

Effects of *Nigella Sativa* and Albendazole alone and in combination in *Toxocara Canis* infected mice

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Abstract

Objective: *Nigella sativa* extract has been used in the Middle East as a traditional medicine for several complaints. We aimed to evaluate the biochemical, histopathological and hematologic changes in *Toxocara canis*-infected mice after treatment with *Nigella sativa* extract, albendazole or a combination of both.

Methods: This comparative study was conducted between June and July 2008 at the Harran University Saniturla Turkey. Sixty healthy adult BALB/c male mice were randomly divided into six groups (D0-D5). Mice in groups D1-D5 received 500 embryonated *T. canis* eggs via esophageal tube. Groups D0 and D1 served as a non-infected sham group and an infected control group, respectively. Groups D2 and D3 received 100 and 200 mg/kg *N. sativa* extract (NSE), respectively. Group D4 received 100 mg/kg albendazole. Group D5 received the combination dose (100+100 mg/kg NSE+albendazole).

Results: Treatment with *N. sativa* of both doses or the combination dose of *N. sativa* and albendazole reduced the degree of inflammation and necrosis, lead to a reduction in the percentage of eosinophils and decreased the elevated liver enzyme levels.

Conclusion: These results indicate that *N. sativa* has a potent effect in protection against organ damage induced by *T. canis* infection.

Keywords: Toxocariasis, Mice, Serum liver enzymes, *Nigella sativa*, Albendazole, Eosinophils (JPMA 61:866; 2011).

Introduction

Herbal medicines are used for treatment of many clinical diseases, but little is known about their mode of action. In recent years, *Nigella sativa* L (Family *Ranunculaceae*) has been used in the Middle East as a traditional medicine for many complaints.¹ The majority of research has focused on its antifungal, antibacterial, antihelminthic, antioxidant and hepatoprotective effects.²⁻⁶ Toxocariasis is a zoonotic disease caused by ingestion of embryonated eggs of the roundworm *Toxocara canis* of which the adult form inhabits the small intestine of infected dogs. About 100% of puppies have ascarid infections during the first 3 weeks of life and 50% at 4 months. During this period, puppies excrete billions of ascarid eggs.⁷ Mammals, including rabbits, rats, mice and monkeys, serve as paratenic hosts. Humans are infected accidentally by ingestion of the embryonated eggs, resulting in visceral larva migrans (VLM) or ocular larva migrans (OLM).⁸ Experimentally, mice infection results from ingestion of the second stage of the larvae.⁹ The second stage larvae hatch in the stomach of the mouse and invade whole small intestine within 2 hours.¹⁰ The larvae reach the liver and lungs within the first 2-3 days, then the muscular system and brain 7 days after infection. The

larvae produce antigen, enzymes and cuticle components which eventually cause tissue damage, necrosis and inflammatory reactions that lead to an increase in eosinophil count.¹¹

No specific drug for the treatment of VLM and OLM has been investigated in well-controlled studies.³ However, a number of other studies concerning the effects of several antihelminthic drugs on human or experimental animal models have been conducted, using different therapeutic procedures. The drugs available for the treatment of toxocariasis presenting as VLM is limited to albendazole, tiabendazole, tinidazole and diethylcarbamazine.^{12,13}

Considering the side effects of these drugs we focused on traditional medicine *Nigella sativa* with the aim to evaluate the biochemical, histopathological and haematologic changes in *T. canis*-infected mice after treatment with *N. sativa*, albendazole or a combination of both.

Methods

Animals Design:

A total of 60 healthy adult male BALB/c mice weighing 32 ± 2 g were maintained in accordance with the

National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were obtained from Istanbul University, Faculty of Science, Department of Biology. Animals were housed individually in polypropylene cages under environmentally controlled conditions (room temperature $22 \pm 2^\circ\text{C}$, humidity 60% and 12/12 light/dark cycle) with free access to standard rodent food (Ankara pellet feed factory, Ankara, Turkey) and water for duration of the study. The Institutional Animal Ethics Committee of Harran University approved animal protocols in advance before the experiment begun. The mice were acclimatized to the laboratory conditions 2 weeks prior to the experiment.

