bark, leaves and peels, have medicinal significance. *P. granatum* is widely used in many countries as a source of therapeutic agents against a variety of pathogenic microbes (ARUN & SINGH, 2012). *P. granatum* peel extract (100 mg/kg) administered for 10 consecutive days was found to stimulate immune systems and enhance cellular immunity in rabbits (ROSS et al., 2001). The constituents of pomegranate include highly hydrolysable tannins (punicalins and punicalagins), ellagic acid (a component of ellagitannins) and gallic acid (a component of gallotannins) (REDDY et al., 2007).

P. granatum has anticestodal and antinematodal activity (ABDELAZIZ et al., 2018; AL-MEGRIN, 2016; AMELIA et al., 2017), antitrepatodal activity against paramphistomes in sheep (VEERAKUMARI et al., 2014).

Therefore, the aims of the present study were to evaluate the scolicidal efficacy (i.e., the mortality rate of the protoscolices) of aqueous extract of *P. granatum* peel and *N. sativa* seed oil on the protoscolices of hydatid cysts, and to determine the exposure time and concentrations of the extracts providing scolicidal activity.

Materials and Methods

Ethical considerations

This study followed the institutional ethical and animal care guidelines. All methods were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Sohag University, Sadat City University and Kafrelsheikh University, Egypt.

Preparation of aqueous pomegranate extracts

Pomegranates were collected and identified as *P. granatum L.* by botanists of the Botany Department, Faculty of Science, Assiut University, based on taxonomy (HYAM & PANKHURST, 1995). Briefly, the aqueous extract of *P. granatum* was prepared as follows. The peel was cut into thin slices, dried and blended until smooth. Then, 25 g of this paste were added to 75 mL water and placed in an extraction bag. This was allowed to boil for 60 min and was then poured into a water bath, which was heated until the extract turned viscous. The extract was then put into an oven at 50-60 °C until dryness was reached. One gram of extract was dissolved in 1 mL of 100% dimethyl sulfoxide (DMSO), and the final concentration of each extract was adjusted to 1,000 mg/mL. Concentrations of 100, 10, and 1 mg/mL were made. The material was kindly donated by the Department of Botany, Faculty of Science, Assiut University (ABDELAZIZ et al., 2018).

Preparation of Nigella. sativa oil extract

N. sativa L. (Ranunculaceae) was identified based on taxonomy (HYAM & PANKHURST, 1995). Dried seeds were purchased from a local market. The seeds were crushed and cold-macerated in petroleum ether (40-60 °C) for three days. After evaporation of the petroleum ether, the extract was taken out and the oil was filtered. The final concentration of each extract was adjusted to 1,000 mg/mL and subsequently concentrations of 100, 10 and 1 mg/mL were made. The extracted oil was kept in screw-capped tubes in the dark at -20 °C until use (AHMAD et al., 2013; SALIH et al., 2009).

Controls used

Niclozamide® (Adwia Inc., Egypt) was used as a positive control at a concentration of 75 mg/mL, while DMSO was used as a negative control solution in a 1% (w/v) formulation (NAGULESWARAN et al., 2006; STETTLER et al., 2003).

Preparation of protoscolices:

Sixty hydatid cysts were collected from the lungs of infected camels that had been slaughtered at Sohag abattoir, Egypt, and were transferred to the laboratory of the Department of Parasitology, Faculty of Veterinary Medicine, Sohag University. Aseptically, the protoscolices were aspirated by 20 mL syring (plastic, single use and sterile) from the cysts washed three times in PBS (pH 7.2). The pooled protoscolices were kept in a concentration of 5×10^3 protoscolices/mL in a normal saline solution

(0.9% NaCl solution), and the viability of these protoscolices was more than 90%. This solution was kept for further use (MAHMOUDVAND et al., 2014a; SADJJADI et al., 2008).

Viability assay

The viability of the protoscolices was ascertained through staining with eosin. A solution containing 0.1 mg of eosin staining powder was added to 100 ml water to form a 0.1% (w/v) concentration stock solution. The viability of the protoscolices was evaluated microscopically by adding 10 µl of eosin stock solution to 10 µL of protoscolices for 15 min. The protoscolices were considered dead if they became stained, and alive if they remained unstained (SADJJADI et al., 2008).

