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Parasitological, pathological and immunological effects of *Nigella sativa* oil in experimentally *Toxocara canis* infected mice

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Abstract. *Nigella sativa* (NS) is a natural plant seed which is used as traditional medicine for treatment of many diseases in the Middle East countries. This study aimed to evaluate commercially prepared NS oil (NSO) in treatment of experimental murine toxocariasis by parasitological, histopathological and immunological parameters compared to albendazole (ALZ). BALB/c mice (n=50) were divided into 5 groups (10 mice, each group). They are divided according to the following groups: group I (normal control mice, NC), group II (infected untreated control mice, IU), group III (infected mice and treated with ALZ), group IV (infected mice treated with NSO) and group V (infected mice treated with ALZ+NSO), all the infected mice were given orally 500 embryonated eggs of *Toxocara* (*T.*) *canis*. In comparison with the mean total number of *T. canis* larvae of IU mice (group II), NSO (group IV) achieved a significant reduction in total *T. canis* larvae (62.2%, P<0.001), ALZ (group III) revealed 66% reduction (P<0.001), while mice treated by ALZ+NSO showed the highest larval reduction percentage with 83.7 (P<0.001). NSO (group IV) reduced the pathological lesions and inflammation caused by *T. canis* infection, whereas mice treated with ALZ+NSO (group V) showed nearly normal histological picture. All the treated mice groups, including that treated with NSO, showed enhancement of immune response demonstrated by increasing specific anti-*Toxocara* IgG, and IgG1 levels, in addition to increment of IL-4 and IFN-γ serum levels. In conclusion, NSO decreased *Toxocara* larvae numbers and reduced the pathological lesions, beside production of immune response characterized by enhanced both Th1 and Th2 responses, so it is recommended to use NSO in treatment of *Toxocara* infection.

INTRODUCTION

Human toxocariasis is a zoonotic disease caused mainly by larvae of *T. canis*, ascarids of dogs, and *T. cati*, ascarids of cats (Lee *et al.*, 2014). Eggs of *Toxocara* shed with feces of the definitive hosts and contaminate the environment, where humans accidentally ingest embryonated eggs of the parasite (Blaszkowska *et al.*, 2015), also, humans may become infected on consuming raw or undercooked meat from other paratenic hosts containing the second-stage larvae of *T. canis* (Noh *et al.*, 2012). Inside intestinal tract of human, *Toxocara* larvae can pass the intestinal walls to migrate to various tissues, where they settle down but cannot develop into adult worms (Clinton *et al.*, 2010).

Children are at higher risk for catching *Toxocara* infection than adults because of their playing habits in soil contaminated with the parasite's mature eggs (Gawor *et al.*, 2014). The disease represents an important public health problem especially in the tropics and sub-tropical regions, where its prevalence rate may reach 40% (Kuenzli *et al.*, 2015). In Egypt, prevalence rates of human toxocariasis records 7.7–12% (El-Shazly *et al.*, 2009; Elshazly *et al.*, 2011).

Persistence of stray dogs and cats is the main cause of continued occurrence of human infection (Kociecki *et al*., 2016).

Albendazole (ALZ) is benzimidazole carbamate compound which is commonly used in treatment of *T. canis* infection (Horton, 2000). It mediates degenerative changes in tegument and intestine of the treated *Toxocara* worms (Lacey, 1990), and it down-regulates genes that express the cell architecture, actin and actin-binding proteins (Lu *et al*., 2014). However, ALZ has a reduced solubility rate, poor intestinal absorption and low bioavailability (Mwambete *et al*., 2004), moreover, it causes many side-effects, such as granulomatous hepatitis (Zuluaga *et al*., 2013) and pancytopenia (Acikgoz *et al*., 2014). So, it is necessary to develop more effective and safe anti-*Toxocara* alternatives drugs (Othman, 2012).

Nigella sativa, *Ranunculaceae* family, is a flowering plant that grows in Middle East, Western Asia and Eastern Europe, and its seed is known as Al-Habba Al-Barakah in Arabic and black seed in English. The mixtures of the seed include many active compounds, like thymohydroquinone (TQ), dithymoquinone, thymol, carotene, vanillic acid and alkaloids. In the recent years, several studies have revealed that NS and its extracts possess many useful pharmacological effects (Tembhurne *et al*., 2014).

The aim of this study was to evaluate the parasitological, pathological and immunological changes in mice infected with *T. canis* after treatment with the commercially prepared NSO compared to ALZ.

MATERIALS AND METHODS

Experimental mice

Fifty male BALB/c albino mice with twelve weeks old and weigh 25±2 grams were used in this study. The mice were kept in the animal house, Faculty of Medicine, Menoufia University. They were bred under environmentally controlled conditions and fed with a standard pellet diet and water *ad libitum*. Handling and treatment of the mice were conducted according to the internationally valid guidelines and ethical conditions (Hau & Van Hoosier, 2003).

Parasite and infection

Stool samples were collected from suspected *T. canis* infected adult stray dogs. The eggs were isolated by straining the samples through a sieve having pores diameter of 0.5 mm. The isolated eggs were washed with saline several times then kept in 0.5% formol saline solution for 4-8 weeks in petri-dishes at 28-30°C under proper aeration to induce embryonation. After maturation, the eggs were transferred and kept at 4°C until use. Each mouse was inoculated orally with about 500 viable *T. canis* eggs, which were counted by hemocytometer (Fan *et al*., 2003).

