

Research Paper

Cite this article: Matar AM, Kora MA, Shendi SS (2023). Evaluation of the therapeutic effect of Olibanum extract against enteric and intramuscular phases of trichinosis in experimentally infected mice. *Journal of Helminthology*, **97**, e44, 1–12
<https://doi.org/10.1017/S0022149X23000214>

Received: 05 January 2023

Revised: 18 March 2023

Accepted: 28 March 2023

Keywords:

T. spiralis; olibanum; albendazole; IL-10; CD8

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Evaluation of the therapeutic effect of Olibanum extract against enteric and intramuscular phases of trichinosis in experimentally infected mice

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Abstract

Trichinosis is a global food-borne zoonotic disease. Most drugs used in its treatment have low bioavailability and reduced activity against larvae. Therefore, there is an urgent need for safe and effective medications. This study aimed to investigate the *in vivo* anti-parasitic and anti-inflammatory efficacy of olibanum (OL) extract, alone or combined with albendazole (ABZ) during both intestinal and muscular phases of trichinosis. Male Swiss albino mice (n = 130) were allocated to seven groups, with 20 mice in each group except for the negative control group (10 mice): negative control (GI), positive control (GII), OL25- treated (GIII), OL50- treated (GIV), ABZ50- treated (GV), OL25 + ABZ25 (GVI), and OL50 + ABZ25 (GVII). For intestinal and muscular phase analysis, each group was divided into two subgroups based on euthanizing day (6 and 35 days post-infection). The drug's efficacy was evaluated through parasitological, biochemical, histopathological, and immunohistochemical studies. OL extract at both concentrations (25 mg/kg/d, 50 mg/kg/d) significantly reduced adult (53.7% and 68.1%, respectively) and larval counts (57.3% and 78.8%, respectively). It improved the histopathological changes in intestine and muscle. The expression of CD8⁺ T cells and the serum level of IL-10 increased significantly during both intestinal and muscular phases ($P < 0.05$) in OL50 treated mice. Additionally, OL decreased abnormal levels of liver enzymes (ALT & AST). Its effects were dose-dependent in both adult and larval stages. In conclusion, OL exhibits promising *in vivo* activity against both stages of *Trichinella spiralis* infection, particularly at the intramuscular phase. It can be safe as an alternative treatment for trichinosis.

Introduction

Trichinosis is a globally food-borne zoonotic disease (Abuelenain *et al.* 2021) caused primarily by *Trichinella spiralis* (*T. spiralis*), the most dangerous human species (Sarhan *et al.* 2021) and ranked among the top ten food-borne parasites worldwide (Abd-elrahman *et al.* 2020). It is estimated that approximately 10,000 cases occur (Gottstein *et al.* 2009) annually, with a 0.2% mortality rate (Etewa *et al.* 2020). Most human infections are caused by accidental ingestion (Yadav & Temjenmongla 2012) of viable infective encysted *T. spiralis* larvae in raw or undercooked pork and pork products (Gottstein *et al.* 2009). In humans, the disease is usually associated with varied symptoms (Ashour *et al.* 2016), and severe complications can seriously jeopardize human health depending on the number of larvae ingested (Ibrahim *et al.* 2019).

Trichinosis has two phases: an intestinal phase and a muscular phase (Sarhan *et al.* 2021). *Trichinella* has the distinct ability to undergo intracellular growth in muscle cells, which constitute a protective nurse cell for the parasite (Elgendy *et al.* 2020). *T. spiralis* larvae can survive successfully in these nurse cells due to their ability to support angiogenesis for nutrition (Allam *et al.* 2021). Chronic trichinosis results in a strong interaction with the host immune system that leads to inflammation of the affected muscles (Elgendy *et al.* 2020).

During infection, trichinosis is characterized by an inflammatory response that occurs in different tissues and organs (Sarhan *et al.* 2021), which aims to destroy the parasite but also poses a threat to the host (Muñoz-Carrillo *et al.* 2017). Therefore, anti-inflammatory drugs are expected to help protect hosts (Kazemzadeh *et al.* 2014). However, the widely prescribed steroidal or non-steroidal anti-inflammatory drugs have several adverse effects that limit their use (Elgendy *et al.* 2020). Accordingly, there is a desperate need to search for new and safe compounds with potent anti-inflammatory and anti-parasitic effects against both the intestinal and muscular stages of trichinosis (Shalaby *et al.* 2010).

Olibanum (OL) is a natural oleo gum resin extracted from *Boswellia* plants (Al-Ghandour *et al.* 2020). The Ebers Papyrus is the earliest written record of OL as a medication (Ammon

2016). Accumulated data indicate that OL possesses several mechanisms of action. It has a predominant anti-inflammatory effect that is mediated through inhibition of inflammatory molecules such as prostaglandin E, cyclooxygenase 2, inducible nitric oxide synthetase, and 5-lipoxygenase, leading to inhibition of leukotriene generation (Efferth & Oesch 2020). The anti-inflammatory effect of OL is related to its content of anti-inflammatory compounds such as boswellic acids (Ammon, 2016). Furthermore, Borrelli *et al.* (2006) observed that the predominant anti-inflammatory activity of OL reduced functional problems in the gut by enhancing motility, inhibiting diarrhea without constipation, inhibiting contraction of intestinal smooth muscles, and controlling acetylcholine and barium chloride-induced diarrhea.

Another mechanism of action added to its anti-inflammatory effect is that it down-regulates pro-inflammatory cytokines (TNF α , IL-1 β , IL-2, INF γ , IL-6, and IL-12) and up-regulates anti-inflammatory cytokines (IL-4, IL-10), suggesting that it has an immunomodulatory effect (Efferth & Oesch 2020). The oil has also shown immunostimulating activity by stimulating the innate and adaptive immune response due to its content of novel polysaccharides, which encourages its use in several immune disorders as an important immunotherapy as well as vaccination purposes (Hosain *et al.* 2019). In addition, the physiological maintenance of the integrity and function of the enterocytes is mediated by its antioxidant activity (Al-Ghandour *et al.* 2020).

Based on the previous data the present study was conducted to determine whether OL extract has any promising therapeutic effects on the intestinal and muscular stages of experimental trichinosis.

Materials and methods

Sample size calculation

The sample size was calculated based on the study's design and objectives. This experimental study evaluated and compared the therapeutic effects of OL extract and ABZ on the intestinal and muscular stages of experimental trichinosis. The sample size was 130 with a 95% confidence interval and 80% statistical power.

Experimental animals

A total of 130 laboratory-bred pathogen-free, Swiss- male albino mice, 6–8 weeks old, weighing 20–25 g each, were acquired from Theodor Bilharz Research Institute's (TBRI) Biology Supply Center (Giza, Egypt). They were kept in the TBRI animal houses, which were maintained at a constant room temperature of (22 \pm 2 $^{\circ}$ C). The animals had free access to the usual food and water.

Parasite and mice infection

The isolate of *T. spiralis* was initially acquired from the diaphragms of diseased pigs slaughtered at a slaughterhouse in Cairo (Cairo, Egypt). By digesting the muscles of infected pigs, infective larvae were recovered (Basyoni & El-Sabaa 2013), collected by sedimentation, and repeatedly washed in 0.85% physiological saline (Sarhan *et al.* 2021). The larvae were routinely stored in the TBRI laboratory by successive passage through mice according to the method outlined by (Gamble, 1996).

The mice were orally infected with *T. spiralis* larvae, as described by Dunn & Wright (1985). Each mouse's inoculum was adjusted to contain approximately 300 larvae (Attia *et al.* 2015).

