Original Article

Efficacy of *Nigella sativa* oil and its chitosan loaded nanoparticles on experimental cystic echinoncoccosis with immunological assessment

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ABSTRACT

Background: Cystic echinococcosis is a frequent worldwide disease. Surgery is the main policy of treatment. The benzimidazoles are the drugs of choice as adjuvant therapy besides surgery, but they have some drawbacks, so there is a pressing need to develop other safe and cheap drugs such as an herbal extract.

Objectives: To increase the efficacy of *Nigella sativa* oil (NSO) administered either as monotherapy or combined with albendazole, and using chitosan nanoparticles as a drug delivery system for both drugs.

Material and Methods: *E. granulosus* protoscoleces were obtained by aseptic puncture of hydatid cysts. Mice infected by intraperitoneal inoculation with nearly 1000 protoscoleces per mouse were subdivided into 7 groups: G2, infected non-treated; G3, treated with albendazole; G4, treated with NSO; G5, treated with NSO and albendazole; G6, treated with albendazole loaded on chitosan nanoparticles; G7, treated with NSO loaded on chitosan nanoparticles; G8, treated with albendazole and NSO loaded on chitosan nanoparticles. G1 included the non-infected, non-treated control mice. Serum was collected for biochemical and serological assay. NO was measured according to nitric oxide assay colorimetric kit. The systemic level of TNF-α and IL-5 was determined in the plasma of mice using commercial enzyme-linked immunosorbent assay kits.

Results: The results revealed that a significant increase of IL-5 occurred in all treated groups to reach the highest value in G8. There was a significant decrease of TNF-α in all treated groups in comparison to G1 and G2 (P<0.001). Mean level of nitric oxide (NO) was significantly increased in groups loaded on chitosan nanoparticles in comparison to G2.

Conclusion: *Nigella sativa* oil showed a promising effect on CE when used alone or in combination with albendazole and its effect was augmented when loaded on chitosan nanoparticles.

Keywords: Hydatid cyst, IL-5, *Nigella sativa* oil, NO level, TNF-α.

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INTRODUCTION

Hydatid cyst, a synonym of the larval stage of *Echinococcus granulosus* (*E. granulosus*) remains a major public health problem that is recurring in several countries[1]. In cystic echinococcosis (CE), surgical resection of the cyst represents the long-established treatment policy and is, in many instances, accompanied by chemotherapy. The risk of intraoperative leakage of the cyst contents (protoscoleces) and subsequently reappearance of CE and secondary cysts due to implantation of scolices in other sites during surgery, were the main problems of this form of treatment. Therefore, the use of effective scolicidal agents is needed, and is obligatory in nearly 10% of the postoperative care[2].

For inoperable cases, treatment with the benzimidazoles and the heterocyclic pyrazine isoquinoline derivative, Praziquantel, remain the only option[3]. Benzimidazoles have various adverse reactions avoidable by constant monitoring of drug serum levels which may not be available in all countries; in addition to the possible teratogenic effect of Mebendazole and Albendazole[4].

Seeds of *Nigella sativa* (*N. sativa*) owe their therapeutic benefits to Thymoquinone (TQ), which is a major active component. Issa[5] found that different extracts of *N. sativa*, in addition to TQ, have an effective anthelmintic action against *Fasciola gigantica*[6]. *Trichinella spiralis* infected rats treated with NSO showed increased antibody production during this parasite's life cycle[7]; and it displayed anti schistosomal effects in murine schistosomiasis *mansoni*[8]. Some studies proved the strong scolicidal effect of *N. sativa* extracts on the protoscoleces of hydatid cysts *in vitro*[9,10]. In our study, we attempted to evaluate NSO extract on hydatid cyst development in experimental mice; and to increase its efficacy we

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used chitosan nanoparticles as a drug delivery system. The evaluation was done by assessment of biochemical and serological parameters of mice serum.