Egg Collection and Preparation:

T. canis worms were collected from naturally infected puppies (taken from a dog shelter in Sanliurfa). The embryonated eggs were removed by dissection of the uteri of the worms according to a method described by Bowman et al.¹⁴ The eggs were diluted with 10% gum arabic suspension to achieve 500 embryonated eggs in 1 ml of suspension.

Plant Material and Drug Preparation:

N. sativa seeds were purchased from a local herbal shop in Sanliurfa Turkey. The seeds were cleaned, washed, dried and grounded into fine powder using an electrical mill. The powder was macerated in methanol for 24 hours and then evaporated in a rotary evaporator and dried by lyophilization. Two different doses of extract were prepared (100 and 200 mg/kg bodyweight) and suspended in 10% gum arabic (Merck, Chemical Company, Darmstadt, Germany). Albendazole was purchased from Sigma (St. Louis, MO, USA) and dissolved in 1% dimethyl sulfoxide (DMSO), prepared as 100 mg/kg bodyweight and suspended in 10% gum arabic.

Experimental Conditions:

A total of 60 healthy adult BALB/c male mice were randomly divided into six groups (n=10). Groups D0 and D1 received orally 1 ml of vehicle (10% gum arabic suspension) and served as a non-infected sham group and an infected control group, respectively. Mice in groups D1-D5 received 500 embryonated *T. canis* eggs via esophageal tube. The treatment was given at 24 hours post-infection in groups D2-D5. Groups D2 and D3 received 100 and 200 mg/kg bodyweight *N. sativa* extract, respectively. Group D4 received 100 mg/kg bodyweight albendazole. Group D5 received the combination dose (100 mg/kg bodyweight *N. sativa* extract+100 mg/kg bodyweight albendazole). The treatment was given for 7 consecutive days to all groups.

At the end of the experiment, all animals were killed by cervical dislocation, and their liver, lungs, brain and muscles were removed. Each liver was weighed on an analytical balance and divided into two portions; one portion was used for histopathological examination.

Worm Recovery and Larvae Count:

The left lung, one portion of liver and muscles were finely minced. The larvae were recovered according to the method described by Parsons and Grieve¹⁵ with little modification. The tissues were weighed and then digested individually in pepsin-HCl solution (pH 1-2, 500 IU pepsin/g tissue) (Sigma-Aldrich, Hyderabad, India) and incubated for 3 hours at 37°C with periodic agitation. Cellular debris was removed after centrifugation. Subsequently, the sedimentation of particles in liquid were poured into a centrifuge tube and stored in a refrigerator at 4°C for 24 hours. The sediment liquid was centrifuged for 2 minutes at 1500 rpm and 2 ml of the sediment was collected and vortexed. Larvae in the sediment were counted under a light stereomicroscope. The brain of each mouse was removed, minced into small pieces, macerated carefully between two slides to ensure a thin layer, and the larvae were counted under a light microscope.

Histological Examination:

Organs, including the liver and lungs, were removed, washed with physiologic solution and fixed separately in neutral formaldehyde overnight, dehydrated in graded ethanol, cleared in xylene, and processed in ParaplastR (Fluka Chemika AG Buchs, Switzerland) at 58°C . 4-5 μm -thick sections were prepared and stained with haematoxylin and eosin (H&E). Three slides were prepared for each tissue and randomly selected for examination by a blinded pathologist. The gross necrotic lesions were histologically identified as large, subcapsular eosinophilic areas of necrotic hepatocytes. Inflammatory foci were defined as discrete clusters of 10 to 80 individual, small, nucleated cells visible throughout the liver. The site of inflammation was taken into consideration only if well-defined lesions and inflammatory foci were present. The degree of inflammation or damage was scored and categorized into four levels: normal (0), no inflammation; mild (1+), <3 foci/area; moderate (2+), 3-6 foci/area and severe (3+), >6 foci/area. Three slides were evaluated for each animal. The mean of all the individual scores was calculated and used for analysis.