Drugs and doses

a) ALZ (Alzental®, EPICO, Egypt) was given orally at dose of 100 mg/kg, diluted in distilled water, once daily for 5 consecutive days, starting on the 1st day p.i. (Yarsan *et al*., 2003).

b) NSO (Baraka capsules®, MEPACO, Egypt) was administrated orally to the infected mice in a dose of 100 mg/kg, the capsules were opened to extract the oil, for 7 successive days, beginning on the 1st day p.i. (Musa *et al*., 2011).

Experimental design

The mice were divided into five groups (10 mice, each) as the followings:

Group I: normal control mice (NC).
Group II: infected untreated control mice (IU).
Group III: infected mice treated with ALZ.
Group IV: infected mice treated with NSO.
Group V: infected mice treated with ALZ+NSO.

At the 45th day p.i., all mice were anaesthetized with ether and euthanized by heart puncture. The blood samples were left till clotted, then centrifuged at 3000 rpm for 5 minutes (min) and the obtained sera were stored at -20°C until use (Fan *et al*.,

2004; Nassef *et al.*, 2014). Also, tissue specimens from the killed mice were taken for parasitological and histopathological studies.

T. canis larval recovery

Small portions from liver, lung, heart and kidney (0.5 g each) were finely minced. The minced tissues were put in 50 ml pepsin-HCl digestion solution at 37°C for 24 hours (h). Then, ethanol was added to the mixture which left for further 24 h. After that, the mixture was centrifuged for 2 min at 1500 rpm and 2 ml of the sediment was taken and thoroughly mixed. Larvae were detected in the sediment by examining under a light microscope (Olympus, Japan) (Wang & Luo, 1998). For counting larvae in brain tissue, small pieces of tissue were carefully squashed between two slides, without using solution of digestion, and examined under the microscope (Chung *et al.*, 2004).

The reduction percentage of the treatment was calculated as following:

Reduction % = (C – T) /C x 100, where C is the mean number of larvae recovered from IU mice (group II) and T is the mean number of larvae recovered from the treated mice groups.

Histopathology of tissues of mice infected with _T. canis_

Specimens of liver, lung, heart, kidney and brain from each mouse included in this study were processed in the Department of Pathology, Faculty of medicine, Menoufia University. They were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin stain (H& E) following the standard procedure.

Preparation of _T. canis_ somatic antigen (TSA)

T. canis somatic antigen (TSA) was prepared according to Sahu *et al.* (2013). Briefly, the adult worms were homogenized on ice after adding protease inhibitors and then exposed to sonicator for 15 cycles at 8-10µ peak to peak with an interval of 30 seconds. The produced mixture was centrifuged at 15000 rpm for 45 min, the supernatant was collected and its protein content was estimated with Coomassie Plus™ (Bradford) assay Kite (Pierce USA). The prepared antigen was aliquoted and stored at -20°C until use.

Immunological parameters

Specific antibody responses to TSA

Detection of *T. canis* specific IgG class and IgG1 and IgG2a subclasses were detected in all mice sera by indirect enzyme linked immunosorbent assay (ELISA) using TSA. Briefly, ELISA microtiter plates were coated overnight at 4°C with 100 µl of the prepared antigen diluted in carbonated buffer, pH 9.4. In the next day, the plates were blocked with 250 µl/well of bovine serum albumin (BSA) diluted in tris-buffer solution (BSA/TBS) for 2 h at room temperature, then they were washed four times with 250 µl of 0.05% phosphate buffer solution/Tween20 (PBS/T20). The serum samples were diluted in the blocking buffer (1:100), loaded into the plates (100 µl/well) and the plates were incubated at room temperature for another 1 h followed by further four washings. After that, 100 µl/well of alkaline-phosphatase (AP)-conjugated anti-mouse IgG, IgG1 or IgG2a (Southern biotech) diluted in PBS/T (1:5000 for IgG and 1:2000 for each IgG1 and IgG2a) were added and left for 90 min. After washing for four times, 100 µl/well of p-nitrophenyl phosphate substrate was added till the appearance of yellow color. Light absorbance was read at 405 nm wave length using ELISA reader (Bio-Rad).

Determination of serum levels of cytokines

Cytokines IL-4 and IFN-γ were measured in the sera of mice by using sandwich ELISA with anti-cytokine antibodies following the manufacturer's instructions (Phar Mingen, San Diego, USA). Recombinant cytokines were used as the standards. Briefly, 96-well flat-bottom plates were coated with 100 µl/well of the capture antibodies diluted in carbonate/bicarbonate buffer, pH 9.6. After incubation overnight at 4°C, the plates were washed three times with washing buffer,

PBS/T, and the nonspecific binding sites were blocked using blocking buffer, PBS/T/1% low-fat dry milk powder. After washing three times, the serum samples or the recombinant cytokines were added in a volume of 100 µl/well. The cytokine standards were serially diluted in the blocking buffer. The plates were incubated for 3 h at room temperature with gentle shaking. The plates were washed and then the appropriate biotinylated anti-cytokine detection antibody was added in a volume of 100 µl/well. After incubation for 1 h with gentle shaking at room temperature, the plates were washed three times. After that, the streptavidin-alkaline phosphatase conjugate was added and the plates were left for 30 min. Finally, the plates were washed for five times and the reaction was developed with para-nitrophenyl phosphate (Sigma), then absorbance at 405 nm was measured using microplate ELISA reader. The cytokine concentration was calculated from standard curve using Microplate Manager Software (Bio-Rad).