Drugs

Reference drug

Albendazole suspension (ABZ) was procured from Pharma Cure Pharmaceuticals Company in Cairo, Egypt (Albendazole 400 mg/10 mL). ABZ was administered orally via gastric tube with an aqueous solution at a dose of 50 mg/kg for three consecutive days in the monotherapy group and at a dose of 25 mg/kg for three consecutive days in the groups that received combined treatment. The importance of the combination lies in reducing the side effects produced by ABZ by reducing its dosage and replacing it with a safer herbal drug (Attia *et al.* 2015).

Preparation of OL extract

Olibanum was purchased as solid, white-colored masses from the website (iHerb.com). The chemical identification was performed in accordance with Abdallah *et al.* (2011). First, OL gum was finely ground, and then 50 gm of the powder was dissolved in 200 mL of 70% ethanol for two days. After centrifuging the solution, the supernatant was evaporated to produce a sticky component. Subsequently, the crude extracts were recovered using a rotary evaporator. Finally, the extract was dissolved in dimethyl sulfoxide, an organic solvent, and stored at 4 $^{\circ}$ C (Jebelli *et al.* 2019). The extract was administered orally through a gastric tube at doses of 25 and 50 mg/kg/d according to Ammon (2010), who reported that oral doses to mice of 50 mg/kg and less were stimulatory while higher doses tended to show the opposite.

Experimental design

The experimental mice were divided into seven main groups as follows: (1) GI (healthy control, 10 mice)—healthy, uninfected, untreated mice; (2) GII (infected control, 20 mice)—infected untreated group; (3) GIII (OL25, 20 mice)—infected and treated with OL at a dose of 25mg/kg; (4) GIV (OL50, 20 mice)—infected and treated with OL at a dose of 50 mg/kg; (5) GV (ABZ 50, 20 mice)—infected and treated with ABZ at a dose of 50 mg/kg; (6) GVI (OL25+ ABZ 25, 20 mice)—infected and treated with OL at a dose of 25 mg/kg in addition to ABZ at a dose of 25 mg/kg; (7) GVII (OL50+ ABZ 25, 20 mice)—infected and treated with 50 mg/kg of OL and ABZ at a dose of 25 mg/kg.

Each of these seven groups was divided into two subgroups according to the euthanizing day: subgroup A [(mice were euthanized on the 6th day post infection (d.p.i.) (Intestinal phase)] and subgroup B [(mice were euthanized on the 35th d.p.i.) (Muscular phase)] (Shoheib *et al.* 2006). Treatment against the adult stage was initiated on the second d.p.i and on day 30 p.i against encysted larvae.

Euthanizing animals and sample collection

On days 6 and 35 p.i., mice were euthanized through cervical dislocation. Previously, the animals had been anesthetized using isoflurane (Etewa *et al.* 2020). The effects of the drugs on adult worms were evaluated using the small intestines of mice sacrificed on day 6 p.i (Intestinal phase). Approximately 1 cm of the mid-intestinal area was preserved in 10% formalin for histopathological analysis. The remaining intestine was utilized to count adult worms of *T. spiralis*. On day 35 p.i. muscle samples were obtained from mice sacrificed to evaluate the effects of the drugs on larvae (Muscular phase). Parasitological, histopathological, and

immunohistochemical (IHC) analyses were performed on intestinal and muscular samples.

Serial blood samples were acquired by the technique of retro-orbital sinus blood extraction using a capillary at 6, 14, 21, and 35 d. p.i. and collected in 2 mL Eppendorf tubes without anticoagulant. The sample was then centrifuged at 10^3 rpm for 10 min. The collected sera were stored at -20 °C until biochemical analysis (Muñoz-Carrillo *et al.* 2021).

Parasitological assessment

Isolation and counting of adult worms in intestines

The small intestines were separated, longitudinally opened, and washed with physiological saline. They were cut into 1-cm pieces and incubated at 37 °C in 10 mL physiological saline for 2 h (Elgendy *et al.* 2020) to permit adult worms to migrate from the intestine to the container (Basyoni & El-Sabaa 2013). Three washes were performed using physiological saline. The fluid was collected and centrifuged at 2000 rpm for 3 min (Elgendy *et al.* 2020). The supernatant was decanted and the sediment was examined in a few drops of saline under a dissecting microscope to count adult worms (Basyoni & El-Sabaa 2013). The worm reduction rate was calculated using the following formula:

$$\text{Worm reduction rate} = \left[\frac{\text{average number of parasites in the control group} - \text{average number in the treatment group}}{\text{average number of parasites in the control group}} \right] \times 100\% \text{ (Huang et al. 2020).}$$

Counting the number of encysted larvae in muscles

On day 35 p.i. mice were euthanized, and their muscles were extracted and cut into pieces. For digestion, these muscles were incubated at 37 °C for 1 h in a solution of 1% HCL, 1% pepsin, and 200 mL distilled water with continuous stirring (Elgendy *et al.* 2020). Next, using a 50- mesh/inch sieve, the digested muscles were filtered to remove large particles. The larvae were collected with a 200-mesh/inch sieve. The *T. spiralis* larvae were recovered and washed twice with distilled water before being suspended in 150 mL of tap water (Ashour *et al.* 2016). Under an inverted microscope, the number of sedimented larvae was determined (Esmat *et al.* 2021).

Biochemical assay

Biochemical assay of liver enzymes

The hepatic enzymes, aspartate transferase (AST) and alanine transferase (ALT), were measured using blood sera. In accordance with the test kit manufacturer's recommendations, the enzyme activity was represented as U/L (Adicon Clinical Laboratories, Shanghai, China).

Measurement of IL-10 in serum samples

Using a sandwich enzyme-linked immunosorbent assay (ELISA), IL-10 was found in serum samples from mice (ab108870-IL-10 mouse ELISA kit, UK). The amounts of IL-10 in serum samples were measured as directed by the manufacturer and expressed as pg/mL.

Histopathological examinations

At 6 d.p.i., intestinal specimens from each mouse (1 cm from the junction of the proximal 1/3 and distal 2/3) were dissected (Nassef *et al.* 2010). At 35 d.p.i., several sections were cut from diaphragm,

thorax, tongue, heart, and leg muscles of each mouse (Attia *et al.* 2015). All specimens were fixed in 10% formalin, dehydrated, and subsequently embedded in paraffin blocks. Formalin-fixed, paraffin-embedded sections (4 µm thickness) were prepared and stained with hematoxylin and eosin for routine histopathological examination.

At both the intestinal and muscular phases, the degree of inflammation was graded as follows: 0 (complete absence of inflammatory cells), 1 (mild inflammation), 2 (moderate inflammation), and 3 (severe inflammation) (Shalaby *et al.* 2010). For statistical purposes, scores 0 and 1 were pooled together as low inflammatory scores while scores 2 and 3 were pooled together as high inflammatory scores.

CD8⁺ IHC staining

Several sections were cut from the paraffin-embedded blocks, followed by deparaffinization in xylene and rehydration in a graded series of alcohol. Antigen retrieval consisted of boiling 10 mL of citrate buffer (pH 6.0) for 20 min, followed by cooling to room temperature. The slides were incubated with a purified rabbit monoclonal antibody to CD8 at room temperature for one night (cat no. ab209775, Cambridge, United Kingdom). Subsequently, using phosphate-buffered saline (PBS), the optimal dilution was 1:2000. All slides were deparaffinized with xylene and then rehydrated in ethanol solutions of decreasing concentration. Antigen retrieval was performed using microwave heating (20 min; 10 mmol/citrate buffer, pH 6.0) after inhibition of endogenous peroxidase activity (15 min of hydrogen peroxidase). After applying the primary antibody to the slides, they were rinsed with PBS overnight at room temperature in a humidity chamber. They were then incubated with the secondary antibody for 15 min before being washed again with PBS. Finally, a modified labelled avidin-biotin reagent was used for 20 min to detect the bound antibody, followed by a PBS rinse. The diaminobenzidine solution 0.1% was utilised as a chromogen for 5 min, and 5–10 min spent counterstaining the slides with Mayer's haematoxylin. Omission of the primary antibody served as a negative control (Attia *et al.* 2015).

