

## Efficacy of Black Seed Oil from *Nigella sativa* against Murine Infection with Cysts of Me49 Strain of *Toxoplasma gondii*

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### ABSTRACT

**Background:** New therapies for toxoplasmosis are critically needed. *Nigella sativa*, commonly known as black seed or black cumin, has been known to include many medicinal properties. It has anti-inflammatory, and immuno-potentiating effects, antihelminthic and antiprotozoal activities.

**Objective:** To study the effect of black seed oil (BSO) from *Nigella sativa* against *Toxoplasma gondii* Me49 strain in a murine model of infection.

**Materials and Methods:** Two separate studies were performed, in which mice were orally inoculated with 10 or 20 *T. gondii* (Me49 strain) brain cysts. In each study, three groups of mice (35 each) were assigned to treatment with BSO for 2 weeks before *T. gondii* infection (BSO prophylactic), day 4 post infection (BSO therapeutic), or left untreated (infected untreated control). The BSO effect on toxoplasmosis was evaluated by the assessment of (1) survival rate and brain cyst burden, (2) brain histopathological lesions and (3) immunohistochemical expression of inducible nitric oxide synthase (iNOS).

**Results:** In infection induced by inoculation of 10, but not 20, cysts/mouse of the Me49 strain, BSO in prophylactic or therapeutic regimens significantly enhanced protection of infected mice against death ( $P = 0.01$ ) and reduced brain cyst burdens at 5, 7 and 12 weeks post infection (PI) ( $P < 0.05$ ) compared to the infected untreated control. The brains of BSO prophylactic or therapeutic groups showed milder meningitis, encephalitis and perivascular cuffing compared to the infected untreated control ( $P < 0.05$ ). Moreover, expression of iNOS was significantly enhanced in both BSO prophylactic and therapeutic groups compared to the untreated infected control. The BSO prophylactic group showed a significant enhanced expression of iNOS, selective to the brain endothelial cells, in the 1<sup>st</sup> week PI. Infection with 20 cysts was more aggressive, resulting in death of all untreated mice by day 35, and 26.7% and 20% protection respectively in BSO prophylactic and therapeutic groups. The estimated probabilities of survival were not significantly different among the 3 groups ( $P = 0.112$ ).

**Conclusion:** BSO showed promising prophylactic and therapeutic effects on murine toxoplasmosis.

**Recommendations:** Further studies are needed to test the anti-*Toxoplasma* effect of *N. sativa* constituents. Additionally, further research work is required to study its prophylactic efficacy against reactivated toxoplasmosis.

**Keywords:** Black Seed Oil, *Nigella sativa*, *Toxoplasma gondii*, iNOS.

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### INTRODUCTION

*Toxoplasma gondii* is an intracellular protozoan parasite of humans and animals with worldwide distribution, including Egypt<sup>(1)</sup>. The type II genotype is most prevalent in immunocompromised patients while strains of type I predominate in congenital infection cases<sup>(2)</sup>.

Toxoplasmosis is a human opportunistic infection and a risk for newborns (causing fetal damage), and for people showing any degree of immunodeficiency, such as HIV carriers or other immunocompromising conditions. Infection of an immunocompetent human host leaves the subject with a life-long latent infection

in the form of quiescent tissue cysts present mainly in brain and muscles<sup>(3,4)</sup>. The host immune response, mainly cell mediated immunity, was found to be critical for controlling acute infection and preventing reactivation of quiescent tissue cysts. Interferon (IFN)- $\gamma$  is a key cytokine in defense against *T. gondii*. In mice, IFN- $\gamma$  induced anti-microbial reaction is mostly credited to production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) expression<sup>(5)</sup>. It was reported that NO has 3 effects in acute toxoplasmosis, the first is a direct antiparasitic effect, the second is an immunoregulatory function which might indirectly influence parasite control, and the third as a molecular trigger of conversion of the replicative tachyzoite stage to the dormant bradyzoite stage<sup>(6)</sup>. During late stage of infection, iNOS expression was found to be critical for prevention of proliferation of tachyzoites in the brain and development of *Toxoplasma* encephalitis<sup>(7)</sup>.

The combination of pyrimethamine and sulphadiazine remains the mainstay for treatment and prophylaxis of most clinical presentations of toxoplasmosis. However, this therapeutic regimen is not always suitable for prolonged treatment because of appearance of adverse side effects, and the potential to contribute to clinical failure by selecting drug-resistant parasite variants. Consequently, new therapies are critically needed<sup>(8,9)</sup>.

There is an increasing awareness of the therapeutic potential of natural products and medicinal plants that are frequently considered to be less toxic and free from side effects than synthetic drugs<sup>(10)</sup>. In this respect, *Nigella sativa*, commonly known as black seed or black cumin, has a great medicinal importance and is known to include many medicinal properties. In addition to its anti-inflammatory action<sup>(11)</sup>, it showed antimicrobial<sup>(12,13)</sup> and antiparasitic<sup>(10,14)</sup> effects. *N. sativa* has been reported to have antihelminthic and anticoccidial activities<sup>(10,14,15)</sup>. It prevents liver damage induced in murine schistosomiasis<sup>(16)</sup>. Importantly, it has an immune stimulating effect to potentiate cellular immune response and enhance interleukins production<sup>(17-19)</sup>. In spite of the large number of studies carried out worldwide on *N. sativa*, there is a need to investigate its anti-*Toxoplasma* activity. The aim of the present work is to study the effect of black seed oil (BSO) from *N. sativa* on murine toxoplasmosis.

## **MATERIALS AND METHODS**

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**Study type:** Case control study.