Analytic statistics

The data were processed on an IBM-PC compatible computer using SPSS version 18 (SPSS Inc., Chicago, Illinois, USA). Comparison relative to the normal control or infected untreated control groups was performed using unpaired Student's t-test. For more than two groups, Tukey HSD Post-hoc Test was used to find means that are significantly different from each other. Data were expressed as mean±standard deviation (SD). The data were considered significant if P value ≤ 0.05.

RESULTS

Recovery of *T. canis* larvae

At end of the experiment, brain was the most parasitized organ revealing 36.3±7.5 larvae in group II (IU), followed by kidney (7±2.1), then heart (3.2±2.4), while total larval burden was 46.5±7.3. Liver and lung did not show any *T. canis* larvae in all the studied mice groups.

In group III, ALZ reduced significantly larvae number by 68.8% (P<0.05), 95.7% (P<0.001) and 60.1% (P<0.001) in heart, kidney and brain, respectively, also, group IV treated with NSO revealed significant reduction of larvae number by 56.3% (P<0.05), 92.8% (P<0.001) and 55.6% (P<0.001) in heart, kidney and brain, respectively. Regarding group V, ALZ+NSO caused 100% reduction in larvae number in both heart and kidney, whereas they reduced larvae counts by 79.6% (P<0.001) in the brain tissues. Mice of group V (ALZ+NSO) showed the highest reduction percentage of total *T. canis* larval burden by 83.7%, while group III (ALZ) revealed 66% reduction which was slightly better than that of group IV (NSO) (62.2%), all these reduction rates were of significant value (P<0.001) (Table 1).

Histopathology results

Herein, there were remarkable pathological lesions in the examined tissues of group II (IU), but larvae were revealed only in heart and brain tissues on histological examination (Fig. 1-5).

Table 1. Effects of NSO alone or in combination on the of number of *T. canis* larvae in tissues of the mice groups

	Heart		Kidney		Brain		Total burden	
	Larval count (mean±SD)	Larval reduction (%)	Larval count (mean±SD)	Larval reduction (%)	Larval count (mean±SD)	Larval reduction (%)	Larval count (mean±SD)	Larval reduction (%)
NC	–	–	–	–	–	–	–	–
IU	3.2±2.4	–	7±2.1	–	36.3±7.5	–	46.5±7.8	–
ALZ	1±1.15*	68.8	0.3±0.8**	95.7	14.5±3.3***	60.1	15.8±3.6***	66
NSO	1.4±1.8*	56.3	0.5±0.9***	92.8	16.1±3.7***	55.6	17.6±3.3***	62.2
ALZ+NSO	0***	100	0***	100	7.4±4.2***	79.6	7.6+4.2***	83.7

Data are expressed as mean±SD. *P<0.05, **P<0.01 and ***P<0.001 are of significant difference in comparison to IU mice (group II).

Liver tissues of group II (IU) had moderate periportal inflammatory cellular infiltrates consisting mainly of lymphocytes and histiocytes. There was congestion of portal blood vessels. There were parenchymal lymphocytic aggregates, hydropic hepatocytic degeneration and focal fatty changes. In group III (ALZ) and group IV (NSO), liver tissues revealed mild periportal inflammatory infiltrates consisted mainly of eosinophils and lymphocytes with minimal portal vascular congestion, whereas that of group V (ALZ+NSO) had minimal periportal inflammatory infiltrates (Fig. 1).

There were intra-alveolar necrotic tissues, many collapsed alveoli and degenerated alveolar septae in lung tissues of group II (IU), also there were heavy cellular infiltrations formed of polymorphonuclear leucocytes, eosinophils and macrophages together with inflammatory exuadtes. Lung tissue of mice belonging to group III (ALZ) and IV (NSO) showed nearly similar pathological improvement presenting with mild peribronchial inflammatory infiltrates and lesser alveolar septae degeneration. Mice of group V (ALZ+NSO) showed the mildest inflammatory response (Fig. 2).

Regarding heart tissue of group II (IU), there were mild inflammatory infiltrates with degeneration of the cardiac muscles and some encapsulated *T. canis* larvae were found. Heart tissues of mice of both groups III (ALZ) and IV (NSO) showed less inflammatory infiltrates than that of group II, while that of group V (ALZ+NSO) revealed very few inflammatory cells (Fig. 3).

Kidney tissues of group II (IU) showed moderate interstitial inflammatory cellular infiltration consisting mainly of lymphocytes and histiocytes with hydropic degeneration of the renal tubules. Blood vessels showed dilatation and congestion. Focal areas of hemorrhage have been seen. Mice of group III (ALZ) had mild inflammatory infiltrates formed mainly of lymphocytes and histiocytes, mild hydropic degeneration affecting renal tubules and some blood