MATERIAL AND METHODS

This experimental case-control study was conducted at Theodor Bilharz Research Institute (TBRI), Giza, Egypt during the period from March 2017 to July 2017.

Materials: NSO was obtained as soft gelatin capsules (450 mg) (Pharco Pharmaceutical, Alexandria, Egypt). The capsule was opened, and oil was dissolved in 2 ml distilled water[11] to obtain a dose of 1.14 gm/kg body weight[12]. Albendazole (Bendax) suspension form (100 mg/5 ml) was purchased from Sigma medical company. Albendazole was given in a dose of 200 mg/kg body weight[13].

Albendazole and NSO loaded on chitosan nanoparticles: The synthesis procedures were carried out according to the ionotropic gelation technique described by Ohya *et al*.[14]. Albendazole loaded on chitosan nanoparticles was given in a dose of 200 mg/ kg body weight and NSO loaded on chitosan nanoparticles was given in a dose of 1.14 gm/kg body weight. Sterile phosphate buffer saline (PBS) and gentamicin were purchased from Sigma Chemical Company, St. Louis, MO, USA, and GIBCO-BRL Life Technology, NY, USA; respectively.

Parasites collection and preparation: *E. granulosus* protoscoleces were obtained by aseptic puncture of cysts[15] obtained from the lung of sheep at El-Warrak slaughter house in Cairo, Egypt. Protoscoleces were prepared according to Amri and Touil-Boukoffa[16] by aseptic aspiration of the hydatid fluid from fertile sheep pulmonary cysts. The fluid was centrifuged and the sediment containing protoscoleces was washed with PBS (pH 7.2–7.4), supplemented with 30 mg/mL gentamicin.

Viability assay: Protoscoleces viability was estimated microscopically by staining with 0.1% eosin prepared by adding 10 µl of eosin stock solution to 10 µL of protoscoleces for 15 min. The unstained protoscoleces showing flame cells movement were considered as viable, while the red-stained and shrunken protoscoleces were considered dead[17]. The percentages of dead/ alive protoscoleces were determined microscopically by counting in a hemocytometer slide[17] to ensure the required number of live protoscoleces for infection.

Mice infection: Each mouse was infected by intraperitoneal inoculation of nearly 1000 viable protoscoleces suspended in 500 µl of sterile PBS[18].

Experimental animals and design: A total of 92 laboratory-bred male Swiss albino pathogen-free adult male Swiss albino mice, six to eight weeks old weighing 25-30 gm, inbred at Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Giza, Egypt were used throughout this study. Animals were housed in TBRI, Experimental Animal Unit. The mice were maintained under standard laboratory care (25° C, with a relative humidity of 40–60 %, normal diet of commercial pellets and potable water. Mice used in this study were classified into 8 groups (G) as shown in table (1).

Drugs were administered orally every other day to mice via tube feeding starting 2 months post protoscoleces inoculation[19] and continued for more 2 months[15]. At the end of the study (4 months post-infection), mice were anesthetized with chloroform, and blood was collected by cardiac puncture[15] in heparinized tubes. Centrifugation of blood at 4000 x g for 15 min was performed to separate plasma, which was placed in plastic tubes and kept at -80°C[20] for serological and biochemical studies.

Assessment of drugs efficacy

Estimation of NO: This was based on the enzymatic conversion of nitrate to nitrite by nitrate reductase, followed by colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is based on two steps of diazotization reaction in which acidified NO2 - produces a nitrosating agent that reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540-570 nm according to nitric oxide assay kit (colorimetric)[21] at www.abcam.com (Version 7b Last Updated 9 August 2017).

Measurement of systemic TNF-α level: The systemic level of TNF-α was detected in the plasma of mice using commercial enzyme-linked immunosorbent assay kits, according to the manufacturer's instructions[15] (USA & Canada | R&D Systems, Inc.).

Measurement of systemic IL-5 level: The systemic level of IL-5 was determined in the plasma of mice using sandwich enzyme immunoassay kits, according to the manufacturer's instructions[22] (USA & Canada, R&D Systems, Inc.).