**Experimental design:** Two separate studies were performed, in which mice were inoculated with 10 and 20

*T. gondii* (Me49 strain) brain cysts. In each study, 3 groups of mice were used (35 mice each): 15 for assessment of survival rate and 20 for assessment of brain cyst burden (at 5, 7, 12 weeks PI). Histopathological changes and iNOS expression were assessed at 1, 3, 5, 7, 12 weeks PI (3 mice in 5 intervals). The brains of the sacrificed mice were divided into 2 halves; one for brain cyst count and the other half for histopathological and iNOS expression assessments. The 1<sup>st</sup> prophylactic group received BSO treatment for 14 days and was then inoculated with *T. gondii* brain cysts. Mice of the 2<sup>nd</sup> therapeutic group were infected and received BSO treatment on day 4 PI for 14 days. The 3<sup>rd</sup> control group was infected and left untreated. Additionally, two negative control groups of 5 mice each were used as uninfected untreated group and uninfected group treated with BSO for 14 consecutive days for assessment of iNOS expression.

**Experimental animals:** Swiss albino mice (5-6 weeks old and 20-25 g weight), were used. Mice were purchased from the Faculty of Veterinary Medicine, Zagazig University and they were housed (5/cage) and offered drinking water and regular mouse feed *ad libitum*. The number of experimental mice was calculated according to similar previous studies<sup>(8,9)</sup>, and within the limited survival time of mice infected with this strain. Our previous experience in maintaining *T. gondii* (strain Me49) in mice showed 20-40% mortality rate over 12 weeks PI (5 extra mice were used).

**Parasites:** Brain cysts of the Me49 strain of *T. gondii* were kindly provided by the Institut de Parasitologie et Pathologie Tropicale de Strasbourg (IPPTS), Université de Louis Pasteur, France. The parasite strain was regularly maintained through Swiss albino mice. To obtain cysts for experimental infections, mice infected at least 8 weeks previously were sacrificed by cervical dislocation and their brains were removed and homogenized with 1 ml saline each. Brain suspensions were used for subsequent experimental infections by intraoesophageal gavage<sup>(8)</sup>.

**Drugs:** Black seed oil (BSO) (El Baraka seed oil, Pharco-Pharmaceuticals, Alexandria, Egypt) was given to the experimental animals in a dose of 5 ml/Kg BW/day orally for 14 consecutive days<sup>(10,16)</sup>. To control drug side effects, a preliminary experiment was conducted in which uninfected mice received BSO in the same dose for 2 weeks. No mortality or clinically significant toxicity in the form of decreased activity, piloerection, lethargy or weight loss, were observed during a period of 1 month.

**Assessment of the BSO effects:** Because of the high mortality rate in mice of the 2<sup>nd</sup> study, assessment of

brain cyst burden, histopathological changes and iNOS expression were only performed in the 1<sup>st</sup> study (infection dose: 10 *T. gondii* brain cysts).

1. **Survival rate:** Mice groups in both studies were monitored daily for 12 weeks PI in the 1<sup>st</sup> study and for 6 weeks PI in the 2<sup>nd</sup> study and deaths were recorded daily.
2. **Brain cyst burden:** This was assessed by direct microscopic examination of brain suspensions from mice sacrificed at 5, 7 and 12 weeks PI. Three mice from each group were sacrificed and their brains were collected and each brain was divided into 2 halves. For estimation of brain cyst burden, one half of each brain was triturated with a mortar and a pestle in 1 ml saline until the suspension appeared homogeneous. The brain suspension (4 drops, 25  $\mu$ l each) was examined for microscopic cyst count (X40), and cyst burden/brain was calculated according to the following equation: cyst count in 100  $\mu$ l  $\times$  10  $\times$  2. If no cysts were observed, the remainder of the brain homogenate was inoculated orally into 2 clean mice ( $\sim$  450  $\mu$ l)<sup>(8)</sup>. In this bioassay, mice were sacrificed after further 6 weeks and their brains were examined for cysts.
3. **Histopathological studies:** The other brain halves were used for preparation of paraffin blocks. Sections of 4-5 $\mu$ m thick were stained with haematoxylin and eosin (H & E). Histopathological examination was used to assess the presence of meningitis, encephalitis and perivascular cuffing using the reported scoring system<sup>(20)</sup> (0: absent, 1: minimal, 2: mild, 3: moderate and 4: severe). The scoring system was applied by two pathologists without prior knowledge of the samples grouping.
4. **Detection of iNOS expression:** As described previously<sup>(21)</sup>, 4-5  $\mu$ m of the deparaffinized brain tissue sections were submitted to antigenic unmasking in a microwave oven. The sections were incubated for 30 min at 37°C in 2% unlabeled sheep serum to reduce nonspecific binding; then incubated in polyclonal rabbit anti-iNOS peptide (Santa Cruz Biotechnology, Inc., CA, USA), at 4°C overnight. Secondary biotinylated antibodies were sheep anti-rabbit antibodies. The reaction was visualized by incubation with 3, 30-diaminobenzidine tetrahydrochloride (Amresco, Solon, OH, USA) for 5 min before counter-staining with Mayer's haematoxylin. The slides prepared from the experimental groups as well as the negative control groups were examined for

iNOS<sup>+</sup> expressing cells with an Olympus microscope and the images were captured in a digital camera. The iNOS positive expression was observed as faint to intense dark brown immune reaction. The iNOS expression was analyzed in 3 sections from each brain of 3 mice in each group. The measurement of the staining intensity was done (in 10 high power fields/section) with Leica Q 500 image analysis system (LICA Microsystem Corporation, England).

**Statistical analysis** was performed using the SPSS 10.0 computer software statistical package. Survival rates were estimated by the Kaplan-Meier product limit method and compared by the two curves and multiple sample tests<sup>(8)</sup>. Differences in brain cyst counts and immunostaining intensities were statistically analyzed using Student's *t*-test. Differences of histopathological changes among groups were compared using Mann-Whitney U test. The level of statistical significance was 0.05.

**Ethical consideration:** Experimental studies in mice were conducted in accordance with the international guidelines and they were maintained under convenient conditions at Parasitology Department, Faculty of Medicine; Suez Canal University, Ismailia, Egypt.