Interpretation of IHC results for CD8⁺ T lymphocytic cells

The presence of a brown membranous stain in inflammatory T cells was considered positive in different studied groups (Eteawa *et al.* 2020). CD8⁺ T cells were quantified per 10 High Power Field and expressed as mean \pm standard deviation (SD). Furthermore, CD8⁺ marker expression was evaluated for: (1) Expression percentage: at 200X magnification, positive cells were counted and assigned a percentage exceeding 200 cells (Bahnassy *et al.* 2004); (2) Intensity of the stain: graded as mild (+), moderate (++) or strong (+++); (3) Histo-score (H score). The following formula was utilised to calculate the H score for all positive specimens. H score = 1 X % of cells with mild staining + 2 X % of cells with moderate staining + 3 X % of cells with strong staining; then a score of 0–300 was assigned (Aeffner *et al.* 2017).

Statistical analysis

All data were analyzed with SPSS version 22 (SPSS Inc., Chicago, IL, USA). All data sets were subjected to Shapiro–Wilk tests to ensure normal distribution. This study included both quantitative and qualitative data. Quantitative data are expressed as mean \pm standard deviation (SD). The Kruskal–Wallis test was used to estimate the difference between means of quantitative variables.

Qualitative data were expressed as number and percent (%). The chi-square test was used for comparison between qualitative data. The significance of differences between groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A *P* value <0.05 was considered statistically significant.

Results

Parasitological assessment results

Parasitological assessment results at 6 d.p.i

The average number of adult worms declined significantly (*P* < 0.05) across all treated groups. The mean adult count in GIIA was 127.7 ± 12.5 . Compared to GIIA, administration of OL50 mg in GIVA significantly reduced the mean number of adult worms (40.7 ± 2.9), with an acceptable drug efficacy of 68.1% (*P* < 0.05). The lowest number of adult worms (9.4 ± 3.1) with the highest drug efficacy of 92.6% was detected in GVIIA, whereas the least reduction was observed in GIIA with a drug efficacy of 53.7 % (Table 1).

Parasitological assessment results at 35 d.p.i

The average number of larvae in GIIB was 1428.3 ± 177.2 . OL 50 mg administration in GIVB resulted in a statistically significant reduction in the mean number of encysted larvae (324 ± 27.2) compared to GIIB (*P* < 0.05), with an acceptable drug efficacy of 77.3%. The most effective treatment with significant reduction in the average number of *T. spiralis* larvae was in GVIIB (72.7 ± 21.4), with 94.9% efficacy (*P* < 0.05). The least reduction was detected in GIIB, with an efficacy of 57.6% (Table 1).

Biochemical results

Liver enzymes measurement results

Throughout the experiment, a significant rise in ALT and AST levels was detected in GII. The administration of OL in GIII & GIV and ABZ in GV on days 6 and 35 p.i. significantly decreased (*P* < 0.05) the abnormal levels of ALT and AST in infected mice. The greatest reduction was realized in GIV (Table 2).

IL10 detection results

In GI, the mean serum concentration of IL-10 was extremely low (15.8 ± 1.2 pg/mL). IL-10 level was augmented starting at 6 d.p.i in all infected and treated groups. In GII, IL-10 was elevated on day 6 (205.1 ± 29.8 pg/mL), gradually increased until it reached a peak on day 21 (511.4 ± 27.5 pg/mL), and then decreased at the end of the study on day 35 (354.7 ± 30.6 pg/mL). In GIV, the mean IL-10 level at 6 d.p.i was 372.8 ± 27.3 pg/mL, and it increased until the end of the experiment at 35 d.p.i. (851.8 ± 24.9 pg/mL). Throughout the experiment, IL-10 levels rose significantly (*P* < 0.05) compared to GII. GV demonstrated elevated levels of IL-10 on day 6 (261.1 ± 23.2 pg/mL), day 14 (494.7 ± 23.1 pg/mL), day 21 (630.1 ± 28.4 pg/mL), and then declined at day 35 (562.6 ± 36.4 pg/mL). Throughout the study, these levels were significantly lower than those detected in GIV mice (*P* < 0.05) but significantly higher than in GII mice. Combined treatment with OL50 mg and ABZ 25mg in GVII showed a significant increase in IL-10 production when compared with all the studied groups (*P* < 0.05). Therefore, the administration of OL led to both a significant increase in IL-10 levels and a change in the pattern of sustained increase throughout the experiment (Figure 1).

Histopathological results

Histopathological results at 6 d.p.i

The histopathological examination of GIA revealed no pathological changes and normal architecture of villi. In the lamina propria and submucosa, GIIA evaluation revealed a dense chronic lymphoplasmacytic inflammatory infiltrate with few eosinophils, and all mice in this group had a high inflammatory score. A total of 40% of the mice had a low inflammatory score (*P* < 0.05) following administration of OL50 mg in GIVA. Compared to GII A, GVA demonstrated marked improvement and a significant reduction in the inflammatory score (*P* < 0.05). Compared to GIIA, GVIIA treated with OL 50 mg and ABZ exhibited significant tissue improvement, with an appearance similar to GIA and a significant reduction in the inflammatory score (*P* < 0.05) (Figure 2 & Figure 4a).

Table 1. Comparison between different studied groups regarding adult worm count at 6 d.p.i. and encysted larvae number at 35 d.p.i.

Studied groups	Adult worm count at 6 d.p.i.			Post Hoc	Encysted larvae number at 35 d.p.i			Post Hoc
	Range	Mean \pm SD	Efficacy (% of reduction)		Range	Mean \pm SD	Efficacy (% of reduction)	
GI (n = 5)	0	0.00 \pm 0.00	-	P1: 0.001**	0	0.00 \pm 0.00	-	P1: 0.001**
GII (n = 10)	110–145	127.7 \pm 12.5	-	P2: 0.001**	4870–7452	6293.5 \pm 981.2	-	P2: 0.001**
GIII (n = 10)	52–67	59.1 \pm 5.2	53.7 %	P3: 0.001**	2055–3148	2689.1 \pm 510.6	57.3%	P3: 0.001**
GIV (n = 10)	37–52	41.8 \pm 4.5	68.1%	P4: 0.001**	900–2075	1333.4 \pm 457.7	78.8%	P4: 0.001**
GV (n = 10)	5–20	12.1 \pm 4.7	90.5 %	P5: 0.001**	617–1360	951.2 \pm 216.2	84.9%	P5 = 0.084
GVI (n = 10)	5–18	11.1 \pm 3.9	91.3 %	P6: 0.001**	325–981	580.6 \pm 178.1	90.8%	P6: 0.001**
GVII (n = 10)	5–12	9.8 \pm 2.8	92.3 %		95–450	218.2 \pm 108.2	96.5%	
K		57.5				60.6		
P value		<0.05*				<0.05*		

n, number of infected mice; SD, standard deviation; K, Kruskal-Wallis test; *, statistically significant; **: Statistically highly significant