Effect of *N. sativa* and *P. granatum* Extracts on Protoscolices

Three concentrations (100, 10 and 1 mg/mL) of each extract were used for 30, 60 and 120 min. 0.5 ml of each concentration was placed in 2 ml tubes, to which 0.5 ml of hydatid sand containing 2500 washed protoscolices was added. The contents of the tubes were gently mixed. The tubes were then incubated at 37 °C for 30, 60 and 120 min. At the end of each incubation time, the upper phase was carefully removed and 0.5 mL of Eosin stain (0.1% w/v) was then added to the remaining settled protoscolices and gently mixed. The percentages of dead/alive protoscolices were determined microscopically by counting in a hemocytometer slide. All experiments were performed in triplicate (SADJJADI et al., 2008).

Statistical analysis

All data were analyzed using of SPSS-IBM (version 20). The protoscolicidal activity was expressed as Mean ± SD (Standard Deviation). Statistical analysis was performed by Two-way ANOVA to compare all groups. Student's T tests (2 tailed) for paired samples and PAERSON test were used to analyze and determine the significance differences of correlation coefficients between mean values of the protoscolicidal efficacy of both extract in relation to concentrations and time exposure, *P values* of less than 0.05 were considered to be statistically significant.

Results

The scolical efficacy (mortality rate among hydatid cyst protoscolices) was recorded for both the extract and the controls at different concentration and different exposure times (Table 1). DMSO treated control did not influence the viability of the parasite during the experiment.

In the present study, *N. sativa* oil showed greater potential scolical efficacy on the protoscolices of hydatid cysts than did *P. granatum* peel extracts, when the extracts were applied at a concentration of 100 mg/mL for an exposure time of 120 min. The maximum mortality rate among the protoscolices (100%) was observed in *N. sativa* oil at 100 mg/mL concentration after 120 min of exposure, while the maximum scolical effect of *P. granatum* peel extract was 89.7% at 100 mg/mL concentration after the same time of exposure.

Statistically, the relationship was not so strong to indicate the association between scolical efficacy and both concentration and exposure time (Tables 2) as the Pearson's correlation coefficient was

Table 1. Scolical effects^a of various concentrations of *Nigella sativa* oil, and *Punica granatum* extract at different exposure time on protoscolices of camel hydatid cysts.

Incubation (exposure) time	Mortality rate% (scolical efficacy) (Mean ± SD)							
	Niclosamide (positive control)	DMSO (negative control)	<i>Nigella sativa</i> oil concentrations*			<i>Punica granatum</i> extract concentrations*		
			100 mg/mL	10 mg/mL	1 mg/ml	100 mg/mL	10 mg/mL	1 mg/ml
30 min	98.8 ± 2.1	0.0	43.9 ± 0.5	35.9 ± 2.4	25.8 ± 0.2	49.3 ± 2.4	38.9 ± 1.4	19.9 ± 1.6
60 min	100 ± 0.0	4.2 ± 2.4	89.9 ± 2.1**	69.9 ± 3.3	45.8 ± 3.6	72.4 ± 1.7**	44.9 ± 3.4	22.3 ± 2.4
120 min	100 ± 0.0	7.6 ± 2.1	100 ± 0.0**	88.4 ± 2.1	49.9 ± 1.1	89.7 ± 0.04**	59.9 ± 2.2	29.2 ± 1.9

All experiments were done in triplicates. SD = Standard Deviation. DMSO = dimethyl sulfoxide. *Concentrations (100 mg/mL, 10 mg/mL and 1 mg/mL) were prepared from the concentrated extract solutions of *Nigella sativa* and *Punica granatum* of 1000 mg/ml; **The values are statistically significant at *P*<0.05; ^aEfficacy indicates the mortality rate of the viable protoscolices due to application of different treatments.

Table 2. Significant correlation between the concentration, and exposure time on the scolical efficacy^a of *Nigella sativa* oil, and *Punica granatum* extract on protoscolices of camel hydatid cyst.