## RESULTS

**Survival rate:** Both BSO prophylactic and therapeutic groups infected with 10 cysts, survived significantly better ( $P = 0.01$ ) than infected untreated control group (66.7%). The therapeutic group showed 86.7% cumulative survival rate. In the prophylactic group, protection was afforded for all mice ( $P = 0.01$ ). The estimated probabilities of survival varied significantly among the 3 groups ( $P = 0.039$ ) (Figure 1). In infection induced with 20 cysts, all untreated mice succumbed by day 35, while 26.7% and 20% of BSO prophylactic and therapeutic groups, respectively, were relatively protected (Figure 2). The estimated probabilities of survival were not significantly different among the 3 groups ( $P = 0.112$ ). BSO slightly prolonged the time of death of both treated groups compared to the control group, (mean time of death was 21.7 $\pm$ 3.4, 21.2 $\pm$ 7.6 and 19.3 $\pm$ 8.7 for BSO prophylactic and therapeutic groups compared to the control group,  $P = 0.4$  and 0.6, respectively).

**Brain cyst burden:** A significant reduction in brain cyst burden occurred in all groups after 7 or 12 weeks PI. In BSO prophylactic group, the reduction in cyst count significantly increased over time. In both prophylactic and therapeutic groups, BSO significantly reduced

the number of brain cysts at 5, 7 and 12 weeks PI compared to the infected untreated control group ( $P < 0.05$ ) (Table 1). Interestingly, no cysts were observed on direct examination of brain tissue in 2 mice from BSO prophylactic group. Bioassay of these brain homogenates did not give rise to *T. gondii* cysts.

**Assessment of histopathological changes:** Generally, the lesions in all groups were characterized by mononucleated cell infiltrates, glial nodules, vascular cuffing by lymphocytes and focal as well as diffuse meningeal mononuclear cells infiltrates. The inflammatory lesions varied from mild to moderate in the brains of animals in all groups with progression of infection until the 5<sup>th</sup> week PI and then decreased. Irrespective of the time PI, the inflammatory scores for the lesions in BSO prophylactic and therapeutic groups compared to the control group, showed less marked meningitis ( $1.6 \pm 0.8$  and  $2.07 \pm 0.9$  versus  $3 \pm 0.7$ ), encephalitis ( $1.9 \pm 0.8$  and  $2.5 \pm 0.8$  versus  $3.07 \pm 0.8$ ), and perivascular cuffing ( $0.9 \pm 0.8$  and  $1 \pm 0.9$  versus  $1.9 \pm 1.2$ ) (Figures 3 and 4 A-D). Comparison of the inflammatory changes between different groups according to time PI revealed no significant difference in the 1<sup>st</sup> week PI. Significant less marked meningitis, encephalitis and perivascular cuffing were observed in the BSO treated groups (prophylactic or therapeutic) compared to the control group at the 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup> and 12<sup>th</sup> weeks PI (Table 2). The BSO prophylactic group showed

less marked pathological lesions compared to BSO therapeutic group.

**Assessment of iNOS expression:** Expression of iNOS was not observed in brain tissue from the uninfected control mice whether treated or untreated (Figure 5A). In infected groups (BSO treated and control), iNOS expression was observed throughout the study period (12 weeks PI) with different intensities. iNOS expression was found to involve different brain cells: glial cells, astrocytes, neurons and endothelial cells. *T. gondii* cysts were frequently and closely surrounded by iNOS<sup>+</sup> cells, but cysts with no surrounding iNOS<sup>+</sup> cells were also observed mainly at 7<sup>th</sup> and 12<sup>th</sup> week PI (Figures 5D, E). Compared with the infected untreated control mice, both BSO treated groups showed a significantly enhanced expression of iNOS at different time points PI. However, different patterns of iNOS expression were observed in BSO treated groups. In BSO prophylactic, a significant higher iNOS expression was observed at 1<sup>st</sup> week PI, mainly involving the brain endothelial cells, compared to BSO therapeutic ( $P = 0.001$ ) and the infected untreated control ( $P = 0.000$ ) (Figures 5B and 6). BSO therapeutic group showed sustained significant higher iNOS expression that started at 3<sup>rd</sup> week PI and continued till the end of the study (Figures 5C-D and 6). In all infected groups, expression of iNOS increased after infection reaching maximum intensity at 5<sup>th</sup> or 7<sup>th</sup> week then decreased at 12<sup>th</sup> week PI.

**Table (1):** Brain cyst counts in mice infected with 10 cysts of *T. gondii* Me49 strain, for BSO prophylactic and therapeutic groups compared to the infected untreated control group.

Mice group	Brain cyst burden (mean ± SD)		
	5 weeks PI <sup>1</sup>	7 weeks PI <sup>2</sup>	12 weeks PI <sup>3</sup>
<b>BSO prophylactic</b> <sup>a</sup>	880 ± 80*	450 ± 50*	200 ± 31*
<b>BSO therapeutic</b> <sup>b</sup>	1080 ± 120**	1140 ± 923	600 ± 68**
<b>Untreated control</b> <sup>c</sup>	3140 ± 790	1110 ± 226	1500 ± 194
* P < 0.01 and ** P < 0.05 compared to infected untreated control group.			
<b>5<sup>th</sup> versus 7<sup>th</sup> week</b>	1a Vs 2a: P=0.001	1b Vs 2b: P=0.9	1c Vs 2c: P=0.01
<b>7<sup>th</sup> versus 12<sup>th</sup> week</b>	2a Vs 3a: P=0.002	2b Vs 3b: P=0.4	2c Vs 3c: P=0.1
<b>5<sup>th</sup> versus 12<sup>th</sup> week</b>	1a Vs 3a: P<0.001	1b Vs 3b: P=0.004	1c Vs 3c: P=0.03

**BSO prophylactic:** Decrease in cyst count is significant in comparison between all week intervals.

**BSO therapeutic:** Decrease in cyst count is significant only in comparison between 5<sup>th</sup> and 12<sup>th</sup> week PI.