P1: Comparison between group II and Group III

P2: Comparison between group II and Group IV

P3: Comparison between group II and Group V

P4: Comparison between group II and Group VII

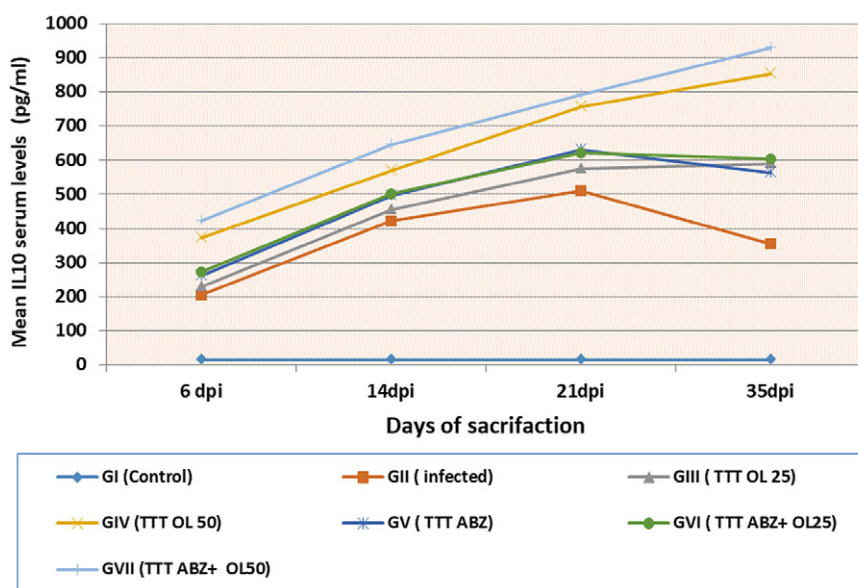
P5: Comparison between group IV and Group V

P6: Comparison between group IV and Group VII

Table 2. Comparison between different studied groups regarding concentrations of serum liver enzymes (ALT & AST) at 6 and 35 d.p.i.

The studied groups	Day 6 p.i.		Day 35 p.i.	
	ALT (μmL) Mean \pm SD	AST (μmL) Mean \pm SD	ALT (μmL) Mean \pm SD	AST (μmL) Mean \pm SD
GI (n = 5)	31.6 \pm 1.0	121.2 \pm 3.4	33.7 \pm 1.2	124.1 \pm 3.2
GII (n = 10)	55.8 \pm 1.9	184.8 \pm 2.7	63.6 \pm 1.4	383.0 \pm 2.3
GIII (n = 10)	45.4 \pm 1.9	153.5 \pm 1.8	44.8 \pm 2.6	256.2 \pm 2.5
GIV (n = 10)	35.8 \pm 2.2	136.7 \pm 2.1	34.2 \pm 4.8	215.5 \pm 3.1
GV (n = 10)	42.5 \pm 2.1	159.3 \pm 1.8	39.9 \pm 2.0	262.9 \pm 2.7
GVI (n = 10)	34.6 \pm 1.3	145.2 \pm 2.9	37.6 \pm 3.7	260.0 \pm 2.2
GVII (n = 10)	34.4 \pm 1.7	140.2 \pm 5.9	34.9 \pm 2.6	262.0 \pm 1.7
K	54.3	53.6	44.2	44.2
P value	<0.05*	<0.05*	<0.05*	<0.05*

n, number of infected mice; ALT, alanine transferase enzyme; AST, aspartate transferase enzyme; SD, standard deviation; K, Kruskal-Wallis; *, statistically significant.

**Figure 1.** IL-10 serum levels of mice in all studied groups at different times throughout the study. The results are represented by the mean \pm SD per each group.

Histopathological results at 35 d.p.i

The histopathological examination of GIB muscle tissues revealed normal muscle tissue. Whereas GIIB revealed many encysted *T. spiralis* larvae diffusely located in the sarcoplasm of muscle bundles and surrounded by a large number of chronic inflammatory cells comprising lymphocytes, plasma cells, eosinophils, and histiocytes. Moreover, 80% of the GIIB mice had a high inflammatory score. Mice administered 50 mg of OL had fewer encysted larvae than mice in GIIB. The majority of these larvae exhibited degenerative changes, including areas of capsule thinning and splitting into thin layers, areas of breakdown, vacuolization, and inflammatory cell infiltration. Compared to GIIB, this group's inflammatory score improved significantly (70% of mice had a low inflammatory score, $P < 0.05$). In contrast, encysted larvae were virtually nonexistent in GVIB. Furthermore, significant improvement in the inflammatory score was observed compared to GIIB (90% of mice had a low inflammatory score, $P < .05$). (Figure 3 & Figure 4b).

CD8⁺ IHC results

CD8⁺ IHC results at 6 d.p.i

GIA revealed a very low density of infiltrating CD8⁺ T cells. GIIA revealed a low CD8⁺ T cell infiltration density, with a mean \pm SD of 220.8 \pm 23.8 positive cells and a mean \pm SD of 105.0 \pm 14.1 for the CD8⁺ H score. GIVA demonstrated a significantly higher density of CD8⁺ T cells than GIIA. The density of CD8⁺ infiltration was significantly higher in GVIIA than in GIIA, with a mean \pm SD of 522.2 \pm 29.3 positive cells ($P < 0.05$) and a mean \pm SD of 239.0 \pm 16.4 for the CD8⁺ H score. The administration of ABZ alone resulted in a significant decrease in CD8⁺ infiltration density in GVA compared to GIIA (Figure 5 & Figure 7a,b).

CD8⁺ IHC results at 35 d.p.i

GIB was completely negative for CD8⁺ IHC. GIIB revealed a low CD8⁺ infiltration density. GIVB demonstrated a significantly higher density of CD8⁺ infiltration than GIIB, with a mean \pm SD