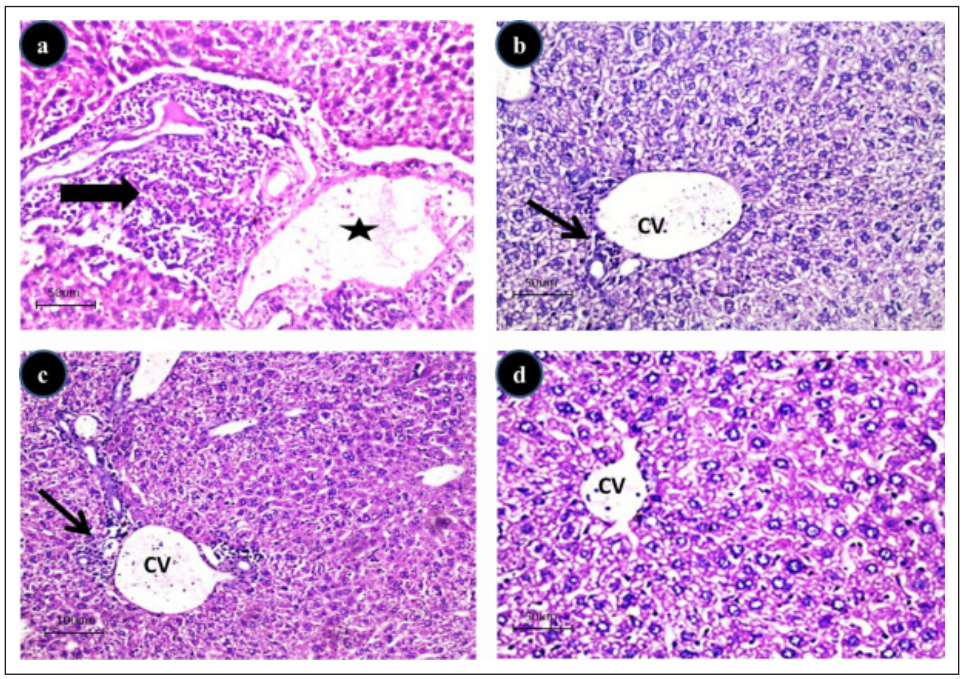

Figure 1. Liver tissue; (a) group II (IU) shows moderate periportal inflammatory cellular infiltrates (thick black arrow) with vascular congestion (star) and focal fatty changes (H&E, scale bar=50µm), (b) group III (ALZ) (H&E, scale bar=50µm) and (c) group IV (NSO) reveal mild inflammatory infiltrates (thin black arrows) with minimal vascular congestion (H&E, scale bar=100µm) and (d) group V (ALZ+NSO) has minimal inflammatory infiltrates (H&E, scale bar=50µm). CV refers to central vein.

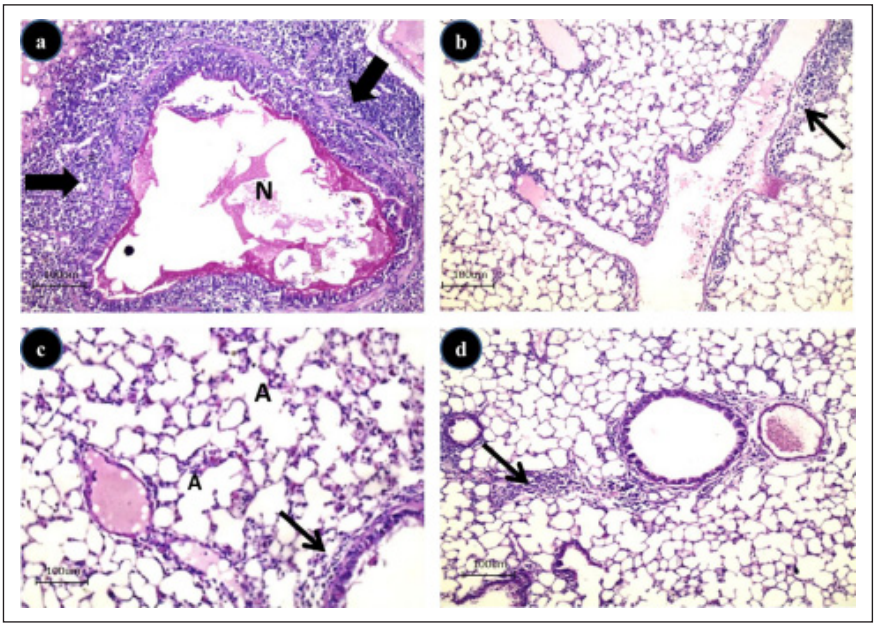

Figure 2. Lung tissue; (a) group II (IU) shows heavy inflammatory cellular infiltration (thick black arrow), intra-alveolar necrotic tissue (N), collapsed alveoli and degenerated alveolar septae with inflammatory exudates (H&E, scale bar=100µm), (b) group III (ALZ) (H&E, scale bar=100µm) and (c) group IV(NSO) have mild peribronchial infiltrate (thin black arrows) with little degeneration of alveolar septae (A) (H&E, scale bar=100µm) and (d) group V (ALZ+NSO) reveals only minimal inflammatory response (thin black arrow) (H&E, scale bar=100µm).