**Untreated control:** Decrease in cyst count is significant in comparison between 5<sup>th</sup> and 7<sup>th</sup> week PI and between 5<sup>th</sup> and 12<sup>th</sup> week PI.

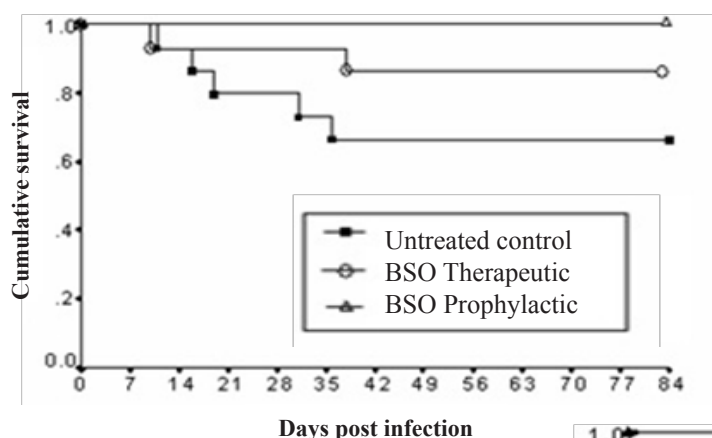


**Table (2):** Inflammatory scores (Mean ± SD) of the study experimental groups infected with 10 cysts *T. gondii* Me49 strain.

Weeks PI	Mice groups	Inflammatory score (Mean ± SD)		
		Meningitis	Encephalitis	Perivascular cuffing
1 <sup>st</sup>	BSO prophylactic	1.7 ± 0.6	2.0 ± 0.0	0.3 ± 0.6
	BSO therapeutic	1.3 ± 0.6	1.3 ± 0.6	0.0 ± 0.0
	Untreated control	2.7 ± 0.6	2.7 ± 1.2	0.3 ± 0.6
3 <sup>rd</sup>	BSO prophylactic	1.7 ± 0.6*	2.0 ± 0.0*	1.0 ± 0.0*
	BSO therapeutic	2.7 ± 0.6	3.0 ± 1.0	0.3 ± 0.6*
	Untreated control	3.0 ± 0.0	3.7 ± 0.6	2.3 ± 0.6
5 <sup>th</sup>	BSO prophylactic	2.3 ± 0.6	2.3 ± 0.6	1.3 ± 0.6*
	BSO therapeutic	2.3 ± 0.6	3.0 ± 0.0	2.3 ± 0.6
	Untreated control	3.3 ± 0.6	3.3 ± 0.6	3.3 ± 0.6
7 <sup>th</sup>	BSO prophylactic	0.3 ± 0.6*	0.7 ± 1.7*	0.3 ± 0.6
	BSO therapeutic	1.0 ± 0.0*	3.0 ± 0.0	1.3 ± 0.6
	Untreated control	2.3 ± 0.6	2.7 ± 1.2	1.7 ± 1.2
12 <sup>th</sup>	BSO prophylactic	2.0 ± 0.0*	2.3 ± 0.6	1.7 ± 1.2*
	BSO therapeutic	3.0 ± 0.0	2 ± 0.0*	2.0 ± 0.0
	Untreated control	3.7 ± 0.6	3.0 ± 0.0	3.0 ± 0.0

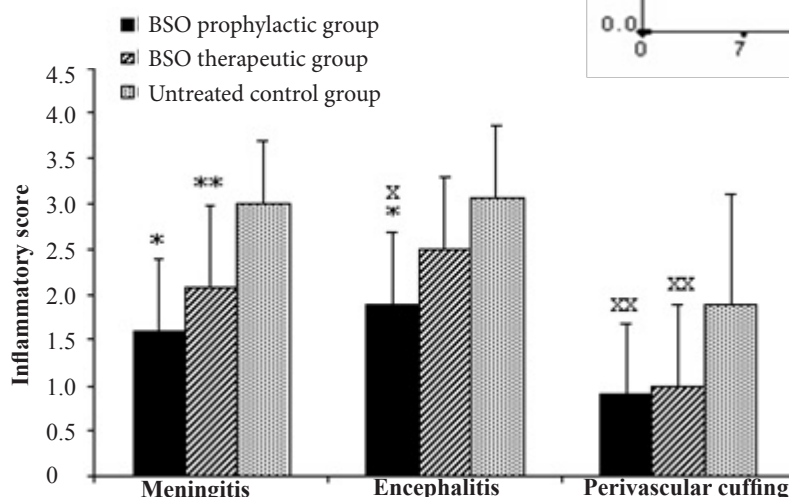
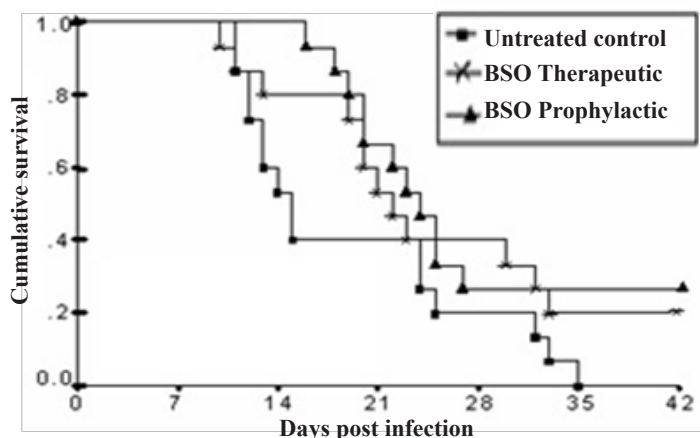
Scoring system: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe.

Results are means of 3 sections per mouse (3 mice per group). \*  $P < 0.05$  compared to the control group.