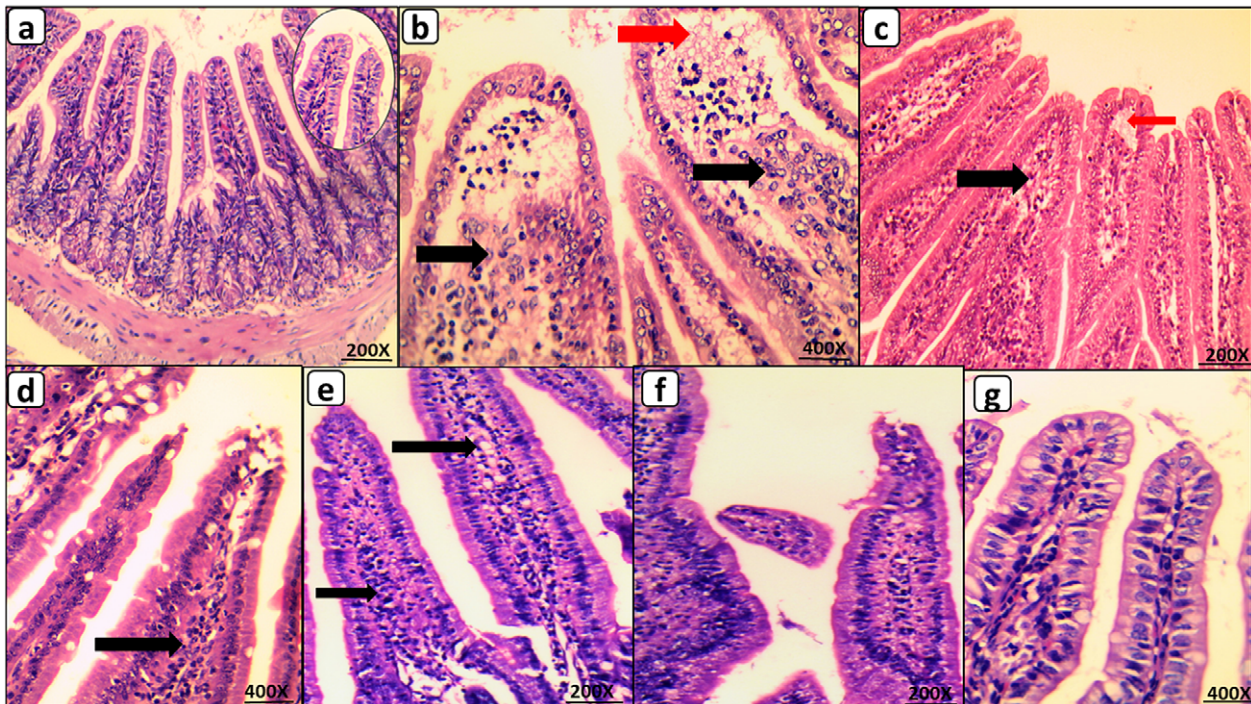


Figure 2. Intestinal phase of *T. spiralis* infected mice at 6 d.p.i. stained by H & E. (a) GI A with normal histology, the inset showed normal villous architecture; (b) GII A showing flattening of villi with widening of its core by oedema (red arrow) and dense chronic lymphoplasmacytic inflammatory infiltrate (black arrow); (c) GIII A with slight histological improvement with widening of core of many villi by oedema (red arrow) and chronic inflammatory infiltrate (black arrow); (d) GIV A exhibiting normal villous architecture with reduced number of inflammatory cells in villous core compared to infected group (black arrow); (e) GV A demonstrated marked improvement and a significant reduction in inflammatory infiltrate (black arrow); (f) GVI A with nearly similar morphology to GV A; (g) GVII A with normal villous architecture and marked reduction of lamina propria inflammatory infiltrate, (200X for A, C, E, & F and 400X for inset of A, B, D, & G).

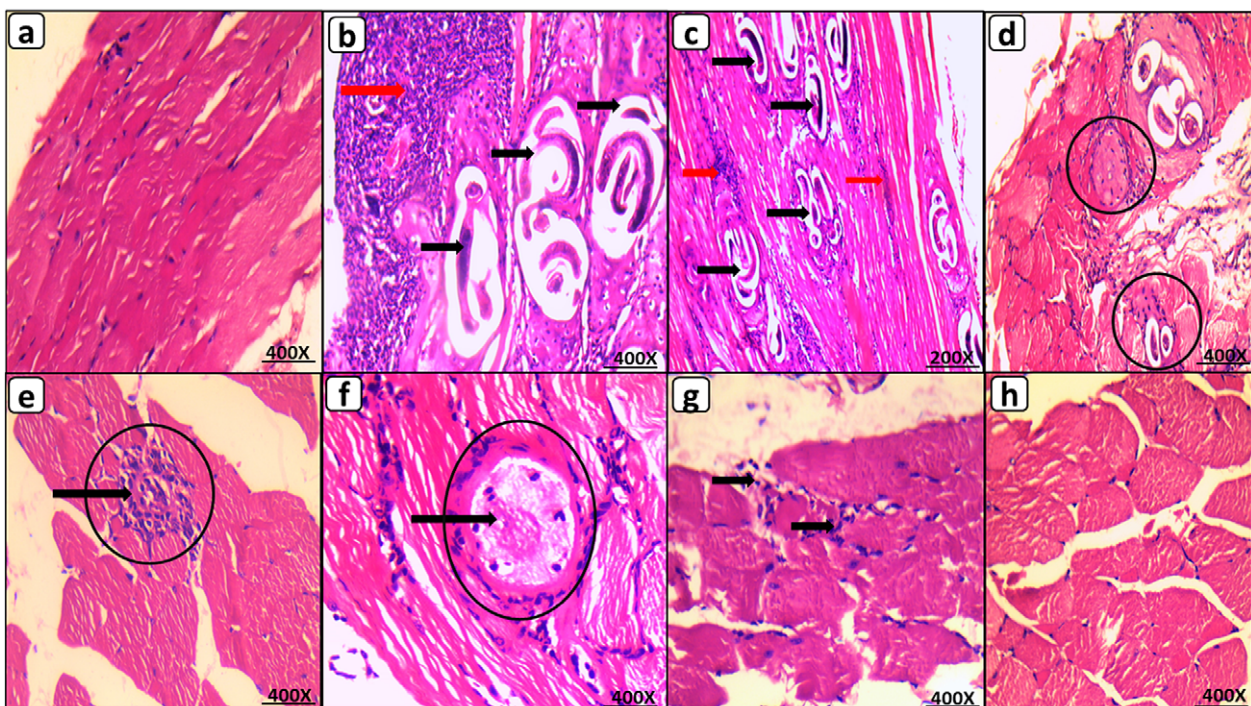


Figure 3. Muscular phase of *T. spiralis* infected mice at 35 d.p.i. stained by H&E. (a) GI B with normal muscle histology; (b) GII B showing multiple encysted *T. spiralis* larvae diffusely present in muscles sarcoplasm (black arrow) surrounded by many chronic inflammatory cells (red arrow); (c) GIII B showing similar histological picture to GII B with many encysted *T. spiralis* larvae (black arrow) surrounded by chronic inflammatory infiltrate (red arrow); (d) & (e) GIV B with reduced number of degenerated larvae (black circles) invaded by inflammatory cells (black arrow); (f) GV B with improved morphology showing markedly degenerated larvae (black circles) invaded by inflammatory cells (black arrow); (g) GVI B with nearly similar picture to GV B showing chronic inflammatory infiltrate within degenerated muscle bundles; (h) GVII B with restoration of near normal muscle histology and complete absence of larvae, (200X for C and 400X for A, B, D, E, F, G, & H).

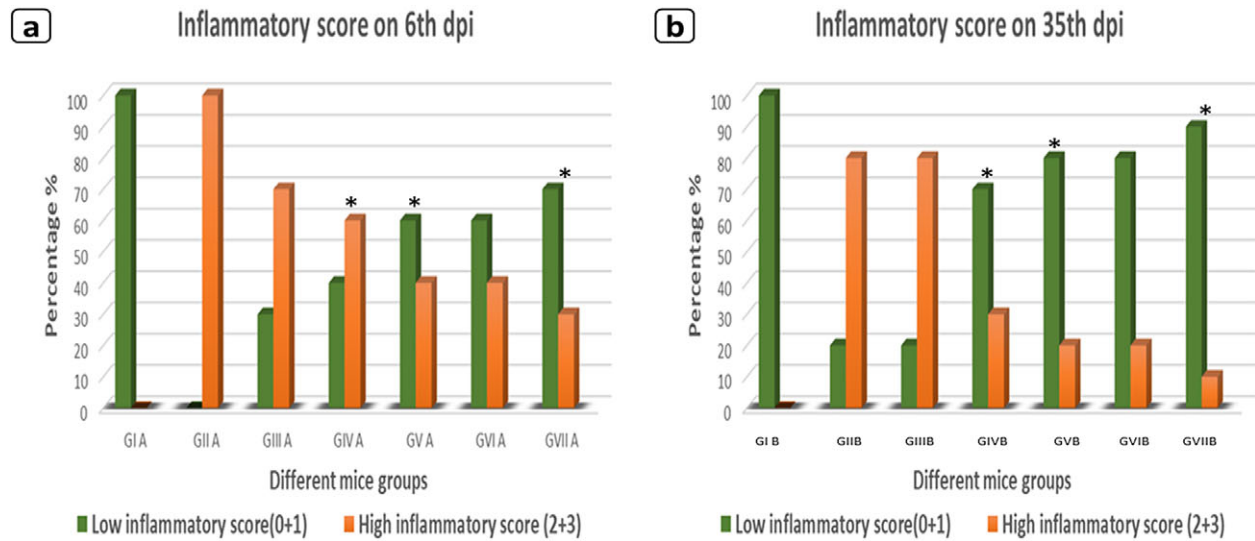


Figure 4. (a) Comparison between the studied groups regarding inflammatory score at 6 d.p.i. * $P < 0.05$ vs GII A; (b) Comparison between the studied groups regarding inflammatory score at 35 d.p.i. Data were expressed as percentage ($n = 5$ in GI B and 10 in all other groups). Post hoc test was used to make group comparison, * $P < 0.05$ vs GII B.