Statistical analysis: In the statistical comparison between the different groups, the significance of difference was tested using ANOVA test (*F* value) to compare mean of more than two groups of quantitative data using LSD as post hoc test for intergroup comparison. *P* value <0.05 was considered statistically significant in all analyses.

Ethical consideration: The study protocol was approved by the Scientific Research Ethical Committee in Faculty of Medicine, Benha University, Egypt, with ethical approval (No: 10549/2016). All the animal

experiments were performed according to the rules of the Scientific Research Ethical Committee, Faculty of Medicine Benha University. As well, animal handling and all procedures were done in agreement with the TBRI ethical guidelines.

RESULTS

Treatment efficacy was 47% in G3 (treated with ABZ) and 57% in G5 (treated with ABZ+ NSO). The lowest treatment efficacy (34%) was obtained in G4 (treated with NSO). The highest treatment efficacy (100%) was obtained in G6 (treated with ABZ loaded on chitosan nanoparticles), G7 (treated with NSO loaded on chitosan nanoparticles) and G8 (treated with ABZ and NSO loaded on chitosan nanoparticles) (Table 1).

Results of NO Level: After 4 months of infection NO level measurement revealed a significant elevation ($P1<0.01$) in groups 2-8 in comparison with G1 (non-infected non treated). NO level in G3 (treated with ABZ) and G5 (treated with ABZ + NSO) was elevated in comparison with G2 (infected non treated) but was not statistically significant ($P2>0.05$). On the other hand, NO level in G4 (treated with NSO) was decreased in comparison with G2 (infected non treated), but was not statistically significant ($P2>0.05$). Furthermore, there was statistically significant elevation ($P2<0.01$) of NO level in G6 (treated with ABZ loaded on chitosan nanoparticles), G7 (treated with NSO loaded on chitosan nanoparticles) and G8 (treated with ABZ and NSO loaded on chitosan nanoparticles) when compared with the NO level in G2 (infected non treated). Besides, the highest level of NO was obtained in G8 (Table 2).

Table 1. Comparison between different tested groups according to treatment efficacy.

G	Groups	Treatment efficacy
1	Non infected non treated (n=10)	-
2	Infected non treated (n=8)	-
3	ABZ (n=12)	47%
4	NSO (n=10)	34%
5	ABZ + NSO (n=12)	57%
6	ABZ loaded on chitosan nanoparticles (n=14)	**100%**
7	NSO loaded on chitosan nanoparticles (n=12)	**100%**
8	ABZ and NSO loaded on chitosan nanoparticles (n=14)	**100%**

ABZ: Albendazole, **NSO:** *Nigella sativa* oil.

Table 2. Comparison between different tested groups according to nitric oxide level (NO).

	Groups	NO level (µmol/l) Mean ± SD	Statistical analysis
1	Non infected non treated (n=10)	104.80 ± 7.55	
2	Infected non treated (n=8)	243.00 ± 13.31	
3	ABZ (n=12)	248.00 ± 2.76	
4	NSO (n=10)	237.00 ± 3.53	***F* = 2021.5**
5	ABZ + NSO (n=12)	243.17 ± 3.69	***P* < 0.001***
6	ABZ loaded on chitosan nanoparticles (n=14)	336.86 ± 6.14	
7	NSO loaded on chitosan nanoparticles (n=12)	337.83 ± 10.81	
8	ABZ and NSO loaded on chitosan nanoparticles (n=14)	433.57 ± 8.46	

	G1	G2	G3	G4	G5	G6	G7	G8
P1		< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
P2			< 0.144	< 0.092	< 0.961	< 0.001*	< 0.001*	< 0.001*
P3				< 0.001*	< 0.114	< 0.001*	< 0.001*	< 0.001*
P4					0.056	< 0.001*	< 0.001*	< 0.001*
P5						< 0.001*	< 0.001*	< 0.001*
P6							0.74	< 0.001*
P7								< 0.001*

ABZ: Albendazole, **NSO:** *Nigella sativa* oil, **SD:** Standard deviation. ***P***: Significance between different tested groups. ***P1:*** Significance between G1 and other groups; ***P2:*** Significance between G2 and other groups; ***P3:*** Significance between G3 and other groups; ***P4:*** Significance between G4 and other groups; ***P5:*** Significance between G5 and other groups; ***P6:*** Significance between G6 and other groups; ***P7:*** Significance between G7 and G8. ***F:*** F value for ANOVA test. * Significant difference.