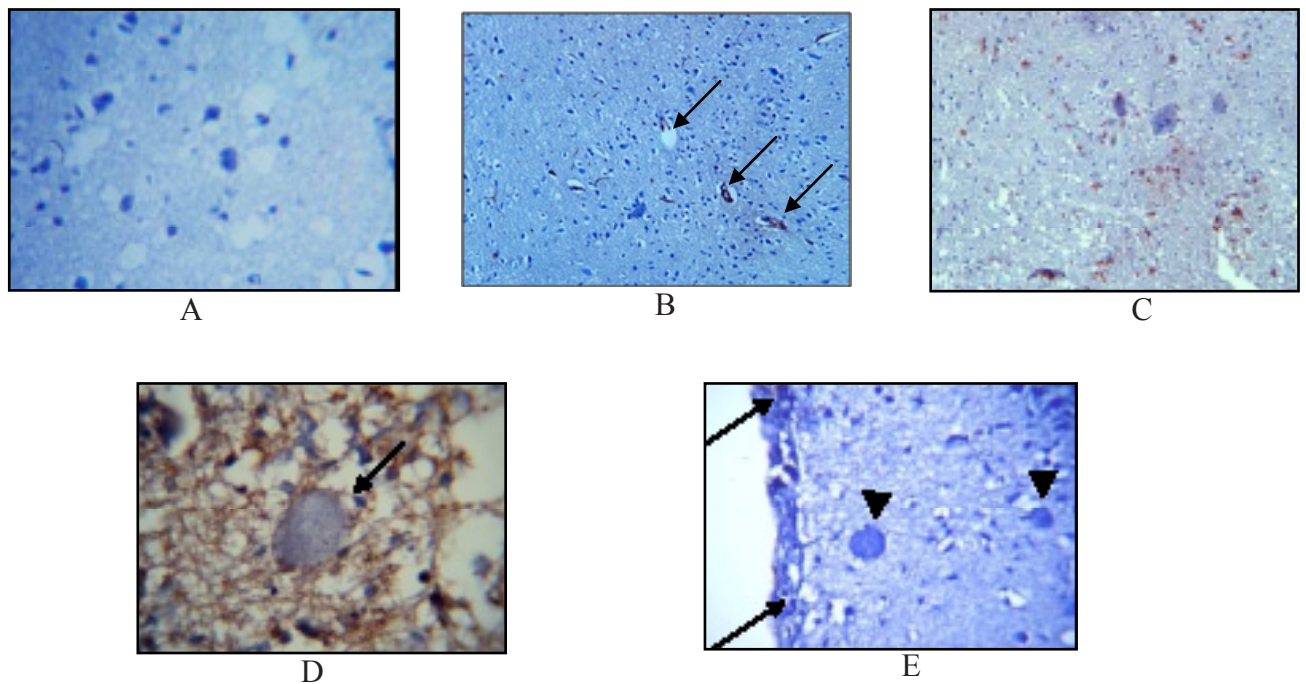
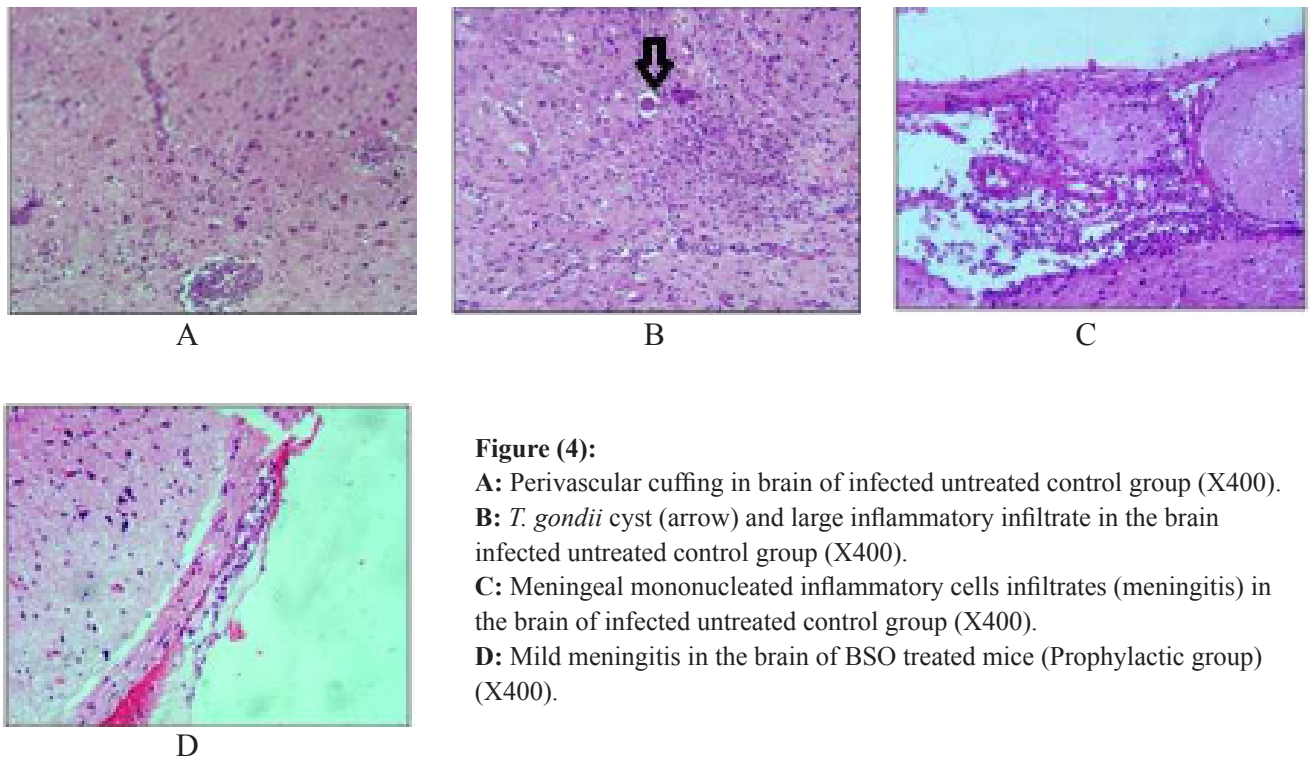
**Figure (1):** Estimated survival rates (%) in mice infected with 10 cysts of *T. gondii* Me49 strain in both BSO prophylactic and therapeutic groups compared to infected untreated control group.