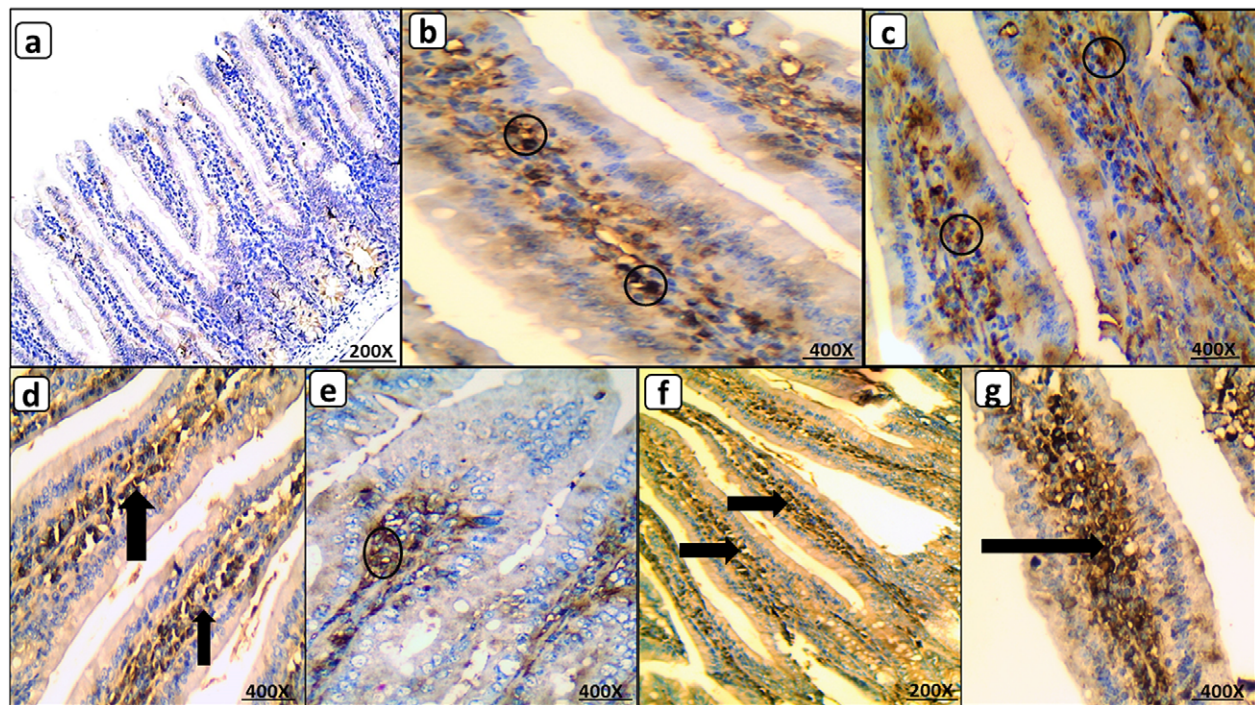


Figure 5. CD8⁺ IHC in intestinal phase. (a) GI A negative for CD8⁺ IHC; (b) GII A with low density of CD8⁺ T cells (black circles); (c) GIII A with few CD8⁺ T cells (black circles); (d) GIV A with high density of CD8⁺ T cells (black arrow); (e) GVA with low density of CD8⁺ T cells (black circles); (f) GVI A with increased CD8⁺ T cells (black arrow); (g) GVII A with dramatic increase of CD8⁺ T cells (black arrow), (IHC 200X for A & F and 400X for B, C, D, E, & G).

of 291 ± 33.1 positive cells and a mean \pm SD of 147 ± 23.5 for the CD8⁺ H score. Compared to GII B, the density of CD8⁺ infiltration increased dramatically and significantly in GVII B, where the mean \pm SD of positive cells was 505 ± 23.6 , and the CD8⁺ H score was 223 ± 25.7 . The administration of ABZ alone resulted in a significant decrease in CD8⁺ infiltration density in GVB compared to GII B (Figure 6 & Figure 7c,d).

Discussion

The standardization of trichinosis treatment has not yet occurred, and the curative efficacy of anti-parasitic drugs has not been convincingly demonstrated (Sun *et al.* 2019). Trichinosis is typically treated with benzimidazole derivatives, such as mebendazole and ABZ (Basyoni & El-Sabaa 2013). However, several limitations have emerged during its clinical use (Shalaby *et al.* 2010) such as

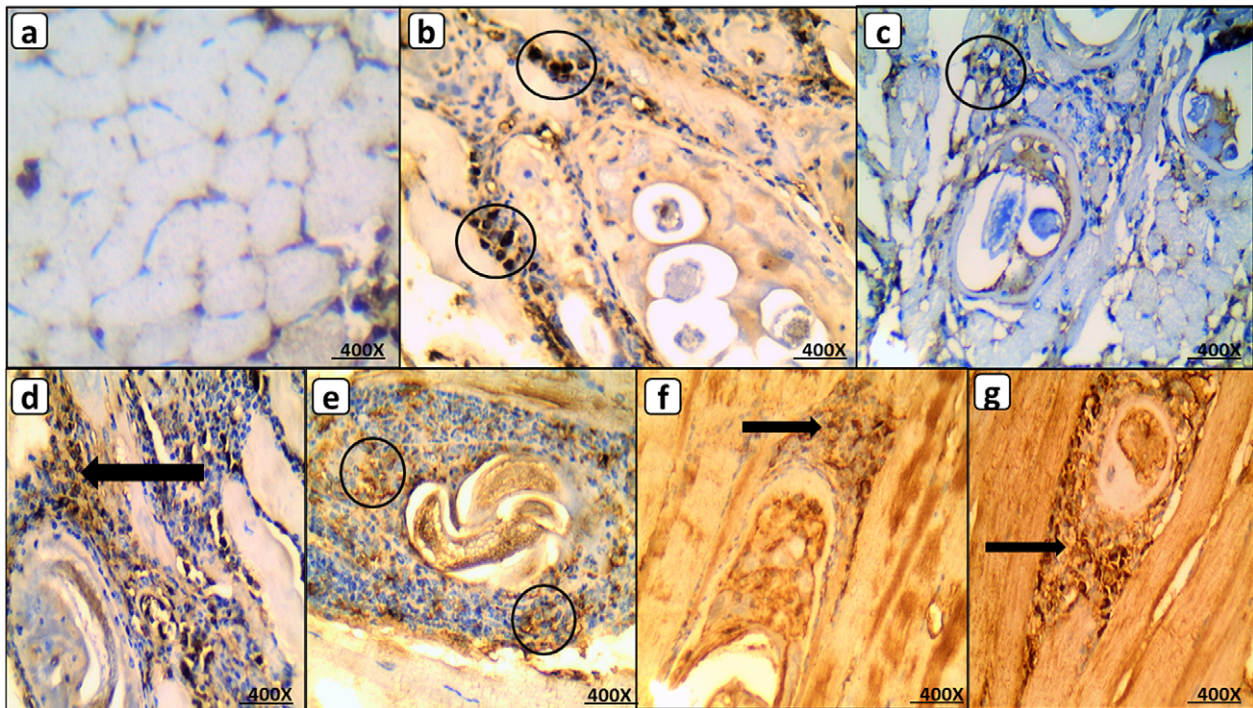


Figure 6. CD8⁺ IHC in muscular phase. (a) GI B negative for CD8⁺ IHC; (b) GII B with low density of CD8⁺ T cells (black circles); (c) GIII B with few CD8⁺ T cells (black circle); (d) GIV B with high density of CD8⁺ T cells (black arrow); (e) GV B with low density of CD8⁺ T cells (black circles); (f) GVI B with increased CD8⁺ T cells (black arrow); (g) GVII B with marked increase of CD8⁺ T cells (black arrow), (IHC 400X for all).