Serum Enzyme Analysis:

Blood was collected from all animals under ether anaesthesia, and by heart puncturing according to the procedure approved by the Institutional Animal Care and

Use Committee (IACUC). The blood samples were allowed to clot at room temperature. Serum was separated by centrifugation at 3000 rpm for 10-15 minutes and utilized for the measurement of biochemical parameters. Assay kits for the measurement of serum enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were purchased from Randox Laboratories Ltd (Ardmore, Crumlin, UK). Measurements were performed with a UV spectrophotometer in accordance with the manufacturer's instructions.

Statistical Analysis:

Inter-group comparisons were conducted using the Kruskal-Wallis test followed by Dunn's multiple comparison of means test. All results are presented as mean±SD. P < 0.05 was considered statistically significant.

Results

The total larval burden in the infected control group D1 was 61. Burden was 28 in the examined lungs (45.9%), 18 in liver (29.5%), 12 in the carcass (19.7%) and 3 in brain (4.9%). Treatment with *N. sativa* alone reduced the total larval burden to 42 and 37 in groups D2 and D3, which indicated a percentage reduction of 31.1% and 39.3%, respectively, compared with the control group D1 (P<0.05). On the other hand, in group D4 treatment with albendazole alone reduced larval burden to 18, which indicated a percentage reduction of 70.5%. Treatment with the combination of albendazole and *N. sativa* in group D5 reduced the larval burden to 8, which indicated a percentage reduction of 87%. Significant difference was observed between the treated groups (D4-D5) and the control infected group (D1) (p<0.01).

Percentage of eosinophils in control group was six-fold higher than in sham group. Treatment with *N. sativa* at doses of 100 and 200 mg/kg reduced the eosinophil percentage from 18% to 7.1% and 7.0%, respectively, while treatment with albendazole alone decreased the percentage of eosinophils to 7.8%. Treatment with the combination of

Table: Activity of ALT, AST, and ALP in study groups on day 7 post-infection with *T. canis*.

Groups	AST U/L*	ALT U/L*	ALP U/L*
D0 Sham (non-infected)	86±6.50	59.4±10.40	963.6±44.89
D1 Control (infected)	203.2±24.24	104.7±5.93	1171.5±84.84
D2 <i>N. sativa</i> 100 mg/kg	107.8±10.04	85.5±14.63	1065.6±105.95
D3 <i>N. sativa</i> 200 mg/kg	98.3±12.51	80.3±12.54	1020.8±123.91
D4 Albendazole 100 mg/kg	92.1±10.69	71.4±9.87	1007.7±117.11
D5 <i>N. sativa</i> 100 mg/kg+Albendazole	89.6±6.25	63.2±11.82	897.2±87.57

*Results were presented as mean ± SD.

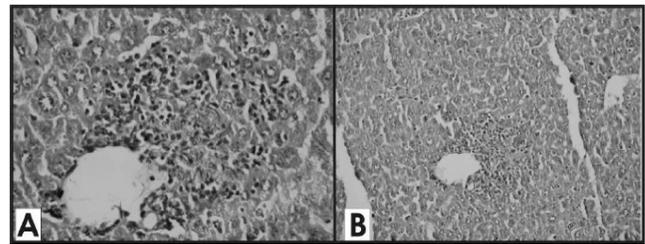


Figure-1: Histopathological changes on the photomicrograph of liver section obtained from control group, sacrificed on day 7 after infection. The site of inflammation and lesions are characterized by polymorphic nucleated granulocytes and multinucleated giant cells with extensive eosinophilic infiltration (A). Section obtained from liver after treatment with *N. sativa* extract reveals fewer inflammatory cells and polymorphic nucleated granulocytes (B) (H&E, 200 X).