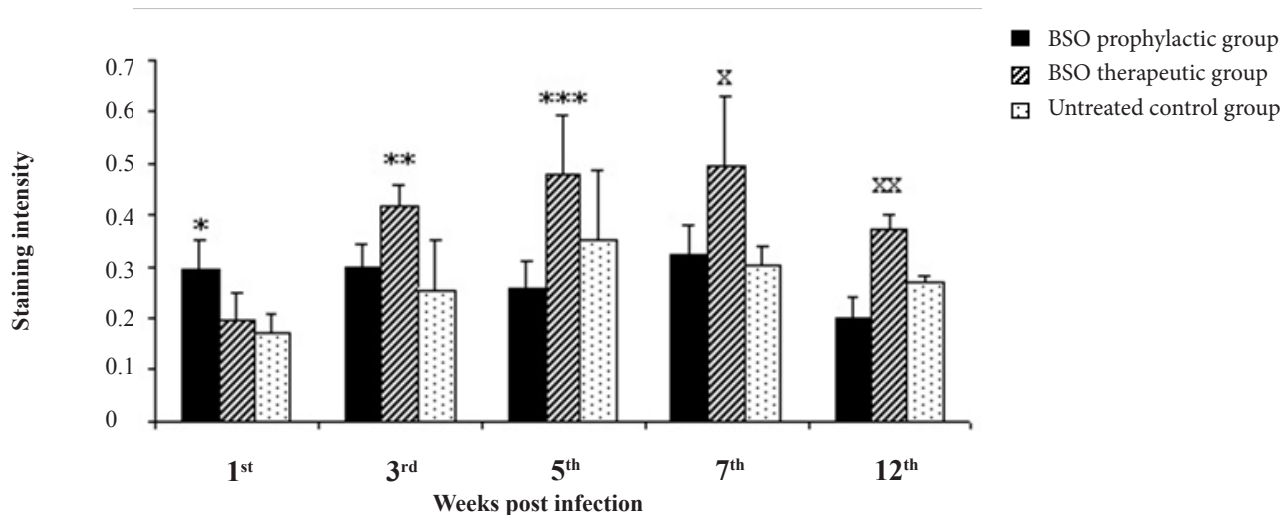
**Figure (2):** Estimated survival rates (%) in mice infected with 20 cysts of *T. gondii* Me49 strain in both BSO prophylactic and therapeutic groups compared to infected untreated control group.



**Figure (3):** Inflammatory score (mean ± SD) in brains of experimental groups infected with 10 cysts of *T. gondii* Me49 strain irrespective of the PI time.

Inflammatory scoring system: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe.





**Figure (6):** Significantly higher expression of iNOS (mean  $\pm$  SD of staining intensities) recorded in brains of the study experimental groups infected with 10 cysts of *T. gondii* Me49 strain.

\* $P = 0.001$  and  $0.000$ : BSO prophylactic versus therapeutic and untreated infected control, respectively,

\*\* $P = 0.000$ : BSO therapeutic versus prophylactic and untreated infected control.

\*\*\* $P = 0.000$  and  $0.04$ : BSO therapeutic versus prophylactic and untreated infected control, respectively.

<sup>X</sup> $P = 0.002$  and  $0.001$ : BSO therapeutic versus prophylactic and untreated infected control, respectively.

<sup>XX</sup> $P = 0.001$ : BSO therapeutic versus prophylactic untreated infected control.

## DISCUSSION

In the present study, Swiss albino mice were highly sensitive to per-oral infection with Me49 strain of *T. gondii*. The mice began dying 2<sup>nd</sup> week after infection with 10 or 20 cysts of the parasite. The overall survival was 66.7% in mice inoculated with 10 cysts after 12 weeks PI while none of mice inoculated with 20 cysts survived after 35 days PI. These findings are in agreement with other studies<sup>(22,23)</sup>. It was previously indicated that the time of mice death and the mortality rate differ according to the mice strain used and the infection dose with the parasite<sup>(22,24)</sup>. The present study results showed that the brains of untreated infected mice contained a larger number of cysts than prophylactic and therapeutic groups by the 5<sup>th</sup> week PI. This was followed by a reduction in cyst count at the 7<sup>th</sup> week PI and up to 12<sup>th</sup> week PI. These findings are in accordance with a previous study using CBA/CA mice<sup>(23)</sup> in which the authors reported that the decrease in the number of brain cysts occurred at 16 weeks PI. In the present study, the brains of infected mice had mild to severe meningitis, encephalitis and cuffing of the blood vessels. These pathological lesions persisted in the chronic stage until the 12<sup>th</sup> week PI. Suzuki and Joh<sup>(25)</sup> examined the brains of C57BL/6 mice infected with the ME49 strain 10 weeks PI. The infected brains showed infiltration of inflammatory mononuclear

cells in both the meninges and the parenchyma associated with a large number of cysts. Moreover, Hermes *et al.*<sup>(26)</sup> observed pathological lesions in brains of infected mice in the form of meningitis, parenchymal inflammation and perivascular cuffing after 12 months PI. The reason for the continuous inflammation and the reduction in brain cyst count observed with Me49 strain infection in the present study was formerly studied<sup>(23,27)</sup>. The authors explained that this parasite strain causes a less stable chronic infection due to the higher rate of cyst rupture associated with a rapid cell-mediated immune response which gave rise to inflammatory nodules. A proportion of inflammatory nodules in the chronic infection was found to contain tachyzoites or bradyzoites; and also many of the cysts were found to contain immature bradyzoites, irrespective of the period PI. They concluded that under immunocompetent conditions there was little evidence of parasite multiplication or new cyst formation in association with cyst rupture leading to a reduction in cyst numbers.

