Evaluation of the effect of Egyptian propolis on experimental trichinosis in mice: *In vitro* and *in vivo* studies

Original Article

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ABSTRACT

Background: Drug resistance to Albendazole (ALB) is common in treatment of trichinosis. Propolis proved an effective safe new therapy for treatment of several parasitic diseases.
Objective: To evaluate the effect of Egyptian propolis ethanolic extract on *T. spiralis* adult and muscle larva *in vitro* and *in vivo*.
Material and Methods: The *in vitro* study utilized adult worms and muscle larvae and included GI (control group), GII (propolis group), and GIII (drug combination group). Parameters used for evaluation were mortality rate and cuticle ultrastructural changes. The *in vivo* study utilized four groups of 160 mice that included GI (control group), GII (propolis group), GIII (drug combination group), and GIV (prophylactic group). Total adult and larval burden in intestine and muscles, biochemical, histopathology, and immunohistochemistry assessments were conducted.
Results: In a dose and time-dependent manner, propolis extract showed potent lethal effect on both adult and muscle larva during *in vitro* 24 h culture with LD90 of 2.58 mg/ml and 58 mg/ml, respectively. Scanning electron microscopy (SEM) showed that propolis extract exerted detrimental cuticle changes of adults and larvae. *In vivo* results showed a statistically significant reduction in the mean adult and larval burden, marked improvement of biochemical parameters, reduced inflammation, and fibrosis in intestinal and muscle specimens with lowered pro-apoptotic Bcl-2-associated X (BAX) protein expression especially when combined with ALB. Compared to propolis group, there was more reduction of the mean adult and larval burden in the prophylactic group with marked improvement of biochemical parameters, reduced inflammation and fibrosis in intestinal and muscle tissues.
Conclusion: Propolis extract is a promising prophylactic and effective anthelminthic alternative.

Keywords: Albendazole; Egyptian propolis; *in vitro*; *in vivo*; trichinosis; prophylaxis; SEM.

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INTRODUCTION

Trichinosis is a global zoonotic disease caused by the nematode *T. spiralis*. It is initiated by ingestion of raw or undercooked meat containing trichina capsules. Pork and its products still are the major infectious source of human trichinosis in developing countries. In fact, trichinosis is not only an important public health problem but also a tremendous threat to meat food safety[1]. The clinical picture reflects the parasite cycle in the human body. The intestinal phase is manifested by gastroenteritis, abdominal pain, diarrhea, nausea and vomiting, that may last up to one week. In 2–3 w after infection, the fertilized female deposits ~1500 newborn larvae that migrate, penetrate, and encapsulate in the host's skeletal muscle. An inflammatory reaction occurs with obvious manifestations that include fever, eyelid/facial oedema, myalgia, and eosinophilia[2]. Severe complications such as myocarditis or encephalitis may happen, particularly in the elderly, leading to death[2].

Host tissue damage in *T. spiralis* muscular phase is caused by inflammatory cells that produced high levels of reactive oxygen intermediates, and stress markers such as cyclooxygenase and transferase leading to muscle necrosis with severe damage and apoptosis. Acceleration of apoptosis transformed the skeletal muscle cell into a nurse cell[3]. Previous immunohistochemical studies showed that the apoptotic and the proapoptotic genes such as *p53* and *bax* genes, respectively were expressed in the nucleoplasm of basophilic cell in the cyst, suggesting involvement of these genes in nurse cell formation. The BAX protein induces apoptosis by forming membrane pore in mitochondria for cytochrome c to be released upon apoptotic signaling[4].

Mebendazole and ALB are the main anti-helminthic drugs used for treatment of trichinosis. They inhibit the microtubule polymerization through selective binding to beta-tubulin monomer of the parasite,

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with little effect on binding of host tubulin. ALB has many adverse effects such as encephalitis, epilepsy, eruptions, and even death[5]. Moreover, it shows diminished effectiveness against *Trichinella* capsule, limited bioavailability and high degree of resistance. Therefore, there is an urgent need to find other new effective and safe alternative treatments[6].

Propolis (bee gum) is a natural, non-toxic resinous substance that has been used worldwide in folk medicine since ancient times[7]. It is safe with a low oral toxicity of LD50 (2,000-7,300) mg/kg in mice; it was also administered orally to mice 4,000 mg/kg/d for 2 weeks with no side effects[8]. It is a complex mixture of botanical exudates collected by the bees from leaves, buds, and other plant tissues, mixed with pollen, wax, enzymes, and saliva of the bees themselves[9]. A typical resinous mixture of propolis is composed of 40–70% balsam (phenolic acids and flavonoids), 20–35% waxes, 1–3% aromatic and essential oils, and 5% other constituents such as vitamins, minerals, proteins, and enzymes[10]. Crude propolis cannot be used directly due to its chemical complexity and should be processed using suitable solvents to remove the inert material and preserve the compounds of interest[11]. The most common solvents used for extraction include water, methanol, ethanol, chloroform, dichloromethane, ether and acetone, with ethanol standing out as particularly effective in producing a propolis extract rich in bioactive compounds, mainly polyphenols which have various biological and therapeutic activities, with low wax content[11].

On the other hand, propolis exhibited pharmacological properties such as anti-inflammatory, antimicrobial, immunomodulatory, antioxidant, antifibrotic, anticancer, and antifungal[12]. It also showed anti-parasitic activity against several parasitic infections such as toxocariasis[13], giardiasis[14], schistosomiasis[15], acanthamoebiasis[16], trypansomiasis[17] and toxoplasmosis[18]. The present study aimed to determine the *in vitro* and *in vivo* efficacy of Egyptian propolis ethanolic extract as a natural component against *T. spiralis* adult and larval stages.

MATERIAL AND METHODS

This experimental case-control study was conducted at the Medical Parasitology Departments, Faculty of Medicine, Ain Shams University, and Theodor Bilharz Research Institute (TBRI) during the period from August, 2021 to March, 2022.

Study design: The study was conducted in two phases; *in vitro* and *in vivo*. The *in vitro* phase included incubation of *T. spiralis* adults and muscle larvae in culture medium with ALB (reference drug), propolis extract at different concentrations, and ALB-propolis combined treatment. In the *in vivo* phase, experimentally infected mice were treated by ALB, propolis extract, and the combined treatment. A fourth group of experimental mice was utilized to investigate propolis efficacy as prophylactic treatment. Parameters used include parasitological, ultrastructural, histopathological, biochemical, and immunohistochemical assessment.