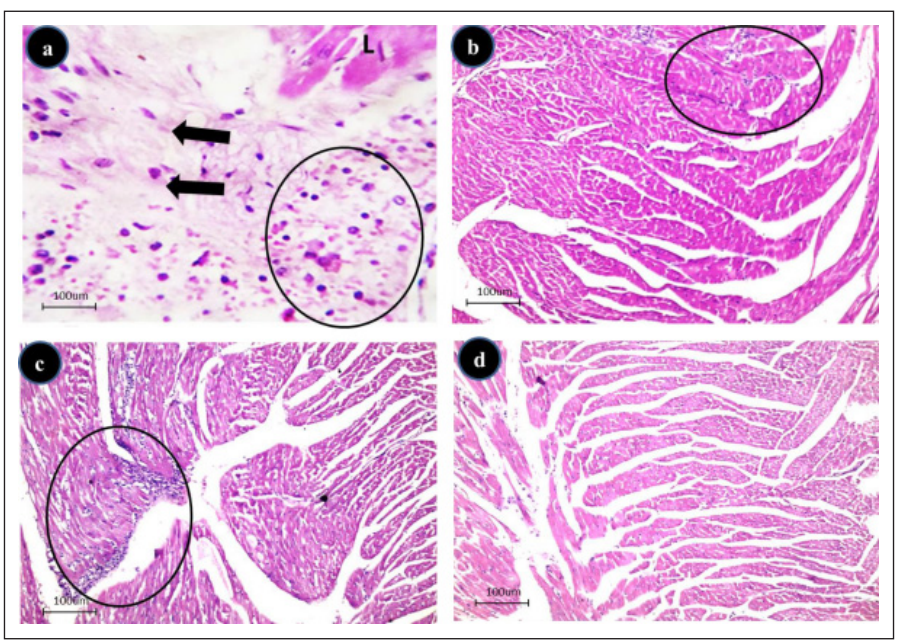

Figure 3. Heart tissue; (a) group II (IU) shows mild inflammatory infiltrates (circle) with degeneration of the cardiac muscles (thick black arrows) and presence of an encapsulated *T. canis* larva (L) (H&E, scale bar=100µm), (b) group III (ALZ) (H&E, scale bar=100µm) and (c) group IV (NSO) (H&E, scale bar=100µm) have less inflammatory infiltrates (circles) than that of group II and (d) group V (ALZ+NSO) reveals very few inflammatory cells (H&E, scale bar=100µm).

vessels revealed dilatation and congestion. Kidney tissues of group IV (NSO) revealed milder inflammatory infiltrate than that of mice treated with ALZ, whereas group V (ALZ+NSO) showed the least inflammatory infiltrate among the mice groups, with presence of wider areas of normal histology (Fig. 4).

Brain tissue of group II (IU) showed many larvae with mild inflammatory infiltrates predominately lymphocytes. All treated mice groups had decreasing in the infiltrates with lowering the number of larvae and this was more pronounced in group V (ALZ+NSO), where brain tissues of mice were of nearly normal histologic appearance and did not reveal any *Toxocara* larvae (Fig. 5).

Specific antibody responses to TSA

Group II (IU) and all the treated mice groups (ALZ, NSO and ALZ+NSO) showed a significant ($P<0.001$) increase in the specific *T. canis* IgG, tested by ELISA using TSA antigen in comparison with group I (NC). Also, all the treated mice groups showed higher IgG level ($P<0.05$) when compared to group II (IU), but there were non-significant difference among IgG levels of the treated mice groups (Fig. 6).

There were significant ($P<0.001$) increment in both specific *T. canis* IgG1 and IgG2a subclasses of group II (IU) and all the treated mice groups in comparison to group I (NC). In all treated mice groups, IgG1 increased significantly ($P<0.001$) when compared to group II (IU), but its level did not vary significantly among the treated mice groups, however, IgG2a level of all treated mice groups did not show significant ($P>0.05$) difference comparing to group II or among the treated mice groups themselves. IgG1 was significantly higher than IgG2a in group

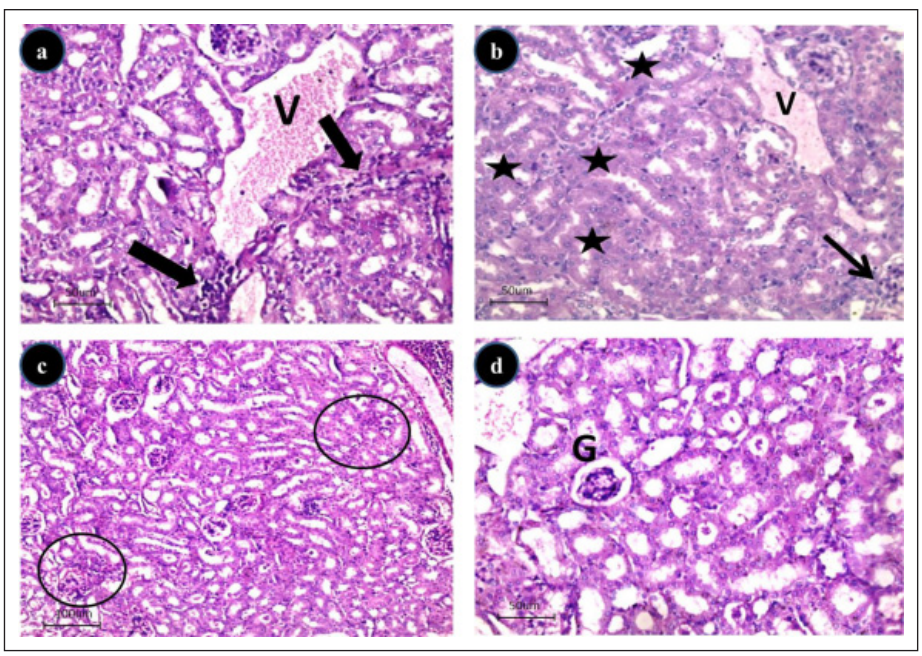

Figure 4. Kidney tissue; (a) group II (IU) shows interstitial chronic inflammatory cellular infiltration (thick black arrows), hydropic degeneration of the renal tubules and the blood vessels show congestion (V) (H&E, scale bar=50µm), (b) group III (ALZ) reveals mild inflammatory infiltrate (thin black arrow) and mild hydropic degeneration (stars) with some dilated congested blood vessels (V) (H&E, scale bar=50µm), (c) group IV (NSO) has milder inflammation (circles) than group III (H&E, scale bar=100µm) and (d) group V (ALZ+NSO) shows the least inflammatory infiltrates with less hydropic degeneration than the other treated mice groups (H&E, scale bar=50µm). G refers to renal glomeruli.