The present results clearly demonstrated the anti-*Toxoplasma* activity of BSO in induced murine infection with the Me49 parasite strain. Whether prophylactic or therapeutic, the BSO treatment was effective in terms of both significantly increasing survival and decreasing brain cyst burden compared with the infected untreated

control. The prophylactic regimen was more effective than the therapeutic regimen as it protected 100% of mice and reduced the brain cyst burden, which remained very low until the end of study. Moreover, absence of cysts in brains of 2 mice from the prophylactic group, associated with absence of cysts in brains of bioassays, demonstrates the potential of BSO given as a prophylactic regimen to prevent brain infection in these mice. In parallel with these findings, both BSO treatments were associated with less pathological changes regarding meningitis, encephalitis and cuffing of blood vessels compared with the infected untreated control group. Additionally, the treatment was associated with enhanced expression of iNOS. In the BSO prophylactic group, the iNOS expression was observed mainly in the brain endothelial cells as early as the 1<sup>st</sup> week PI. In the BSO therapeutic group, iNOS expression was significantly enhanced at the 3<sup>rd</sup> week PI and throughout the study period.

*N. sativa* has been in use in many Middle Eastern and Far Eastern countries as a natural remedy for over 2000 years. It is commonly eaten alone or in combination with honey and in many food preparations without any undesirable effects<sup>(13)</sup>. Crude extracts and essential oil of *N. sativa* were proved to have many therapeutic effects<sup>(19,28,29)</sup>. It was found to exert an anti-malarial effect as it significantly decreased the parasitaemia and increased the survival times of mice infected with *Plasmodium berghei*<sup>(30)</sup>. The *N. sativa* aqueous extract was found to exert an inhibitory action on the growth and motility of *Trichomonas vaginalis* *in vitro*, as well as a potential therapeutic effect against *Blastocystis hominis*<sup>(31,32)</sup>. Moreover, its alcoholic extract was found to be as effective as metronidazole in the cure of giardiasis<sup>(15,33)</sup>. *N. sativa* oil was found to afford protection and prevent liver damage induced by *Schistosoma mansoni* infection in mice by modulating the immune response and reducing inflammation<sup>(16)</sup>.

The anti-*Toxoplasma* potential of BSO found in the present study extends its anti-parasitic effects. The anti-*Toxoplasma* action exhibited by BSO may be attributed to the presence of different classes of alkaloids and phenolics<sup>(34)</sup> that are known for their antiprotozoal activity against *P. falciparum*, *Leishmania* and *Trypanosoma* spp., *E. histolytica* and *C. parvum*<sup>(30,35,36)</sup>. It may have also been a result of the immunomodulatory properties of BSO. In murine toxoplasmosis, interferon- $\gamma$ -dependent cell-mediated immunity plays the major role in resistance against toxoplasmosis. Protection against *T. gondii* infection requires the prompt development and persistence of an active type 1 cytokine response characterized by the production of inflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ <sup>(37)</sup>. Pro-inflammatory cytokines at infection site are

important for recruitment and activation of leukocytes, which mediate local host defenses<sup>(38,39)</sup>. Although the inflammatory reaction induced by pro-inflammatory cytokines during *T. gondii* infection is crucial for control of the parasite, a critical immunoregulation is required to prevent host immunopathology<sup>(21)</sup>. The immune responses are also essential for maintaining the latency of chronic infection<sup>(40)</sup>. Several studies point to the effect of *N. sativa* on the immune system. The *N. sativa* seeds were found to produce an increase in the ratio of helper to suppressor T cells and to enhance natural killer cell activity in normal volunteers<sup>(41)</sup>. *In vitro* studies showed that *N. sativa* enhanced the production of IL-3 by human lymphocytes and had a stimulatory effect on macrophages<sup>(17)</sup>. Besides, the immunomodulatory effect of *N. sativa* purified proteins was studied in mixed lymphocyte cultures, and was found to cause changes in the levels of cytokines with an enhancing effect on production of IL-1 $\beta$  and TNF- $\alpha$ <sup>(42)</sup>. The *in vitro* enhancing effects of *N. sativa* on the T cell immunity were confirmed by *in vivo* studies<sup>(43,44)</sup>. Moreover, *N. sativa* oil was found to exhibit a striking antiviral effect against murine cytomegalovirus infection, that coincided with elevation of IFN- $\gamma$  in serum which lasted for a prolonged time<sup>(13)</sup>.

In the present study, BSO prophylactic and therapeutic regimens were associated with increased expression of iNOS in brain, compared to untreated infected control. This finding may be explained by the enhancement of IFN- $\gamma$  and TNF- $\alpha$  production by BSO, as reported in other studies<sup>(13,42)</sup>. *In vitro* experiments have shown a crucial role for both IFN- $\gamma$  and TNF- $\alpha$  in the induction of reactive nitrogen intermediates (RNI) including NO, which emerges as an important regulatory molecule involved in the minimization of the immunopathological alterations induced by the parasite<sup>(3)</sup>.