Parasite and animals: The strain of *T. spiralis* was obtained from infected pork collected from Cairo abattoir and maintained in the animal house of TBRI by consecutive passages in mice. Parasite-free male Swiss albino mice, 6–8 weeks old, weighing 25–30 g each, were used. The animals were housed in proper cages and kept on a standard commercial pelleted diet with free accessible water. Mice were orally infected with 200 *T. spiralis* larvae[19].

Isolation of adult worms and muscle larva: Adult worms were obtained from the small intestine of infected mice 7 days post-infection (dpi). Overnight starved mice were sacrificed, the entire small intestine was removed, cut into 2-cm sections, and placed on gauze in a beaker containing 250 ml 0.85% saline for 3h at 37°C. After incubation, adult worms collected from the bottom of the beaker were washed three times by PBS and counted under a dissecting microscope[20]. Muscle larvae were recovered from the carcasses of infected mice 30 dpi by the artificial digestion technique. Muscles were immersed overnight in artificial digestive fluid composed of 100 ml saline, 1 ml concentrated HCl and 1 g pepsin at 37˚C under continuous mixing with a mechanical stirrer. The suspension was sieved then centrifuged at 1,000 rpm for 2 min to sediment the larvae. The collected larvae were washed several times with PBS and centrifuged. Sedimented larvae were microscopically counted using light microscope[6].

Propolis and reference drug: Propolis was purchased from honeybee colonies located in the Apiary of Beekeeping Research Department, Plant Protection Research Institute, Agriculture Research Center at Dokki, Giza, Egypt, while ALB was purchased as Alzental®, produced by the Egyptian International Pharmaceutical Industries, EIPICO, Egypt. For the *in vitro* study, ALB tablet was dissolved in PBS at a concentration of 20 µg/ml[21]. For the *in vivo* study, ALB was given in a dose of 50 mg/kg orally for 3 successive d starting from the 3rd dpi[6].

Preparation of propolis ethanolic extract: One hundred grams of the resinous material of propolis was cut into small pieces and extracted with 250 ml of 80% ethanol. The alcoholic extract was evaporated under vacuum at 50°C until dryness. Dried ethanolic extract of propolis (14 g yield) was suspended in PBS (pH 7.2) [22]. For the *in vitro* study, different concentrations of propolis (0.5, 1, 2.5, 5, 10, 20 mg/ml) were prepared[22]. For the *in vivo* study, propolis was given in a dose of 250 mg/kg/d orally using gavage tube for 45 dpi[23].

***In vitro* culture medium:** The collected *T. spiralis* adults and muscle larvae were incubated separately in a 24-well tissue culture plate containing an incubation medium consisting of Rapid Prototyping and Manufacturing Institute (RPMI-1640) medium (composed of 20% fetal bovine serum, 200 U/ml Penicillin and 200 µg/ml Streptomycin). Adults and muscle larvae were counted by light microscope and adjusted in RPMI-1640 medium at a density of 25 adults and 50 larvae per well[24].

Study groups: The following table demonstrates the study groups and subgroups.

***In vitro* lethal effect of propolis:** The experiment was carried out in triplicate, and data recorded from treated groups were compared with the results of the control groups. Samples were incubated at 37°C and 5% CO_2 for 48 h and examined at 1, 4, 6, 24, 36, and 48 h using an inverted microscope. At the end of the incubation intervals dead and living adults and larvae were counted. Dead worms and larvae appear C-shaped or linear with no movement[25]. The mortality percent (%) was calculated as follows: Mortality% = [M (Test) /M (Control)] X 100, where M (Test) and M (Control) were the number of dead parasites in the examined well and the total number of parasites in the control well (untreated parasite), respectively[26].

***In vitro* (Culture incubation)**
Adult
I**A (Control group)**
IAa (Infection control): Cultured in RPMI-1640.
IAb (ALB control): Treated with ALB (20 µg/mL) dissolved in PBS[21].
IAc (Solvent control): Cultured in PBS only.
IIA (Propolis extract-treated group): Treated with propolis extract dissolved in PBS at 4 different concentrations[22].
IIAa: 0.5 mg/ml
IIAb: 1 mg/ml
IIAc: 2.5 mg/ml
IIAd: 5 mg/ml
IIIA (Combined treatment group): ALB (20 µg/ml) plus propolis extract (5 mg/ml)[24].
Muscle larva
IM (Control group)
IMa (Infection control): Cultured in RPMI-1640.
IMb (ALB control): Treated with ALB (20 µg/ml) dissolved in PBS[21].
IMc (Solvent control): Cultured in PBS only.
IIM (Propolis extract-treated group): Treated with propolis extract dissolved in PBS at 4 different concentrations]22[.
IIMa: 2.5 mg/ml
IIMb: 5 mg/ml
IIMc: 10 mg/ml
IIMd: 20 mg/ml
IIIM (Combined treatment group): Treated with ALB (20 µg/ml) and propolis extract (20 mg/ml)[24].

***In vivo* (Experimental mice)**
Mice (n=160) were divided into four groups; I, II, III, and IV

	No.
I (Control group)	
Ia (Negative control): Non infected group.	10
Ib (Infection control): Infected control group.	30
Ic (Drug control): Treated with ALB (50 mg/kg) for 3 successive d starting from the 3rd dpi[6].	30
II: Treated with propolis extract (250 mg/kg/d) for 45 d starting from the 3rd dpi[23].	30
III: Treated with a combination of ALB (50 mg/kg) for 3 successive d starting from the 3rd dpi and propolis extract (250 mg/kg/d) for 45 d starting from the 3rd dpi[24].	30
IV: Propolis extract was given as prophylaxis 250 mg/kg/d starting 5 d before infection and continued till 45 dpi[23].	30

Ultrastructural assessment (SEM): At the end of the incubation period (24 h and 48 h) samples of worms and larvae were added to a fresh fixation solution of 2.5% glutaraldehyde (w/v) and incubated overnight at 4°C, then washed in 0.1M sodium cacodylate buffer for 5 min and post-fixed in 2% osmium tetroxide for 1 h. The samples were dehydrated in ascending grades of alcohols and dried using a critical point of carbon dioxide drying. After sputter coating with gold, they were examined by SEM (Jeol-Jsm-IT200)[27].