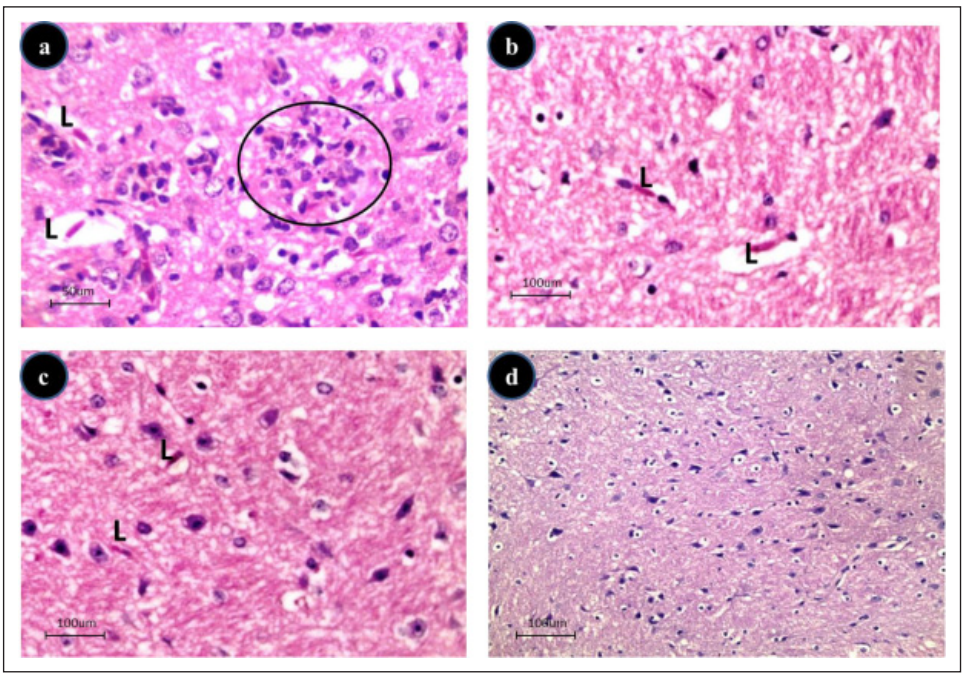

Figure 5. Brain tissue; (a) group II (IU) shows multiple deposited *T. canis* larvae (L) with mild inflammatory infiltrates (circle) in the form of lymphocytes (H&E, scale bar=50µm), (b) group III (ALZ) and (c) group IV (NSO) have few L with low inflammatory infiltrates (H&E, scale bar=100µm) and (d) group V (ALZ+NSO) shows normal histologic appearance (H&E, scale bar=100µm).

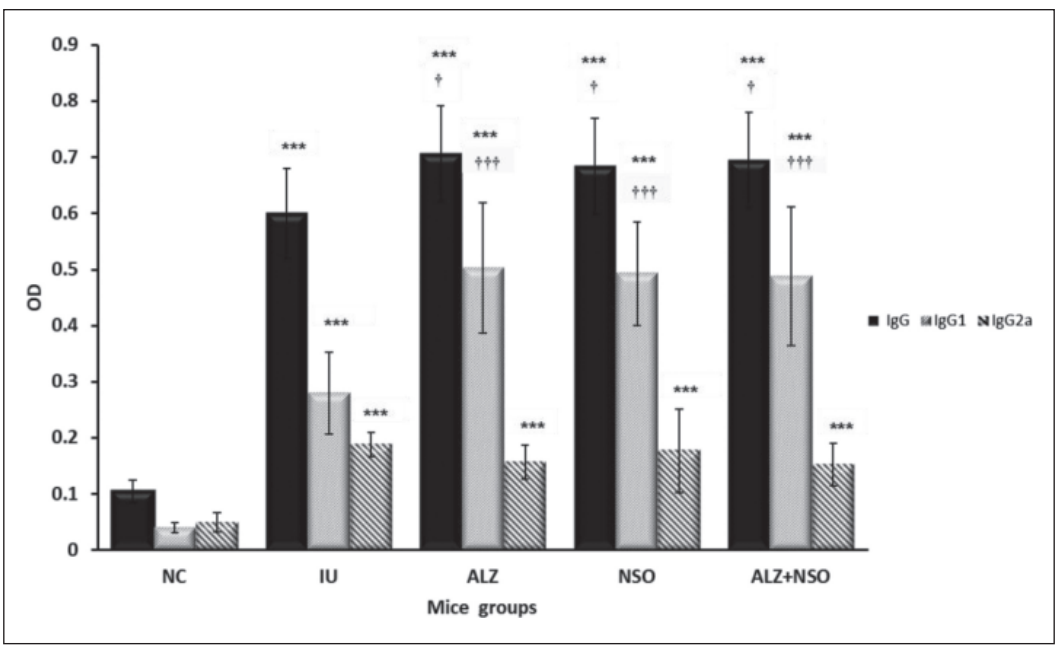

Figure 6. Effects of NSO on specific antibodies response against TSA antigen measured by ELISA. Data are expressed as mean OD±SD. ***P<0.001 is of significant difference in comparison to group I (NC). †P<0.05 and †††P<0.001 are of significant difference in comparison to group II (IU). IgG1 is significantly higher than IgG2a (P<0.01 in group II, P<0.001 in all treated mice groups).