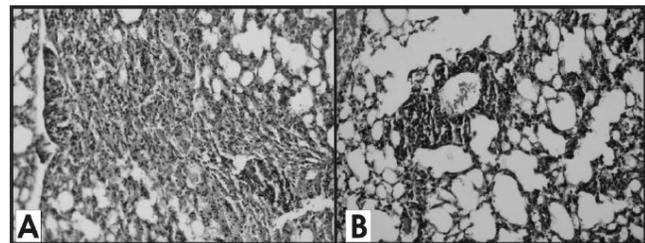


Figure-2: The site of damage surrounded by inflammatory lymphocytes, and severe bleeding and extensive eosinophilic cells on the photomicrograph of lung section obtained from infected control group sacrificed on day 7 after infection (A). After *N. sativa* treatment alveoli are clearer with less bleeding and fewer sites of inflammation (B) (H&E, 200 X).

albendazole and *N. sativa* reduced the percentage of eosinophils to 6.6%.

The histopathological changes observed in the lung and liver sections of the control group compared with the sham group are shown in Figures 1A and B and Figures 2A and B. Histopathological examination revealed inflammatory cell infiltration in the liver (Figure 1A). Severe inflammation and bleeding associated with massive infiltration of inflammatory cells into the interstitial and alveolar tissues was observed in lung parenchyma (Figure 2A). Determination of pathogenicity assessed by inflammatory as well as histopathological changes revealed that *T. canis* larvae were able to induce varying degrees of histopathological changes in the host organs. Mild (1+) and, moderate (2+) to severe (3+) histopathological changes were observed in the brain, lung and liver of infected mice. Significant difference between the infected control (D1) group and non-infected sham (D0) group were observed with respect to histological changes in brain, lung and liver (p<0.05). Treatment with *N. sativa* alone decreased the degree of damage and the degree of inflammation in the brain, liver and lung, in a dose-dependent manner. There was significant difference between the *N. sativa* treated groups (D2-D3) and the control infected group (D1) regarding the histological changes in brain, lung and liver (p<0.05).

Treatment with Albendazole alone also reduced the

degree of damage and necrosis compared with *T. canis*-infected mice tissues. Significant difference between the Albendazole treated group (D4) and infected control (D1) group were observed with respect to histological changes in brain, lung and liver ($p < 0.05$). Moreover, treatment with the combination dose of *N. sativa* and albendazole reduced the degree of necrosis and inflammation and resulted in a reduction in the percentage of eosinophils (Figures 1 and 2). There was significant difference between the combination dose of *N. sativa* and albendazole treated group (D5) and the control infected group (D1) regarding the histological changes in brain, lung and liver ($p < 0.05$).

The results with regard to the effects on serum levels of liver enzymes including ALT, AST and ALP are presented in Table. It was found that infection with *T. canis* in D1-D5 groups elevated the liver enzymes of ALT, AST and ALP when compared with the non-infected group (D0). A significant increase in serum levels of ALT and AST was found in control infected group (D1) compared with the non-infected group ($p < 0.01$). No significant difference was found between the infected control and non-infected groups regarding serum level of ALP ($p > 0.05$).

Treatment with *N. sativa* with 100mg/Kg and 200mg/Kg were observed to reduce serum liver enzyme levels as compared to infected control group and albendazole alone treated group. Treatment with combination dose markedly prevented the elevation of liver enzymes. Liver enzyme levels of ALT and AST in the treated groups (D2-D5) were significantly lower compared with the infected control group ($p < 0.05$, $p < 0.05$, $p < 0.05$, $p < 0.01$, respectively) (Table). However, no significant difference was found between the treated groups and infected control group regarding serum level of ALP ($p > 0.05$). Also, there was no significant difference between the treated groups and the non-infected sham group regarding serum levels of ALT, AST and ALP ($p > 0.05$).