Mice sacrifice and obtained specimens

- On the 7th dpi, 10 mice from each infected group were anesthetized and sacrificed by cervical dislocation. The small intestine of each mouse was removed, opened longitudinally, and washed. About 1 cm from the intestine was preserved in 10% formalin and the rest of the intestine was used for *T. spiralis* adult worm count.
- On the 30th dpi, 3 ml venous blood sample from each mouse (10 mice in each group) was withdrawn for biochemical assessment.
- On the 45th dpi, 10 mice from each infected group were sacrificed, muscle samples (diaphragm) were removed and preserved in 10% formalin for the histopathological and immunohistochemical studies and the rest of the muscles were digested for total larval count.

Intestinal adult and muscle larval burdens: Collected adult worms[20] and muscle larvae[6] were counted under dissecting microscope. The treatment efficacy was calculated from the percentage of reduction in the adult worm or larval count using the following equation: Efficacy of treatment (%) = [(Nc-Nt) /Nc] x 100, where Nc is the mean number recovered in controls and Nt is the mean number recovered in treated mice[28].

Biochemical assessment: Sera were separated and used to determine therapeutic effect of propolis extract and ALB on serum biochemical parameters as renal parameters (urea and creatinine), liver parameters (AST and ALT), and muscle enzymes creatine phosphokinase (CPK).

Histopathological examination[29]: One cm from mid intestinal region (taken at the 7th dpi), and a piece of skeletal muscle (taken at 45th dpi), were fixed in 10% formol-saline, dehydrated in ascending grades of ethanol, and cleared in xylol. Impregnation was done in pure soft paraffin for 2 h at 55^{o}C then hard paraffin sections of 5 µ thickness were cut by microtome. Sections of intestine were stained by H&E stain to evaluate the intensity of the inflammatory cellular infiltrate within intestinal villi and the submucosa, and the ratio of villus/crypt. The small intestinal inflammatory response was assessed by examination of 10 high power fields (HPF×200) in each tissue section. The scoring criteria were as follows: 0: none, +1: less (up to 10 cells/HPF), +2: moderate (11–40 cells/HPF), +3: severe (more than 40 cells/HPF). Sections of muscle were stained by both H&E and Masson trichrome stains. The intensity of the inflammatory reaction around the muscle capsule was assessed as follow: +1: mild, +2: moderate, and +3: intense reaction[25]. The examination considered fibrous capsule integrity and thickness, larval appearance, and adjacent cellular inflammatory infiltrates.

Immunohistochemistry staining[30]: To investigate the expression of pro-apoptotic BAX protein, paraffin sections were cut into 5 µ thickness on poly L-lysine-coated slides. Sections were deparaffinized in xylene for 1 h, rehydrated in descending grades of alcohol, then incubated in hydrogen peroxide 3% for 5 min to allow blocking of endogenous peroxidase activity. Next, they were washed twice in PBS. Sections were placed in 0.01 mol/L citrate buffer (pH 6) and into a microwave for 5 min for antigen retrieval. This was followed by incubation with the primary antibody, rabbit polyclonal anti-BAX (ab53154) (1:100, IHC-P); purchased from Abcam®, Cambridge, MA, USA. The slides were then washed twice in PBS for 5 min each. The goat anti-rabbit IgG H&L (HRP) (ab205718) secondary antibody was applied, and the sections were again incubated for 20 min, then washed 3 times in PBS for 5 min each. Diaminobenzidine tetra hydrochloride solution was applied to the sections, and they were further incubated for 10 min. Sections were washed in distilled water and counterstained with Mayer's Hematoxylin for 2 min, followed by washing in distilled water, dehydration, clearing, and mounting by DPX. Paraffin-embedded mouse spleen tissue was used as a positive control. Negative controls were processed according to the same protocol, except for the use of the primary antibody.

Statistical analysis: Values of the measured parameters in this study were expressed as mean±SD. Statistical analysis was performed using the SPSS 22.0 software package. Student's *t*-test was performed to compare the means of treated groups with the control group, and analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD) as a post-hoc test in multiple groups were performed. The LD50 and LD90 were calculated using Probit regression analysis. The difference was considered statistically significant when $P<0.05$.

Ethical considerations: Mice were maintained under convenient conditions at the animal house in TBRI, following the recommendations of the National Institutes of Health Guidelines for Animal Experimentation. The research was approved from the Scientific Research Ethical Committee, Faculty of Medicine, Ain-Shams University, Cairo, Egypt.

RESULTS

***In vitro* effects of propolis extract on *T. spiralis* adult worms and muscle larvae:** The lethal effect of propolis extract on adult worms was both dose and time dependent. Propolis extract concentration from 0.5 to 5 mg/ml significantly increased adults mortality compared to the parasite control ($P<0.05$). *T. spiralis* adult worms 100% death rate was achieved after 24 h for IIIA, after 36 h for IIAb, IIAc, and IIAd, and after 48 h for IIAa. While LD50 and LD90 values after 6 h were 6.83 mg/ml and 19.25 mg/ml, respectively, and after 24 h they were 0.89 mg/ml and 2.58 mg/ml, respectively (Table 1).

The larval mortality rate of *T. spiralis* was both dose and time dependent. Compared with that of the controls, propolis extract had a statistically significant effect ($P<0.05$) on larval mortality rate at 2.5 mg/ml to 20 mg/ml. Complete death rate (100%) of muscle larvae was achieved after 24 h for IIIM. The LD50 and LD90 values after 6 h were 35.7 mg/ml and 137.37 mg/ml, respectively, whereas their values after 24 h were 15.54 mg/ml and 58 mg/ml, respectively (Table 2).

Ultrastructural findings: Results showed normal structure (Fig. 1A), and cuticle (Fig. 1B) of control IAa adult worms, and severe damage and ruptured cuticle in IAc (Fig. 1C). Minimal changes with small blebs were noticed in the cuticle of IIAa (Fig. 1D), while IIAb revealed moderate cuticular changes with

Table 1. *In vitro* effect of the Egyptian propolis on the mortality rate of *T. spiralis* adults.