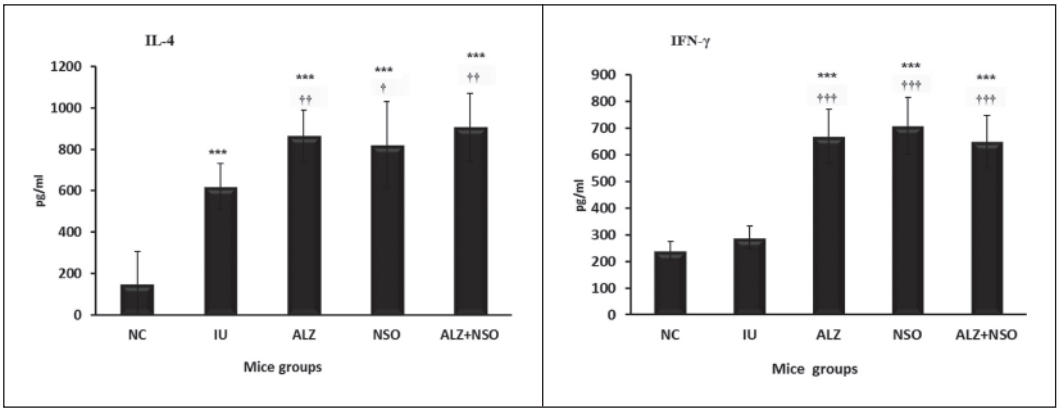

Figure 7. Effect of NSO treatment on serum levels of IL-4 and IFN-γ which their levels were measured by sandwich ELISA. Data are represented as mean±SD. ***P<0.001 is of significant difference in comparison to group I (NC). †P<0.05, ††P<0.01 and †††P<0.001 are of significant difference in comparison group II (IU).

II (P<0.01) and it boosted more than IgG2a (P<0.001) in all the treated mice groups (Fig. 6).

Effect of NSO on serum levels of IFN-γ and IL-4

Group II (IU) showed significant (P<0.001) elevation of IL-4 level, but non-significant (P>0.05) increase of IFN-γ level in the mice sera comparing with group I (NC) (Fig. 7).

In comparison with group II (IU), group V (ALZ+NSO) revealed the highest increase (P<0.01) in IL-4 level, followed by groups III (ALZ) (P<0.01) and finally group IV (NSO) (P<0.05) (Fig. 7). IFN-γ level was the highest in group IV (NSO), then in group III (ALZ), and finally in group V (ALZ+NSO), all with high significant value (P<0.001) in comparison with group II (IU). IL-4 or IFN-γ did not show significant difference among the treated mice groups (Fig. 7).

DISCUSSION

Human toxocariasis is the most common zoonotic infection which is of a chronic nature. ALZ is commonly used for treatment of human toxocariasis, however, it is of low solubility, low bioavailability on the tissues, and needed to be supplied frequently with high doses. Also, it is not very effective (Marquez-Navarro *et al.*, 2009). Prolonged use of anti-parasitic drugs may lead to resistance against these drugs (Geerts & Gryseels, 2001; Kappagoda *et al.*, 2011). For the above mentioned causes, pursuit for developing new drugs against *T. canis* infection is urgently needed.

Nowadays, the natural products are recommended to replace the traditional anti-parasitic drugs including that for *Toxocara.* Herein, we evaluated the efficacy of the herbal NSO as anti-*Toxocara canis* agent in experimentally infected mice.

In this study, NSO (group V) achieved 62.2% reduction in the total larval burden and this result was better than that revealed by Musa *et al.* (2011) who reported only 31.1% and 39.3% reduction in total *T. canis* larval count on using 100 and 200 mg/kg of NS, respectively. When NSO combined with ALZ (group V), the reduction percentage of total larvae number increased to 83.7 which was nearly close to that reported by Musa *et al.* (2011) (87%). The differences between both studies might be related to using diverse NS preparations and different infectious eggs load.

T. canis larvae were not found in liver and lung of the treated mice groups and this may be due to the introduction of treatment on the same day of infection when the larvae are at hepatopulmonary phase of migration when they are more susceptible to the treatments (Bardon *et al.*, 1994). Also, mice

of group II (IU) did not show *T. canis* larvae in liver and lung as the larvae continuously migrate (Othman, 2012). The incomplete disappearance of the larvae in some tissues of mice using either ALZ (group III) or NSO (group IV) might be due to their low bioaviability in these tissues, moreover *T. canis* has been proven to possess efficient strategies to escape the immune attack that elicited by the treatments (Maizels, 2013).