	<i>Nigella sativa</i> extract		<i>Punica granatum</i> extract		N
	Correlation Coefficient	<i>P</i> value	Correlation Coefficient	<i>P</i> value	
Efficacy ^a & exposure	.398	.006*	.286	.036*	27
Efficacy ^a & concentration	-.490-	.062*	-.298-	.022*	27

*Correlation is significant at the 0.05 level (2-tailed); ^aEfficacy indicates the mortality rate of the viable protoscolices due to application of different treatments. N = number of samples 27 (represent for each extract: 3 replicates × 3 concentrations × 3 three exposure intervals).

smaller than ± 0.5 . The scolical efficacy of both extracts became higher after 60 and 120 min than after 30 min of incubation, and this difference was highly significant ($P < 0.05$). At the same, the mean efficacy was proportionally increased with the extract concentrations that were applied, given that as the concentration increased, the scolical efficacy also increased significantly ($P > 0.01$).

Discussion

Surgery is currently the mostly successful and effective treatment approach for cases of hydatid cysts (BELGHITI et al., 1986; BRUNETTI et al., 2010; JUNGHANSS et al., 2008; PRASAD et al., 1991). Moreover, scolical drugs are injected into the hydatid concurrently with surgery preoperatively to kill the protoscolices and avoid occurrences of leakage of hydatid fluid into the peritoneum and fatal anaphylactic shock (BESIM et al., 1998). However, the absence of objective evidence about the efficacy and toxicity of these drugs may lead surgeons to miss out this procedure during the operation (CIFTCI et al., 2007; HOSSEINI et al., 2006; MOAZENI & LARKI, 2010).

Recently, several studies have been conducted with a view to finding natural scolical products in plant extracts that would provide lower toxicity than that of chemical drugs. In-vitro studies using different *Allium sativum* extracts showed effectiveness on hydatid cyst protoscolices and scolices (MOAZENI & LARKI, 2010; SADJJADI et al., 2008). Zibaei et al. (2012) tested the scolical effects of *Olea europaea* and *Satureja khuzestanica* extracts on the protoscolices of hydatid cysts. This information from previous studies has shown that *N. sativa* contains a large variety of substances that possess scolical activity. *N. sativa* was mentioned in several studies by Forouzanfar et al. (2014), in which its active constituent thymoquinone was investigated regarding its antimicrobial effects. Akhtar & Riffat (1985) screened the antiparasitic activity of *N. sativa* seeds in relation to cestode worms and stated that these seeds had high efficacy against adult cestode worms.

P. granatum has been widely evaluated in different countries as a source of therapeutic agents with anticestodal and antinematodal activity (ABDELAZIZ et al., 2018; AL-MEGRIN, 2016; AMELIA et al., 2017).

Recently, some studies have been conducted to investigate the efficacy of *N. sativa* extract against the protoscolices of hydatid cyst (MAHMOUDVAND et al., 2014a,b) where applied both methalonic and aqueous extracts of *N. sativa* at concentration of 50 mg/mL were lethal to all CE protoscolices after 10 and 30 min of incubation; respectively which agreed with our results.

The present study had confirmed the scolical efficacy of the oil of *N. sativa* seeds, and of the ethanolic extract of *P. granatum* peel at different concentrations with different exposure times, against the protoscolices of hydatid cysts from camels. The oil extract of *N. sativa* demonstrated greater efficiency than that of *P. granatum*, in that it killed all the protoscolices examined (100%) at a concentration of 100 mg/mL after 120 min of exposure, while *P. granatum* gave rise to a maximum death rate (89.66%) at 100 mg/mL after 120 min.

Statistically, there were moderate significant correlations between efficacy and concentration and between efficacy and exposure time (Tables 1 and 2). Thus, all of the experiments of the current study revealed dose-dependent and time-dependent scolical effects from extracts of both *N. sativa* and *P. granatum*, on the protoscolices of camel hydatid cysts.

Conclusion

N. sativa had greater scolical effect against hydatid cysts than *P. granatum*, and the efficacies of both extracts were of moderate significant correlations to exposure time and concentrations. These extracts have a useful effect and may be useful for the PAIR (Puncture-Aspiration-Injection-Re-Aspiration) method for treatment of hydatid disease because of their rapid and highly scolical activity. However, further studies are needed to clarify the mechanism of action of these plant extracts. Moreover, in-vivo studies are mandatory for evaluating the toxic effects of these plant extracts, and to study the possible adverse effects on both humans and domestic animals.

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