In the present study, higher iNOS expression in brains of BSO treated mice was associated with less inflammatory changes and lower parasitism. It was found that low or no iNOS expression contributed to uncontrolled parasite multiplication that in addition to inflammatory and necrotic lesions in the CNS induced the mortality of TNFRp55<sup>-/-</sup> and iNOS<sup>-/-</sup> mice<sup>(21)</sup>. Moreover, studies directly addressing the role of NO in murine acute toxoplasmosis revealed that an inhibition of iNOS with aminoguanidine beginning at the day of infection leads to an increased intracerebral parasitic load and exacerbated inflammation of the brain tissue<sup>(45)</sup>. Furthermore, iNOS-deficient mice had impaired parasite control and developed rapidly fatal acute cerebral toxoplasmosis<sup>(7)</sup>. The precise mechanism for NO-mediated anti-*Toxoplasma* effect remains undetermined. Presumably, intracellular generation of



NO and metabolites could result in metabolic inhibition of mitochondrial and nuclear enzymes essential for parasite respiration and replication<sup>(37)</sup>. In addition to this direct antiparasitic effect, NO exerts an important immunoregulatory role in acute toxoplasmosis. The production of high levels of RNI has been associated with suppression of lymphocyte proliferation in normal mice<sup>(46)</sup>. This regulatory mechanism may work through the induction of apoptosis in a variety of cell types, including T cell clones<sup>(47)</sup>. Previous experiments have shown that, RNI produced by Th1 cells activated by specific antigens or Con A can inhibit the secretion of IL-2 and IFN- $\gamma$ , exerting a self-regulatory effect on Th1 cells<sup>(48)</sup>. Reactive nitrogen intermediates can also modulate leukocyte recruitment and accumulation<sup>(49)</sup>. Furthermore, NO possibly also serves as a molecular trigger of conversion of the replicative tachyzoite stage to the dormant bradyzoite stage at least in certain cell types<sup>(50)</sup>.

In the present study, enhanced iNOS expression in the brain endothelial cells at the 1<sup>st</sup> week PI in BSO prophylactic group could have contributed to the continued significant lower parasitism in this mice group. Since one of the first steps in the development of cerebral toxoplasmosis is the penetration of blood-brain barrier, the induced inhibition of parasite replication in brain endothelial cells may prove important in limiting entry of the parasite into the brain<sup>(51)</sup>. It was observed that stimulation of human brain microvascular endothelial cells with IFN- $\gamma$  resulted in the induction of toxoplasmosis<sup>(52)</sup>. Additionally, the proinflammatory cytokine IL-1 $\beta$  alone, or a combination of TNF- $\alpha$  and IFN- $\gamma$  were found to induce production of large amounts of NO by iNOS in mouse and rat brain microvascular endothelial cells under inflammatory conditions<sup>(53,54)</sup>. *N. sativa* was found to enhance the production of these cytokines<sup>(13,17,42)</sup>.

In conclusion, BSO showed promising prophylactic and therapeutic effects on *Toxoplasma gondii* infection. Further research work is required to study its prophylactic efficacy against reactivated toxoplasmosis. Additionally, further studies are needed to test the anti-*Toxoplasma* effect of the constituents of *N. sativa*, which might represent a promising area for anti-*Toxoplasma* remedy.

**Author contribution:** Rayan HZ proposed the research idea, performed the experiments, and shared in assessment of the immunohistochemical findings. Rayan HZ and Wagih HM shared in the study design. Wagih HM and Atwa MM performed the histopathological and immunohistochemical studies. All authors shared in writing the manuscript.

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## تأثير زيت حبة البركة على عدوى الفئران بطفيلي التوكسوبلازما جوندى سلالة Me49

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**الهدف من البحث:** دراسة تأثير زيت حبة البركة على العدوى بطفيلي التوكسوبلازما جوندى سلالة Me49 فى فئران التجارب.

**الطرق المستخدمة:** شملت الدراسة تأثير اعطاء زيت حبة البركة كجرعات وقائية وعلاجية على عدوى الفئران بعدد 10 و 20 من أكياس الطفيلي، وتمت متابعة الفئران على مدار 12 أسبوع بعد العدوى. وكان تقييم هذا التأثير عن طريق دراسة نسبة الوفيات بين المجموعات المختلفة من الفئران، عدد أكياس الطفيلي فى مخ الفئران فى الأسبوع 5، 7، 12 بعد العدوى، كما تمت دراسة التغيرات الباثولوجية ووجود انزيم iNOS فى قطاعات من مخ الفئران فى الأسبوع 1، 3، 5، 7، 12 بعد العدوى.

**النتائج:** كانت نتائج البحث جيدة فى نموذج العدوى بعدد 10 أكياس للطفيلي، وجد أن كان علاج الفئران بزيت حبة البركة فى جرعات وقائية أو علاجية قد قلل من نسبة وفيات الفئران نتيجة العدوى بالطفيلي، كما قلل من عدد أكياس الطفيلي فى مخ الفئران. وأثبتت نتائج البحث أن الجرعات الوقائية لزيت حبة البركة أدت الى عدم وجود أى من أكياس الطفيلي فى مخ عدد من الفئران مما قد يدل على قدرته على الوقاية من العدوى. كما قلل زيت حبة البركة من حدة التغيرات الباثولوجية مثل التهاب السحايا والتهاب أنسجة المخ الناتجة عن العدوى. ولقد أوضحت نتائج الدراسة أن العلاج بزيت حبة البركة أدى الى زيادة فى انزيم iNOS فى أنسجة مخ الفئران، وكانت هذه الزيادة واضحة فى خلايا المخ brain endothelial cells فى الأسبوع الأول بعد العدوى فى مجموعة الفئران التى تم اعطائها جرعات وقائية من زيت حبة البركة مما قد يفسر قلة عدد الكياس الطفيلي أو عدم وجودها فى هذه المجموعة من الفئران.

**الاستنتاجات:** ويتضح من هذه الدراسة أن زيت حبة البركة له تأثير وقائى وعلاجى على العدوى بالتوكسوبلازما جوندى سلالة Me49.

**التوصيات:** دراسة التأثير الوقائى لزيت حبة البركة على اعادة تنشيط طفيل التوكسوبلازما جوندى، ودراسة مكونات زيت حبة البركة وتأثير كل منها على حدة على العدوى بالتوكسوبلازما جوندى.