Results of TNF-α Level: There was significant reduction (*P*1<0.01) in TNF-α level in G2-8 in comparison with G1 (noninfected non treated). Also, treatment in G3-8 revealed significant reduction (*P*2 < 0.01) in TNF- α expression in comparison with G2 (infected non treated). Furthermore, the highest reduction in TNF- α expression was in G8 (treated with ABZ loaded on chitosan nanoparticles and NSO loaded on chitosan nanoparticles) (Table 3).

Results of IL-5 Level: There was significant elevation (*P*1<0.01) in IL-5 level in G2-8 in comparison with G1 (non-infected non treated). Also, treatment in G3-8 revealed significant elevation (*P*2< 0.01) in IL-5 expression in comparison with G2 (infected non treated). Furthermore, the highest elevation in IL-5 expression was in G8 (treated with ABZ loaded on chitosan nanoparticles and NSO loaded on chitosan nanoparticles) (Table 4).

Table 3. Comparison between different tested groups according to TNF- α level.

	Groups	TNF- α (Pg/ml) Mean ± SD	Statistical analysis
1	Non infected non treated (n=10)	428.80 ± 5.41	
2	Infected non treated (n=8)	404.75 ± 3.58	
3	ABZ (n=12)	384.33 ± 3.11	
4	NSO (n=10)	370.00 ± 4.57	***F* = 8552.5**
5	ABZ + NSO (n=12)	328.83 ± 2.79	***P* < 0.001***
6	ABZ loaded on chitosan nanoparticles (n=14)	206.71 ± 3.58	
7	NSO loaded on chitosan nanoparticles (n=12)	210.00 ± 2.89	
8	ABZ and NSO loaded on chitosan nanoparticles (n=14)	173.57 ± 3.80	

	G1	G2	G3	G4	G5	G6	G7	G8
P1		< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
P2			< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
P3				< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
P4					< 0.001*	< 0.001*	< 0.001*	< 0.001*
P5						< 0.001*	< 0.001*	< 0.001*
P6							0.029*	< 0.001*
P7								< 0.001*

ABZ: Albendazole, **NSO:** *Nigella sativa* oil, **SD:** Standard deviation. ***P***: Significance between different tested groups. ***P1:*** Significance between G1 and other groups; ***P2:*** Significance between G2 and other groups; ***P3:*** Significance between G3 and other groups; ***P4:*** Significance between G4 and other groups; ***P5:*** Significance between G5 and other groups; ***P6:*** Significance between G6 and other groups; ***P7:*** Significance between G7 and G8. ***F:*** F value for ANOVA test. * Significant difference.

Table 4. Comparison between different tested groups according to IL-5.

	Groups	IL-5 (Pg /ml) Mean ± SD	Statistical analysis
1	Non infected non treated (n=10)	10.13 ± 0.189	
2	Infected non treated (n=8)	26.25 ± 0.240	
3	ABZ (n=12)	29.37 ± 0.310	
4	NSO (n=10)	31.64 ± 0.400	***F* = 6194.2**
5	ABZ + NSO (n=12)	35.42 ± 0.330	***P* < 0.001***
6	ABZ loaded on chitosan nanoparticles (n=14)	36.84 ± 0.520	
7	NSO loaded on chitosan nanoparticles (n=12)	42.43 ± 1.000	
8	ABZ and NSO loaded on chitosan nanoparticles (n=14)	47.87 ± 0.260	