Study subgroups	Time intervals					
	1 h	4 h	6 h	24 h	36 h	48 h
IAa	0.0±0.0	0.0±0.0	0.0±0.0	8.0±2	35.0±1.53	40.0±2
IAb	0.0±0.0	0.0±0.0	0.0±0.0	6.7±1.15	36.0±1.73	41.3.±2.08
IAc	1.3±0.58	36.0±2**	52.0±2**	93.3±1.15***	100±0.0***	100±0.0***
IIAa	0.0±0.0	0.0±0.0	0.0±0.0	22.7±1.53	96±1.73***	100±0.0***
IIAb	0.0±0.0	0.0±0.0	2.7±0.58	61.3±1.52**	100±0.0***	100±0.0***
IIAc	0.0±0.0	0.0±0.0	5.3±0.58*	86.6±3.05**	100±0.0***	100±0.0***
IIAd	0.0±0.0	18.7±2.08*	38.7±3.06**	98.7%±0.58***	100±0.0***	100±0.0***
IIIA	26.7±0.58*	66.7±1.53**	78.0±0.58***	100±0.0***	100±0.0***	100±0.0***
LD50			6.83	0.89		
LD90			19.25	2.58		

Data were expressed as the mean ± SD. **IAa:** Infection control; **IAb:** Solvent control; **IAc:** ALB (20 µg/ml); **IIAa:** Propolis extract (0.5 mg/ml); **IIAb:** Propolis extract (1.0 mg/ml); **IIAc:** Propolis extract (2.5 mg/ml); **IIAd:** Propolis extract (5.0 mg/ml); **IIIA:** ALB (20 µg/ml) and propolis extract (5 mg/ml); **LD:** lethal dose; ***:** Compared with the corresponding infection controls (P<0.05); ****:** Compared with the corresponding infection controls (P<0.01); *****:** Compared with the corresponding infection controls (P<0.001).

Table 2. *In vitro* effect of the Egyptian propolis on the mortality rate of *T. spiralis* muscle larvae.

Study subgroups	Time intervals					
	1 h	4 h	6 h	24 h	36 h	48 h
IMa	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
IMb	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
IMc	1.33±0.58	14.67±1.53*	30.67±2.51**	74.0±2.65***	83.33±3.06***	88.67±3.22***
IIMa	0.0±0.0	0.0±0.0	0.0±0.0	6.0±2	11.33±2.52*	32±3.61**
IIMb	0.0±0.0	0.0±0.0	4.67±0.58	10.67±1.15*	32.67±3.51**	49.33±4.73**
IIMc	0.0±0.0	1.3±0.58	10.0±2*	30.67±2.08**	49.33±2.52**	69.33±3.21***
IIMd	0.67±0.58	8.6±1.15*	29.33±1.53**	62.67±2.08***	72±3.61***	85.33±2.08***
IIIM	32±2.65**	52±2.65***	82.67±3.06***	100.0±0.0	100±0.0	100±0.0
LD50			35.7	15.54	9.77	4.91
LD90			137.37	58	46.21	28.45

Data were expressed as the mean ± SD. **IMa:** Infection control; **IMb:** Solvent control; **IMc:** ALB (20 µg/ml); **IIMa:** Propolis extract (0.5 mg/ml); **IIMb:** Propolis extract (1.0 mg/ml); **IIMc:** Propolis extract (2.5 mg/ml); **IIMd:** Propolis extract (5.0 mg/ml); **IIIM:** ALB (20 µg/ml) and propolis extract (5 mg/ml); **LD:** lethal dose; ***:** Compared with the corresponding infection controls (P<0.05); ****:** Compared with the corresponding infection controls (P<0.01); *****:** Compared with the corresponding infection controls (P<0.001).

body swelling (Fig. 1E). In addition, IIAc showed more erosions and erupted cauliflower mass (Fig. 1F). Severe erosions were observed in IIAd (Fig. 1G). Combined therapy in IIIA showed the most considerable damage causing rupture of the worm (Fig. 1H). On the other hand, IMa showed normal control larva (Fig. 2A) and normal cuticle (Fig. 2B). Cuticular severe damage occurred in IMc (Fig. 2C), while IIMa showed flattening of transverse creases (Fig. 2D), and IIMb revealed mild erosion with wide furrow spaces (Fig. 2E). Moreover, IIMc showed more widening in the longitudinal ridge and moderate destruction in the cuticle (Fig. 2F). Rupture and severe erosion of the cuticle were observed in IIMd (Fig. 2G). Combined therapy (IIIM) showed the most considerable damage (Fig. 2H).

***T. spiralis* adult worm count in the small intestine at 7 dpi:** The results showed a statistically significant (P<0.0001) reduction in the mean adult worms counts in all treated groups when compared to the infected control group (Table 3). The highest reduction (99.34%) was observed in Gr. III that received combined Propolis extract and ALB.

***T. spiralis* total larval count in muscles at 45 dpi:** Results of larval count in muscles and their percentage of reduction are shown in table (4). A statistically significant reduction occurred in the mean larval counts in all treated groups when compared to the infected control group (P<0.0001). The highest reduction was observed in Gr. III that received combined Propolis extract and ALB (98%).

Effect of drugs on serum biochemical parameters: All treated groups showed significant (P<0.001) decrease in serum level of AST, ALT, urea, creatinine and CPK when compared with their corresponding infected control group (Table 5).

Histopathological examination: Results revealed normal structure of the small intestine 7 dpi in Ia (Fig. 3A), while Ib showed severe inflammatory cellular infiltrate (+3) with increased villous/crypt ratio (Fig. 3B). Intestinal lesions in Ic showed significantly improved mild inflammatory infiltrate (+1) (Fig. 3C). In addition, mice of II and IV groups showed improved intestinal architecture with (+2) inflammatory cells (Fig. 3D and F), while III showed the most evident improvement of inflammatory infiltrate (+1) (Fig. 3E).

Normal muscle tissue morphology with thin fibrous streaks in Ia at 45 dpi (Fig. 4A H&E stained and 5A Masson trichrome stained). In Ib, there was intense inflammatory cellular infiltrations (+3) around intact