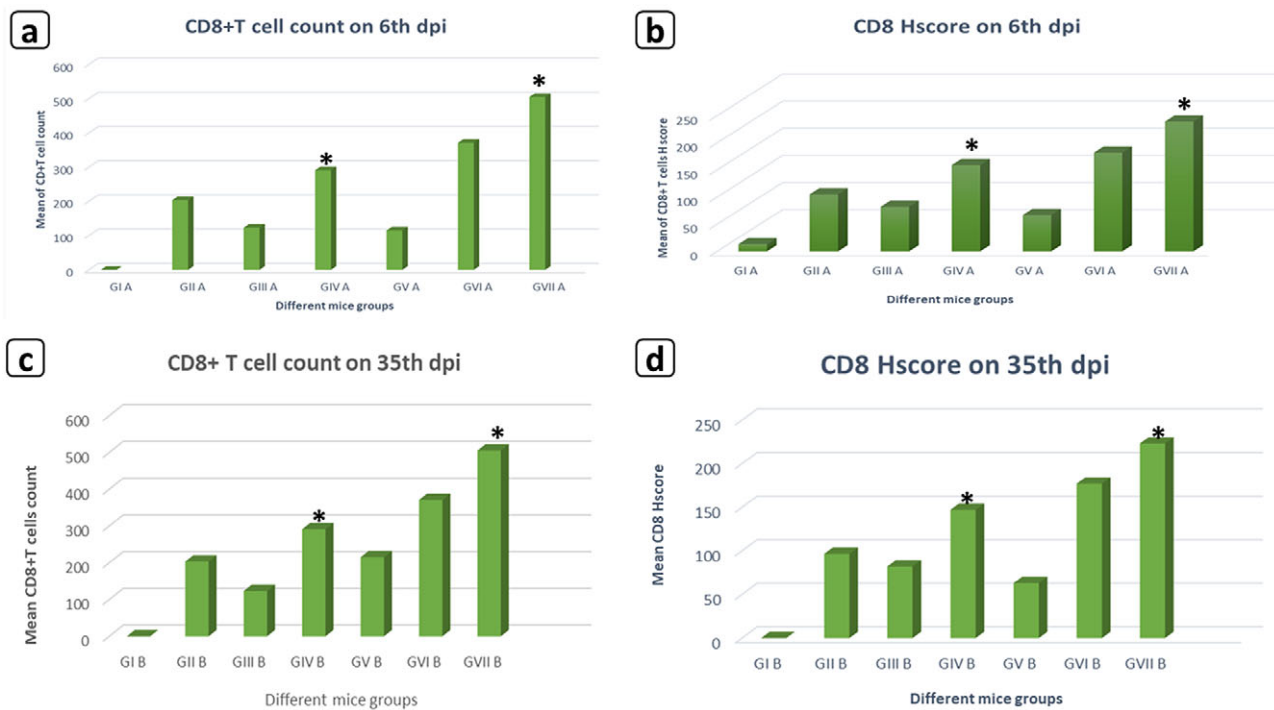


Figure 7. (a) Comparison between the studied groups regarding CD8⁺ T cells count at 6 d.p.i.; (b) Comparison between the studied groups regarding CD8⁺ H score at 6 d.p.i. * $P < 0.05$ vs GII A; (c) Comparison between the studied groups regarding CD8⁺ T cells count at 35 d.p.i.; (d) Comparison between the studied groups regarding CD8⁺ H score at 35 d.p.i. Data were expressed as mean \pm SD ($n = 5$ in GI B and 10 in all other groups). Post hoc test was used to make group comparison. * $P < 0.05$ vs GII B.

limited absorption and emerging resistance (Prichard 2007). In addition, severe systemic adverse drug reactions have been reported, while others suspect it to be a carcinogen (Shalaby *et al.* 2010; Yadav & Temjenmongla 2012).

Several studies have advocated using natural products (Yadav & Temjenmongla 2012), particularly those derived from well-tolerated herbal sources with minimal side effects (Gilleard & Beech 2007). One such product is OL, which has been used since antiquity as a treatment for many inflammatory conditions such as arthritis, asthma, and inflammatory bowel disease (Ammon 2016). In addition, it has antiparasitic activities against many protozoa, such as *Trypanosoma brucei rhodesiense* (East African Human Trypanosomiasis, sleeping sickness), *Plasmodium falciparum* (Tropical Malaria) (Schmidt *et al.* 2011), and *Giardia lamblia* trophozoites (Al-Ghandour *et al.* 2020). Nevertheless, very little is known about its anthelmintic activities. As far as we know and after a comprehensive review of literature, the effect of OL on *T. spiralis* has not been clarified. In the present study, the *in vivo* anti-parasitic and anti-inflammatory effects of OL alone or in combination with ABZ at different graded concentrations, 25 and 50 mg during both the intestinal and intramuscular phases of *T. spiralis* infection, was investigated.

A significant reduction in the parasite burden in intestinal and muscle tissues was observed in treated mice compared to infected-control mice. The highest efficacy of the drug with the lowest burden of adult worms and muscle larvae was observed in the group that received the combination of OL 50 mg and ABZ 25 mg. OL administration significantly reduced the number of adult worms and muscle larvae with higher efficacy in the muscular phase than the intestinal phase. In addition, stronger findings for OL 50 mg relative to OL 25 mg strongly suggests that OL has dose-dependent anti-trichogenic effects. In addition, the group treated with ABZ 50 mg also resulted in a significant decrease in the number of adult intestinal worms and the number of muscular larvae. Interestingly, the efficacy of the OL extract was more prominent in the muscular phase than in the intestinal phase, in contrast to ABZ, which showed a more prominent effect in the intestinal phase than in the intramuscular phase.

Prior literature from other disease models is consistent with our findings. Al-Ghandour *et al.* (2020) reported that the mean count of *Giardia* trophozoites in the intestinal wash of the OL-treated group was significantly less than the control-infected group. Abdallah *et al.* (2011) discovered that OL doses of 10, 15, and 20 mg/kg/day inhibited the multiplication of *G. lamblia* *in vivo* in a dose-dependent manner. In addition, Schmidt *et al.* (2011) reported that serratal (Oleoresin of *B. serrata*) has *in vitro* antiprotozoal activity against *Trypanosoma brucei rhodesiense* (East African Human Trypanosomiasis, sleeping sickness) and *Plasmodium falciparum* (Tropical Malaria).

Regarding liver function tests, the current research revealed that *T. spiralis* infection led to a significant elevation of ALT and AST levels throughout the course of the experiment. Elevated ALT and AST indicate liver damage, and this may be a consequence larval migration (Gamble *et al.* 1997) or detoxification of the drug and metabolites of the parasite stages (Abuelenain *et al.* 2021). Similar results were reported by Basyoni & El-Sabaa (2013) and Abuelenain *et al.* (2021).