	G1	G2	G3	G4	G5	G6	G7	G8
P1		< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
P2			< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
P3				< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
P4					< 0.001*	< 0.001*	< 0.001*	< 0.001*
P5						< 0.001*	< 0.001*	< 0.001*
P6							< 0.001*	< 0.001*
P7								< 0.001*

ABZ: Albendazole, **NSO:** *Nigella sativa* oil, **SD:** Standard deviation. ***P***: Significance between different tested groups. ***P1:*** Significance between G1 and other groups; ***P2:*** Significance between G2 and other groups; ***P3:*** Significance between G3 and other groups; ***P4:*** Significance between G4 and other groups; ***P5:*** Significance between G5 and other groups; ***P6:*** Significance between G6 and other groups; ***P7:*** Significance between G7 and G8. ***F:*** F value for ANOVA test. * Significant difference.

DISCUSSION

Medical treatment in CE is effective against small cysts less than 4 cm in diameter with thin walls. It is indicated in patients who are at high risk for surgery, in patients with multiple peritoneal cysts, cysts in multiple organs, bone cysts, cysts in the brain and to prevent secondary echinococcosis after spillage during surgery[23]. However, McManus *et al*.[24] reported that there is no ideal agent that is both effective and safe. It is essential, therefore, to identify other efficient protoscolecidal agents, that would overcome the severe drawbacks of the artificial pharmaceuticals[25]. In this context, herbal extracts have been recognized as safe substitute agents[26]. Diversity of associated cellular immune responses determine the outcome of the disease. Motivation and action of Th1 cytokines produce IFN-γ and IL-12 that lead to protection of host and destruction of the cyst; whereas, the activity of Th2 cytokines response induces secretion of ILs 4, 5, 10 and 13 which protect the parasite from protective immune reaction and cause the evolution of the disease[27]. The early Th1 response is gradually replaced by a Th2 response, dominated by IL-5 and IL-10[28]. In our study, there was a significant increase in IL-5 in G2 (infected not treated) in comparison to the healthy group, due to stimulation of Th2 as explained by Rahdar *et al*.[27]. Our results confirm the view of Zhang *et al*.[29] who reported that large quantities of IL-5 were produced by hydatid disease patients. Also, this increase endorses Juyi *et al*.[30] report on the increase of serum levels of IL-5 in primary and secondary hydatid cyst infections. The increase of IL-5 in ABZ treated G3 agrees with Dvoroznáková *et al*.[22] 22 who recorded a high level of IL-5 in serum of mice treated with free ABZ after infection with *E. multilocularis*. Our recorded increase in G4 treated with NSO contradicts with the report of Koshak *et al*.[31] who stated that in an *in vitro*/*in vivo* model of bronchial asthma NSO extract and/or its active constituents (including TQ, nigellone, and alpha-hederin) showed anti-histaminic, anti-eosinophilic, anti-leukotrienes, anti-immunoglobulin effects, and reduced pro inflammatory cytokines (ILs 2, 4, 5, 6, 12, and 13). The greater cytokine response recorded in our study may be attributed to immunological boosting by antigens liberated from dead parasites[32].

Concerning the serum value of TNF-α in our study, there was a significant reduction in G2 (infected non-treated) and all treated groups. Moreover, the most significant reduction was G8 (treated with ABZ and NOS loaded on chitosan nanoparticles). It appears that this reduction is strongly linked to the inhibition of cyst development and reflects the good efficacy of NSO, ABZ, their combination, and their loaded chitosan nanoparticles on experimentally hydatid cyst infected mice. In addition, this recorded TNF-α level could be related to the second immune response phase of experimental echinococcosis which happens after one month when the hydatid cyst is totally formed and it shows more Th2 response, with reduction in TNF- α[14]. The reduction of TNF- α in G2 also agrees with Mondragón-De-La-Peña *et al*.[33] who reported that infection with hydatid down-regulates TNF- α as a method of parasitic evasion from the immune system. In G3 treated with ABZ the recorded reduction of TNF- α was also reported by Zhang and Jing[34] who likewise found that treatment with ABZ decreased TNF- α in *E. multilocularis* infected mice.