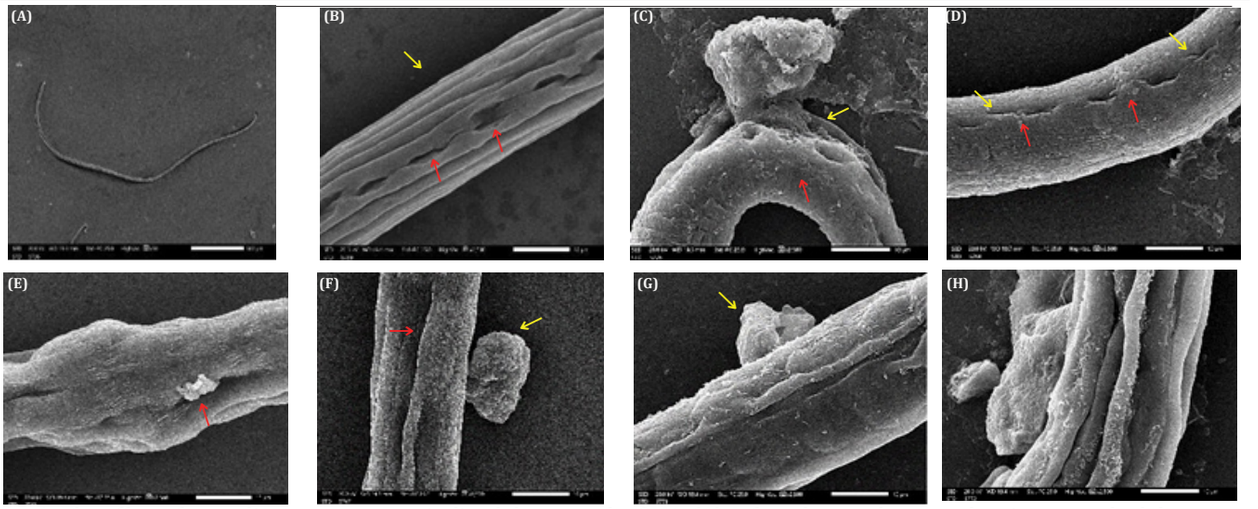

Fig. 1. The effect of propolis extract on *T. spiralis* adult worms by SEM. **(A)** Infected control group, showing normal adult worm. **(B)** Infected control group, showing normal cuticle with longitudinal ridges (yellow arrow) and hypodermal glands openings (red arrows). **(C)** ALB-treated group, showing severe destruction and rupture of the cuticle (yellow arrow) with sloughing (red arrow) and loss of normal annulations. **(D)** Propolis extract 0.5 mg/ml treated group, showing small blebs (red arrows) with disappearance of longitudinal ridges and narrow hypodermal glands pores (yellow arrows). **(E)** Propolis extract 1 mg/ml treated group, showing swelling of the body and sloughing of the cuticle (red arrow). **(F)** Propolis extract 2.5 mg/ml treated group, showing widening of longitudinal furrow space with erosions along the worm surface and large cauliflower mass (yello arrow). **(G)** Propolis extract 5 mg/ml treated group, showing multiple erosions, complete loss of the normal annulations and large vesicle (yellow arrow). **(H)** Group treated with Propolis extract 5 mg/ml and ALB combination, showing severe damage, fissures and rupture of the worm. Except for figure 1A (X50), all photos are X2500.

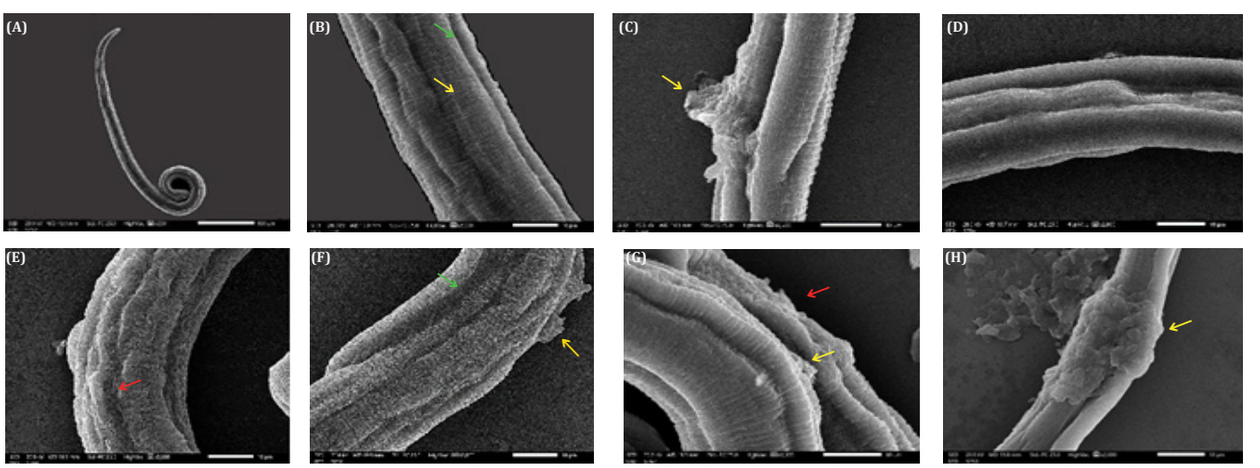

Fig. 2. The effect of propolis on *T. spiralis* muscle larva by SEM. **(A)** Infected control group, showing normal muscle larva. **(B)** Infected control group, showing normal cuticle with transverse creases (yellow arrow) and longitudinal ridges (green arrow). **(C)** ALB- treated group, showing rupture of the cuticle with large blebs (yellow arrow). **(D)** Propolis extract (2.5 mg/ml) treated group, showing flattening of the cuticle with loss of the transverse creases. **(E)** Propolis extract (5 mg/ml) treated group, showing erosion of the cuticle and widening of the longitudinal ridge's spaces (red arrow). **(F)** Propolis extract (10 mg/ml) treated group, showing destruction of the cuticle (yellow arrow) and wide longitudinal furrow space (green arrow). **(G)** Propolis extract (20 mg/ml) treated group, showing rupture and erosion of the cuticle (red arrow) with small blebs (yellow arrow). **(H)** Group treated with propolis extract (20 mg/ml) and ALB combination, showing severe destruction, rupture of the larva with large area of erosion (yellow arrow) and complete loss of normal annulations. Except for figure 2A (X250), all photos are X2000.

Table 3. Mean adult count of *T. spiralis* in the study groups in the small intestine at 7 dpi.

Study subgroups	Adult count		Reduction percent		
	Mean ± SD	P^a values	%	*F* test	P^b values
Ib	101 ± 5.3	-	-		
Ic	2.7 ± 0.6	<0.0001*	97.36		
II	19 ± 2.6	<0.0001*	81.19	691.039	<0.0001*
III	0.7 ± 0.6	<0.0001*	99.34		
IV	13.7 ± 1.5	<0.0001*	86.47		

Ib: Infected control; **Ic:** ALB-treated; **II:** Propolis extract-treated; **III:** Combination-treated; **IV:** Prophylactic group; P^a**:** Significant difference comparing with control using Student *t*-test; P^b**:** Significant difference comparing between groups using ANOVA test.

capsules of encysted larvae (Fig. 4B), and severe fibrosis with accumulation of collagen fibers in the capsules (Fig. 5B). Mice of both Ic and IV showed mild inflammatory reaction and fibrosis with degenerated capsules (Fig. 4C, 4F, 5C and 5F), while II showed moderate inflammatory reaction and fibrosis (Fig. 4D and 5D). The most evident reduction in inflammation and fibrosis was observed in III (Fig. 4E and 5E).