The anti-*Toxocara* effects obtained by NSO might be attributable to many factors. We suggest that NSO may have a direct lethal effect on the *Toxocara* larvae *in vivo*, as Shalaby & El-Moghazy (2013) found that NSO caused vacuolization and disorganization of the cuticle of adult *Toxocara vitulorum in vitro*. TQ which is the major bioactive component of the essential oil of NS may induce oxidative stress against *Toxocara* larvae causing suppression of some important antioxidant enzymes like glucose-6-phosphate dehydrogenase as in case of *Schistosoma* treated experimentally with NS (Mohamed *et al*., 2002). Moreover, NS has documented stimulatory effects on immune system of the host (Majdalawieh & Fayyad, 2015) and these could help in destroying the migrating *Toxocara* larvae.

In mice of group IV, NSO caused regression of inflammation and other pathological lesions in different organs and these effects were nearly similar to that occurred by ALZ. When NSO was administrated with ALZ (group V), examined organs of the infected mice revealed approximately normal histological appearance. Our results were in parallel with Musa *et al*. (2011) who found that treatment with NS extract alone, in a dose dependent manner, decreased the degree of damage and inflammation in liver, lung and brain, as well they noticed more improvement when combining NS extract with ALZ.

Ameliorating the inflammatory lesions after treatment with NSO in our study may be due decreasing the number of *T. canis* larvae in the tissues. Also, TQ has anti-inflammatory properties mediated through diminishing level of inducible nitric oxide synthase enzyme and inhibiting generation of eicosanoids which are potent inflammatory promoters (Darakhshan *et al.*, 2015).

Herein, group II (IU) showed Th2 polarization immune profile indicated by increasing IgG1, and high IL-4 serum level. This immune response of *Toxocara* infection was in accordance with other studies (de Avila *et al.*, 2016; Mazur-Melewska *et al.*, 2016), whereas on introduction of NSO, ALZ or ALZ+NSO, the mice groups showed significant increment of each IgG1, IL-4 and IFN-γ serum levels demonstrating activation of both Th1 and Th2 cells.

The increment of specific *T. canis* IgG and IgG1 in mice treated with NSO could be due to shedding of antigens from the larvae which can stimulate immune cells (Lescano *et al*., 2015). These specific antibodies may play a protective role against larvae of *Toxocara* representing one anti-*Toxocara* mechanism that induced by NSO. It seems that IFN-γ level was not enough to stimulate B lymphocytes to secrete IgG2a or this antibody production may need prolonged lymphocyte stimulation by the cytokine.

Various NS preparations caused stimulation of different immune cells with production of antibodies and cytokines. For example, NS extracts enhanced IFN-γ secretion *in vitro* and *in vivo* (Keyhanmanesh *et al*., 2010; Boskabady *et al*., 2011). Recently, Mady *et al*. (2016) found that *Toxoplasma* infected mice treated with NSO had higher IFN-γ level more than that treated with NSO+ pyremetherine. Also, mice splenocytes treated with extract of NS produced significant larger amount of IL-4 than non-treated cells (Majdalawieh *et al*., 2010).

In contrast to our results, some studies showed that the extracts of NS did not have any significant effects on IL-4 or IFN-γ serum levels (Buyukozturk *et al*., 2005; Majdalawieh *et al*., 2010) and these inconsistent immunological effects of NS might be due to different experimental conditions, host species, dose of NS preparations, and mode of administration (Majdalawieh & Fayyad, 2015).

IFN-γ, a Th1 cytokine, may play important roles in controlling the *Toxocara* infection in this study. IFN-γ promotes secretion of IgG2a and IgG3 antibodies (Collins, 2016), and it enhances macrophage differentiation (Martinez and Gordon, 2014). Pilarczyk *et al.* (2008) found that increasing IFN-γ, in addition to IL-12 and TNF-α, enhanced the host protection against *T. canis* in mice treated with sodium selenite. Treatment of the experimental *T. canis* infection with probiotic yeast, *Saccharomyces boulardii*, reduced the number of *T. canis* larvae through increasing IL-12 which stimulates the production of IFN-γ and this was considered the basis for the protective mechanisms of this probiotic against *T. canis* (de Avila *et al.*, 2016). Also, IL-4, a Th2 cytokine, stimulates the differentiation of lymphocytes into plasma cells promoting production of specific and protective antibodies (Ho & Miaw, 2016) and these antibodies may have role in combating *Toxocara* infection.

In our study, ALZ caused immunological profile in the treated mice similar to NSO. There are few reports studied the immunological effects of ALZ in mice infected with *Toxocara*. Free ALZ, given to healthy mice, stimulated T and B cells and the level of specific antibodies increased significantly after exposure to *T. canis* infection (Boroskova *et al.*, 1998). In contrast to our result, Reis *et al.* (2010) found that ALZ did not significantly affect the specific anti-*Toxocara* IgM or IgG levels in experimentally infected CD-1 mice. These inconsistence immunological effects of ALZ may be related to its dose, the therapy duration and type of the host (Jacobs *et al.*, 1996).

In conclusion, NSO, a commercially prepared available natural extract, succeeded in reduction of the *T. canis* larvae count and decreased the pathological lesions in tissues of the infected mice and these were close to that achieved with the traditionally used ALZ, added to that it manipulated the immune response towards a protective direction. NSO combined with ALZ achieved the most effective results. So, the safe natural NSO is recommended to replace or combine with ALZ to enhance its anti-*Toxocara* effects and combat its side effects.

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