The anti-inflammatory properties of NSO lead to a systemic decrease in TNF-α level in G4 (treated with NSO), G5 (treated with ABZ and NSO), G7 (NSO loaded on chitosan nanoparticles) and G8 (treated by ABZ and NSO loaded on chitosan nanoparticles). Our recorded decrease of TNF-α conforms with the report by Umar *et al*.[35] who found that in an animal model of rheumatoid arthritis, oral administration of TQ, the most abundant constituent of NSO, resulted in significantly reduced levels of TNF-α. Also, it was reported that TQ leads to a reduction of TNF-α in experimentally induced arthritis in rats[36], and reduced pancreatic ductal adenocarcinoma cell synthesis of TNF-α[37]. Furthermore, the secretion of TNF- α was blocked by *N. sativa* ethanolic extract[38], and aqueous extract[39]. As with Viktorov and Yurkiv[40] who found that ABZ inhibited secretion of TNF-α in a primary culture of rat Kupffer cells, we recorded significant reduction of the serum TNF-α level in G3 (treated with ABZ). This result also conforms with Zhang and Jing[33] who reported that ABZ decreased TNF-α in mice infected with alveolar echinococcosis. However, our results differ from those of Labsi *et al*.[41] who declared an increase in expression of TNF-α in hepatocytes and Kupffer cells in liver sections of mice from ABZ treated group. Also in contrast to our results for treatment by NSO in G4, Sheir *et al*.[42] reported that NSO increased TNF-α in mice infected with *S. mansoni*.

The elevated NO level in G2 (infected non treated) may be attributed to the aggressive cellular immune response against the protoscoleces of *E. granulosus* which is responsible in 90% of initial parasite death[43]. This elevation in NO level agreed with Labsi *et al*.[15] who showed raised NO levels in different tissues in CE infection. It was reported that high amounts of NO produced by activated peritoneal macrophages inhibited the growth and spread of *E. granulosus* cysts in mice; also, it has been shown in another *in vitro* study that NO produced by mouse-derived active macrophages deactivates protoscoleces of *E. multilocularis*[44]. These results are significant in terms of exhibiting the anti-parasitic effects of NO over the physiological amounts produced by iNOS [inducible nitric oxide synthase] as a result of the immune response of the host. There was an elevation in serum level in groups treated with ABZ only (G3) and when combined with NSO (G5) but was not significant in comparison to untreated G2. This elevation was also noted by

Locatelli *et al*.[45]. The authors reported that ABZ is a strong reactive oxygen species generator, provoking oxidative stress by elevating thiobarbituric acid-reactive substances and depleting antioxidant enzymes in a time-dependent manner. In G4 treated with NSO, the increase in serum level is in parallel with that reported by Kanter *et al*.[46] and Ashraf *et al*.[47] who indicated that NSO has antioxidative stress effect. In groups treated with drugs loaded on chitosan nanoparticles there was a significant increase in the serum level. This elevation might be related to the stimulatory effect of chitosan on macrophages NO production[48]. In support of this finding is the report by Pattani *et al*.[49] who confirmed that chitosan nanoparticles increase NO production *in vivo*.

In our study, we concluded that the efficacy of ABZ and NSO increases by loading on chitosan nanoparticles. This was verified by their 100% efficacy and their modifying effect on NO, TNF- α and IL-5 levels. Further studies are needed to evaluate the effect of chitosan nanoparticles alone on hydatid cyst *in vitro* and *in vivo*.

Authors contribution: HSM Ali and SM Kishik conceived the study, and wrote the manuscript; IM Nagati, IR Ali, NSM Aly, MM Fawzy shared in the study design. IR Ali performed the experiments and analyzed the data; all authors revised the manuscript.

Conflict of interest: The authors declare that they have no conflict of interest.

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