Table 4. Mean larval count of *T. spiralis* in the study groups in the muscle at 45 dpi.

Study subgroups	Larval count		Reduction percent		
	Mean ± SD	P^a values	%	*F* test	P^b values
Ib	9678.6±1690.5	-	-		
Ic	774.0±189.0	<0.0001*	92.00		
II	3316.4±461.9	<0.0001*	65.73	99.5	<0.0001*
III	194±65.8	<0.0001*	98.00		
IV	1782±766.9	<0.0001*	81.60		

Ib: Infected control; **Ic:** ALB-treated; **II:** Propolis extract-treated; **III:** Combination-treated; **IV:** Prophylactic group; P^a**:** Significant difference comparing with control using Student *t*-test; P^b**:** Significant difference comparing between groups using ANOVA test.

Table 5. Serum biochemical parameters (AST, ALT, urea, creatinine and CPK) in the study groups at 30 dpi.

Biochemical parameters	Study subgroups						Statistical analysis	
	Ia	Ib	Ic	II	III	IV	*F* test	*P* values
AST (U/ML)	123±2.2	194.4±11.3	140±7.9	164.2±13.7	137.4±8	158.4±7.4	38	<0.001
ALT (U/ML)	31.6±2.07	61.2±8.4	41.6±2.7	47.6±5.6	39.8±1.9	45.8±4.7	21	<0.001
Urea (mg/dl)	49.4±2.7	68.8±2.3	54.2±1.3	57.8±1.8	54±2	54.6±1.1	56.2	<0.001
Creatinine (mg/dl)	1.4±0.17	2.8±0.21	2.2±0.16	2.1±o.6	1.9±0.17	2.3±0.16	11.6	<0.001
CPK (IU/L)	52.4±1.5	338.8±121.7	123.2±2.8	135.4±11	122.6±2.5	135.2±10	18.8	<0.001

Ia: Non-infected non treated; **Ib:** Infected control; **Ic:** ALB-treated; **II:** Propolis extract-treated; **III:** Combined-treated; **IV:** Prophylactic group.

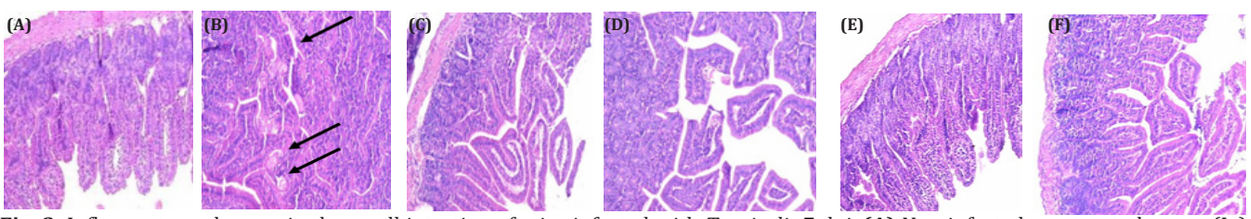

Fig. 3. Inflammatory changes in the small intestine of mice infected with *T. spiralis* 7 dpi. **(A)** Non infected non treated group (Ia) showing well preserved villi and normal inflammatory cellular pattern. **(B)** Infected control group (Ib) showing dense inflammatory mononuclear cellular infiltrate (lymphocytes, plasma cells and eosinophils) mainly in the core of the villi and extending into the submucosa with score (+3). Villi are flattened, distorted with focal erosions. Crypts hyperplasia, increase villous/crypt ratio, goblet cell hyperplasia and many cut sections in adult worms (arrows) are also noted. **(C)** ALB-treated group (Ic) showing more villous preservation and mild inflammatory infiltrate score (+1). **(D)** Propolis extract treated group (II) showing fewer cut sections in adult worms (arrows) and reduced inflammatory cells compared to the infected control group score (+2). **(E)** Group treated with propolis extract and ALB combination (III), showing absence of adult worms and well-preserved villi with evident reduction in the intensity of the inflammatory infiltrate score (+1). (F) Group that received Propolis extract as prophylaxis (IV) showing mild to moderate inflammatory infiltrate score (+2) (H& E×200).

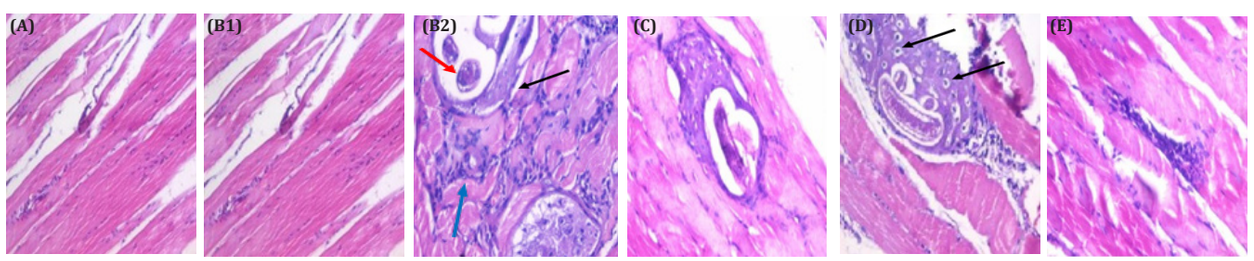

Fig. 4. Histopathological changes in the skeletal muscles (H&E) of mice infected with *T. spiralis* 45 dpi. **(A)** Non infected non treated group (Ia) showing normal muscle tissue morphology (x400). **(B1)** Infected control group (Ib) showing diffuse degenerative changes all over the muscle bundles with multiple encysted *T. spiralis* larvae (x100). **(B2)** Infected control group (Ib), showing a cyst with preserved capsule (black arrow) and larva (red arrows) surrounded by intense inflammation and massive inflammatory cellular infiltration (blue arrow) (x400). **(C)** ALB-treated group (Ic) showing smaller cyst surrounded by mild inflammation and small amount of inflammatory cell infiltration (x400). **(D)** Propolis extract-treated group (II) showing fewer *Trichinella* cysts with degenerated capsule, infiltrated by many macrophages (black arrow), and surrounded by moderate inflammatory cellular infiltrates (x400). **(E)** Combined treatment (III) showing remnants of degenerated cysts replaced by mild inflammatory infiltrate (x400). **(F)** Prophylactic group (IV) showing small cyst with degenerated capsule, dead degenerated larva, and mild inflammatory reaction (x400).