Discussion

T. canis is considered to be primarily responsible for VLM and OLM. The route of larval migration in the experimental mouse can be divided into an early hepatopulmonary or visceral phase during the first week after infection, followed by a brain, heart and kidney phase.¹⁰ Hepatic damage resulting from *T. canis* larvae infection is of major importance among liver diseases worldwide.¹⁵ Previous work by Lai et al.¹⁶ demonstrated that larval antigens are primarily responsible for host inflammatory reactions associated with the infection. The larvae in the liver, elicit T-lymphocyte responses leading to macrophage activation, which seem to play a role in

phagocytic removal of unwanted debris at the site of inflammation.^{16,17} The severity of the disease is determined by the extent of inflammation and amount of larval debris deposited in the tissues.¹⁷ In our study, elevated serum levels of AST, ALT and ALP were observed in all infected groups (D1-D5). This seems to be associated with damage of hepatic tissue and impaired cell membrane permeability or is due to deposition of *T. canis* larvae in tissues. The present study showed reduction of larval burden in treatment groups by both doses of *N. sativa* (D2, D3) was 31.1% and 39.3%, respectively compared with infected control group (D1). Also, the treatment reduced the degree of damage and inflammation in the liver and the percentage of eosinophils, in comparison to the *T. canis*-infected control group. The changes observed in the percentage of circulating eosinophils indicated that eosinophil count is an important index of parasite infection and parasitic incidence. Albendazole has been shown to be effective in ocular and visceral larval infections.^{18,19} The proven efficacy of albendazole in such infections is associated with its nematocidal activity, though the individual immunity has an important role in the response to *T. canis* infection. Moreover, the hexane extract of *C. ambrosioides* which is a herbal medicine has been reported to have an anthelmintic activity in vitro by reducing the inflammatory reaction produced by the infection of *T. canis* larvae in vivo.²⁰

The enhanced liver functions as reflected by a significant decrease in elevated serum levels of the enzymes ALT and AST after treatment with albendazole alone or combination treatment of albendazole and *N. Sativa* in the present study supports previous studies reporting the efficacy of albendazole in *T. canis* infections.

On the other hand, the effect of *N. sativa* extract can be partially attributed to modulation of immune response via a variety of inflammatory mediators, of which expression has been shown to be increased in response to *T. canis* infection.²¹ Many researchers have studied the effects of *N. sativa* or its derived thymoquinone on the immune system, and found that the extract stimulates the production of some certain cytokines, and leads to alterations in the levels of these mediators.^{22,23} It has been stated recently that *Nigella sativa* and thymoquinone have a therapeutic and protective effect by decreasing oxidative stress in streptozotocin-induced diabetic rats.³ Moreover, *N. sativa* has been reported to have a stimulatory effect on human lymphocytes and polymorphonuclear leukocyte phagocytic activity.²⁴ It has also been suggested that fixed oil of *N. sativa* inhibits the generation of eicosanoids which play an important role in inflammation.²⁵ We suggest that the decrease in the degree of inflammation observed in both liver and lungs of mice treated with *N. sativa* might be associated with this anti-eicosanoid activity.

In the present study, larval recovery levels in larval toxocariasis were higher in liver and lungs and lower in muscles and brain on day 7 after infection, which is in line with a study by Lai et al.¹⁶ This result may be attributable to proteolytic activity of enzymes produced by larvae that leads to moderate to severe histopathological changes in liver and lungs, and mild changes in the muscles and brain.

Conclusion

Our data indicate that the treatment with *N. sativa* at both doses or the combination dose of *N. sativa* and albendazole reduced the degree of inflammation and necrosis in liver, lung and brain induced by *T. canis* infection, lead to a reduction in the percentage of eosinophils, and markedly decreased the elevated liver enzyme levels.

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