A significant reduction in ALT and AST levels was observed in treated mice compared to infected-control mice. However, slightly different results were recorded by Abuelenain *et al.* (2021), who found that ALT was decreased in ABZ-treated mice, while AST was still elevated when compared to control-infected mice. In contrast,

Arise & Malomo (2009) noted that ALT and AST activities were significantly elevated in ABZ-treated animals and hypothesized that repeated administration of ABZ might impair hepatocyte integrity and affect their normal functions.

Notably, a greater decrease in the abnormal activities of ALT and AST was observed in mice treated with OL 50 mg than the decrease obtained by ABZ monotherapy. These results indicate that OL was less irritating to hepatocytes than ABZ, and this is inconsistent with Eltahir *et al.* (2020), who reported that *Boswellia serrata* (BS) may have hepatoprotective properties.

Histopathological examination revealed marked inflammation in intestinal and muscular tissues caused by *T. spiralis* infection. Several previous studies have reported similar results (Abou Rayia *et al.* 2017; Elguindy *et al.* 2019; Etewa *et al.* 2020; Nassef *et al.* 2018; Shalaby *et al.* 2010). The present study revealed an improvement in histopathological changes with both treatments in both phases of trichinosis as evidenced by a significant decrease in the number of inflammatory cells, a low inflammatory score, and normal villous architecture at the level of the intestine and a reduced number of degenerated larvae with their surrounding cellular infiltrates and increased regenerative muscles at the muscular phase. The most significant improvement was observed in mice treated with a combination of OL 50 mg and ABZ 25 mg, whose appearance was comparable to that of the non-infected control group in both phases. Of note, OL 50 mg had a superior effect to OL 25 mg. Interestingly, the efficacy of OL was greater in the muscular phase than in the intestinal phase, in contrast to ABZ, which demonstrated a greater effect in the intestinal phase than in the muscular phase, as demonstrated by previous ABZ studies (Caner *et al.* 2008; Prichard 2007).

Previous studies are consistent with our findings in other disease models. Al-Ghandour *et al.* (2020) reported that the histopathological changes caused by *Giardia* infection in the duodenum and jejunum improved in the OL-treated group. Liu *et al.* (2014) reported that a boswellic acid-containing extract reversed the *S. japonicum* egg-induced hepatic granulomatous inflammatory reaction and fibrosis in treated mice. Likewise, other authors have demonstrated that boswellic acid extracts mitigate pulmonary and colonic fibrosis in rats (Ali & Mansour 2011; Latella *et al.* 2008).

Notably, besides the anti-inflammatory effect of OL (Ammon 2016), another mechanism of action has been reported in that OL downregulates T helper1 (TH1, CD4⁺ T cells) and the secretion of pro-inflammatory cytokines (IL2, IFN- γ), while T helper2 (TH2, CD8⁺ T cells, also known as cytotoxic T cells) and the secretion of anti-inflammatory cytokines (IL-4, IL-10) are upregulated (Huang *et al.* 2022). The primary function of CD8⁺ T cells is believed to be the prevention of immunopathology during infection (Stäger & Rafati 2012). In addition, its secretory anti-inflammatory cytokine, IL-10, is required for an effective intestinal immune response as well as the reduction of local and regional inflammation during the early stages of muscle infection (Beiting *et al.* 2004).

For IL-10 serum level, measurements were taken on days 6, 14, 21, and 35 p.i. The present study demonstrated that post-infection IL-10 rose on day 6, gradually increased until it reached a peak on day 21, and then decreased on day 35 p.i. Similar results were observed by other authors who reported a significant increase in IL10 serum levels during early phase of *T. spiralis* infection (Bakir *et al.* 2017; Muñoz-Carrillo *et al.* 2021; Song *et al.* 2019). These observations contradict those of Dvorožňáková *et al.* (2011), who discovered that the production of IL-10 cytokine was moderately inhibited during the intestinal phase of *Trichinella* infections from day 5 to day 10 p.i. This disparity may be because IL-10 was

measured *in vitro* using splenocytes from mice infected with a low dose of larval infection (ten larvae of *T. spiralis*).

Administration of OL 50 mg significantly altered serum levels of IL-10 in the form of a significant increase that persisted until the end of the experiment. This pattern differed from that observed in the infected group. On the other hand, the ABZ-treated group also exhibited a significant increase in serum IL-10 levels, but these levels were significantly lower than those detected in OL-treated mice throughout the study and showed a similar pattern to those detected in the infected group. In contrast, the group that received a combination of OL 50 mg and ABZ 25 mg had the highest serum concentration of IL-10 in a similar pattern to the OL 50 mg monotherapy group.

Our findings of an increase in serum IL-10 levels in OL treated mice were supported by additional research. Marefati *et al.* (2020) demonstrated that pre-treatment with AKBA, one of the active ingredients in *Boswellia serrata* resin (OL), improved hippocampal IL-10 levels. Chevrier *et al.* (2005) stated that the delivery of the resin extract dissolved in sesame oil displays immunomodulatory characteristics *in vitro* and encourages a change from a TH1 response to a TH2 response in the cytokine production profile by murine splenocytes.

For CD8⁺ T cells IHC results, *T. spiralis* infection resulted in increase in CD8⁺ T cells expression in the infected tissues (intestine and muscle) compared to the healthy control mice. Likewise, Karmansaka *et al.* (1994) showed that numerous CD8⁺ T cells were observed in the intestines of mice infected with *T. spiralis*. Additionally, Gomez-Morales *et al.* (2002) observed *in vitro* increase in CD8⁺ T cell expression during the muscular phase of trichinosis in humans. However, Dvorožňáková *et al.* (2011) reported that the number of splenic CD8⁺ T cells in *T. spiralis* infected mice significantly elevated until day 10 p.i., i.e., during the intestinal phase, while the increase at the muscle phase did not occur until day 60 p.i.

Olibanum 50 mg administration either alone or in combination with ABZ 25 mg, significantly increased the mean number of CD8⁺ T cells in the affected tissues (intestine and muscle). However, this increase was greater during the muscular phase than during the intestinal phase. In contrast, ABZ monotherapy significantly reduced the density of CD8⁺ T cell infiltration compared to the infected group. This finding cannot be explained and warrants further study. According to Gomez-Morales *et al.* (2002), an increase in the number of CD8⁺ T cells is accompanied by an increase in the number of memory cells. Therefore, these findings could indicate that OL may have a protective effect against *T. spiralis* infection, a possibility which needs more research.

Conclusion and recommendations

Olibanum's anti-inflammatory and immunomodulatory properties make it an effective anti-parasitic agent against *T. spiralis* infection. OL is more effective at the muscular phase than the intestinal phase. OL has dose-dependent anti-trichogenic effects; as such, it may be considered a promising future therapy for *T. spiralis* infection. Therefore, additional research is required to standardize the dosage for an optimal anti-trichogenic effect. Also, OL induced an increase in CD8⁺ T cells and production of the anti-inflammatory cytokine IL-10. Thus, we suggest it may have an immunomodulatory effect in trichinosis, which warrants further investigation.

Acknowledgments. The authors wish to thank the Faculty of Medicine and National Liver Institute, Menoufia University, for providing most of the required facilities.

Financial support. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors, and all costs were paid by the authors.

Competing interest. The authors declare none. All authors have approved the submission of this manuscript. The results have not been previously published and are not being considered for publication in another journal.

Ethical standard. Following the approval of TBRI's institutional ethical committee, all animal care and procedures were conducted per the National Research Council's Guide for the Care and Use of Laboratory Animals. IRB No. 5/2022PATH8 was granted on behalf of the Ethics Committee for Scientific Research at the Faculty of Medicine, Menoufia University, Egypt.

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