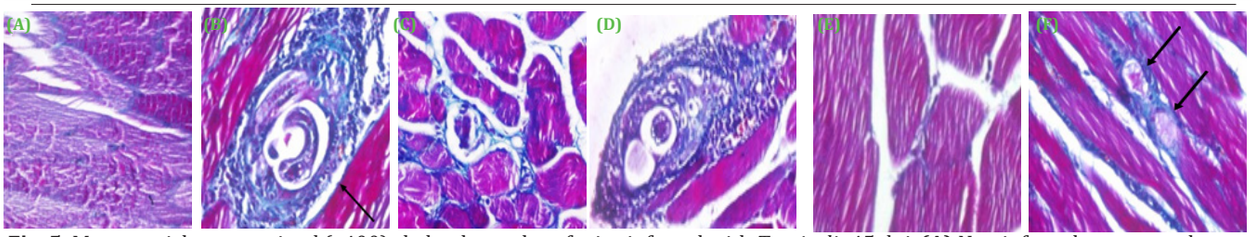

Fig. 5. Masson trichrome stained (x400) skeletal muscles of mice infected with *T. spiralis* 45 dpi. **(A)** Non infected non treated group (Ia) showing normal muscle tissue morphology with thin fibrous streaks between muscle fibers. **(B)** Infected-control group (Ib) showing preserved fibrous capsule (green to blue colored) (black arrow) with more collagen fibers accumulated. **(C)** ALB- treated group (Ic) showing less accumulated collagen fibers. **(D)** Propolis extract-treated group (II) showing degenerated capsule, infiltrated by many macrophages and moderate fibrosis. **(E)** Group (III) treated with combined propolis extract and ALB, showing evident decrease in fibrosis up to normal morphology. **(F)** Group (IV) received that propolis extract as prophylaxis showing degenerated cysts (black arrows) surrounded by mild fibrosis.

Immunohistochemical evaluation: After 45 dpi skeletal muscles revealed strong cytoplasmic BAX protein staining around capsule of encysted larva of Ib compared to the negative expression of BAX protein in Ia (Fig 6A, B). While mice of Ic and II showed moderate cytoplasmic expression of BAX protein with degenerated and infiltrated capsule (Fig. 6C, D), groups III and IV showed mild cytoplasmic BAX protein expression around degenerated cyst (Fig. 6E, F).

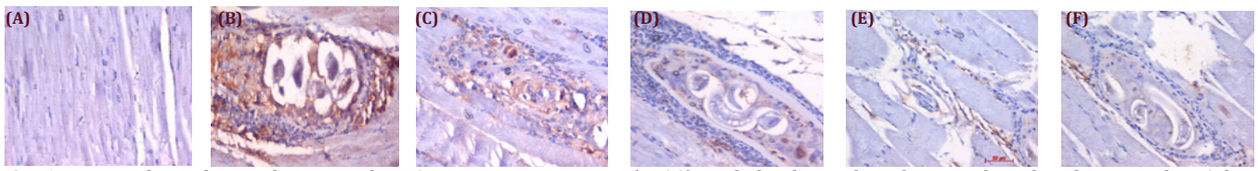

Fig. 6. Immunohistochemical staining for BAX protein expression (x400) in skeletal muscles of mice infected with *T. spiralis* 45 dpi. **(A)** Non infected non treated group (Ia) showing negative expression of BAX protein. **(B)** Infected control group (Ib) showing strong cytoplasmic expression of BAX protein staining in cellular infiltrates around encysted larva (brownish discoloration of cytoplasm). **(C)** ALB-treated group (Ic) showing degenerated cyst with moderate cytoplasmic expression of BAX protein in cellular infiltrates. **(D)** Propolis extract-treated group (II) showing encysted larva with mild to moderate cytoplasmic expression of BAX protein. **(E)** Group (III) treated with combined propolis extract and ALB, showing remnant of *Trichinella* cyst with degenerated capsule and contents surrounded by few inflammatory cells and macrophages with mild expression of BAX protein. **(F)** Group (IV) that received propolis extract as prophylaxis showing encysted larva with degenerated capsule infiltrated by few inflammatory cells with mild cytoplasmic expression of BAX protein. (x400).

DISCUSSION

The *in vitro* test with helminths is a valuable tool for discovering the anthelminthic properties and the mode of action of a specific agent[31]. In the present *in vitro* study, propolis extract showed potent lethal effect on adult *T. spiralis* with the concentration range used being based on multiple attempts to reach the optimum range, *i.e.*, as observed on adult *Toxocara vitulorum in vitro*[22]. The ethanolic extract of Foumban propolis (source: Cameroon) also showed remarkable dose-dependent lethal effect on *O. ochengi in vitro*[32]. Besides, Silva *et al.*[15] reported a complete reduction in the motility of adult *S. mansoni* incubated *in vitro* with Brazilian red propolis.

On the other hand, Siheri *et al.*[33] tested twelve samples of propolis (P1-P12) from different parts of Libya against *T. spiralis* adults, finding that samples P6 to P12 were inactive against the worms, while samples P1 to P5 inhibited adults' viability. These inconsistencies might be due to differences in the type of propolis, which mainly depend on the geographical origin and the different doses used. The bioactive compounds present in different propolis samples vary according to the botanical source, their geographic regions, and the period/season in which they are collected, all contribute to propolis' chemical composition[11]. Alday-Provencio *et al.*[34] reported that samples of propolis obtained during different seasons showed different levels in the biological activity against *G. lamblia* trophozoites, highlighting the value of the sample collected in the summer. Similar results were obtained when Regueira-Neto *et al.*[35] observed that sample of Brazilian red propolis collected in Pernambuco during the rainy season had better anti-leishmanial and anti-trypanosomal activities.

Regarding propolis effect on muscle larvae in our study, it also increased their mortality in a dose- and time-dependent manner. Essential oil from Brazilian red propolis showed larvicidal activity *in vitro* against larvae of *T. cati*[13]. Sama-Ae *et al.*[16] observed the anti-*Acanthamoeba* encystation, excystation and anti-adhesion activity of propolis ethanolic extract.

It is noticeable that propolis doses used against adult worms were lower than those used against muscle

larvae, which can be explained by finding that the adults were more susceptible to the propolis extract than larvae as they completely degenerated within 3 h at high doses (10 and 20 mg/ml). Consequently, we used lower propolis doses in the next attempt to calculate LD and examine the samples by SEM. This finding was also observed by El-Sayad *et al.*[36] who found that methanol extract of the flowers of *Chrysanthemum coronarium* (species of flowering plant in the family Asteraceae) showed a potentially higher lethal activity against *T. spiralis* adult worms than muscle larvae *in vitro*.

Moreover, propolis extract showed a synergistic effect when combined with ALB. Most of the pharmacodynamic studies suggested that propolis extract enhanced the drug efficacy. The synergistic action was related to strengthened action on interfering cell wall integrity and protein synthesis and reduction of chemoresistance via promoting the intracellular permeability of the chemotherapy[37]. Accordingly, the powerful synergistic effect of Spiramycin/propolis loaded chitosan/alginate nanoparticles on acute murine toxoplasmosis was reported[38].

We conducted a SEM analysis to determine whether propolis extract affected the cuticle of *T. spiralis* adult and larva after *in vitro* incubation. Results showed that propolis extract exerted detrimental structure changes in the cuticle of the incubated adults and larvae that were directly proportional to drug concentration. This destructive effect was the highest in propolis/ALB combination. Similarly, propolis extract exerted destructive effect on the tegument of adult *T. vitulorum*[22], facilitating penetration of the propolis to deeper-lying tissues leading to worm death. Sousa *et al.*[39] also suggested that vestitol, an isolated isoflavan compound from red propolis, had anti-*T. cruzi* activity through loss of membrane integrity. Similar results, *i.e.*, significant reduction in both adults and larvae, were recorded in the present *in vivo* study as well as propolis prophylactic effects. Issa[23] reported that immunosuppressed mice that received prophylactic treatment with Egyptian propolis, five days before infection with *S. mansoni* and 45 dpi, showed a significant reduction in the number of lung' schistosomula, adults and eggs in the feces, liver, and intestine. Sarhan *et al*.[40] also reported that the combination of selenium nanoparticles and propolis caused a significant reduction in adult and larval counts in murine trichinosis. It also had antiparasitic, anti-inflammatory and anti-angiogenic effects on trichinosis.

The antiparasitic activity of propolis extract is apparently related to the presence of saponins, tannins, flavonoids, terpenoids, polyphenols and alkaloids[32]. Prenylated flavonoids are responsible for improving the interaction with biological membranes and proteins[32]. The tannins would react directly with the surface proteins of the parasite resulting in a physiological dysfunction in the nematodes such as the mobility and the absorption of food, leading to death of the worm[32]. In contrast, Mahmoud *et al*.[41] observed that ethanolic extract of Egyptian propolis has no schistosomicidal activity *in vivo* when administered alone in mice infected with *S. mansoni*. However, a significant reduction in both lymphocytic infiltrates, and the number and diameter of parasite egg-induced granulomata were observed. In addition, the investigators reported that propolis administration combined with praziquantel, achieved a significant reduction in worm burden compared to groups treated with either compound alone.

The present results showed significantly increased levels of AST, ALT, urea, creatinine and CPK in infected control group as compared with non-infected healthy mice, which might be due to hepatic and renal damage caused by the migrating larvae. Unbalanced muscle metabolism, together with increased permeation through the muscle fiber membranes, cause a leakage of enzymes into the serum. The most common and marked increase is that of CPK[6]. The present study showed marked biochemical parameters shift toward normal values in all treated groups, that was previously reported[42].

The worm penetration of the gastrointestinal mucosa causes deformed villi, hyperplasia of the crypts of Lieberkühn and massive cellular infiltrates in the mucosal sublayer[43]. Moreover, during muscle-invasion stage, *T. spiralis* larvae destroy muscle fibers partially and stimulate the infiltration of inflammatory cells[2]. Our results of histopathological examination in propolis extract-treated group agreed with several studies[2,16,44-49]. Wang *et al.*[44] reported that flavonoid extract from propolis inhibited cardiac fibrosis through downregulation of fibrosis-related factors including collagen I, collagen III, matrix metalloproteinase-2 (MMP- 2), MMP-9 and TGF-β1. It possesses various mechanisms of action in modulating inflammation towards the regulatory balance, *i.e.*, balance between pro-inflammatory and anti-inflammatory activity[46]. Its anti-inflammatory activity can be explained by the presence of active flavonoids and cinnamic acid derivatives that can significantly suppress the lipoxygenase pathway of arachidonic acid metabolism and pro-inflammatory cytokines (such as IL-1β, IL-6, IFN-γ, and TNF-α)[46]. It also reduces the migration of immune cells such as macrophages and neutrophils by downregulating the chemokines CXCL9 and CXCL1[47], inhibits classical and alternative complement pathways and diminishes C3 protein functional activity[45].

In contrast, it exhibits immunostimulatory effects on macrophages, by increasing the ratio of CD4:CD8 cells in mice, stimulating TNF-α and IL-1β production, increasing cell receptors expression such as TLR-2 and TLR-4 and hydrogen peroxide generation by murine macrophages[48]. Thus, propolis extract and

its isolated components demonstrate a dual nature as immunostimulant, to prevent the infection, and immunomodulator to dampen the inflammatory state and counter the immune dysfunction after the onset of the disease, this is the most fascinating and promising effect[46]. This dual effect may be attributed to caffeic acid phenylethyl ester, an active component of propolis, that can suppress NF-β activation and reduce the level of pro-inflammatory cytokines. At the same time, it can increase antibody production, T-lymphocyte proliferation and IL-4 and IL-2 secretion, thus restore the balance between pro-inflammatory and anti-inflammatory mediators[16,49]. Similarly, when Rebouças-Silva *et al*.[48] evaluated the leishmanicidal and immunomodulatory properties of Brazilian green propolis extract, they observed reduction in most of the inflammatory mediators, increased expression of the antioxidant enzyme heme oxygenase-1 (HO1) and enhanced TNF-α level, which aids in controlling parasite load, suggesting the potential of propolis extract in orchestrating an effective and balanced innate immune response in the treatment of cutaneous leishmaniasis.

Our immunohistochemical study showed lowered BAX protein expression in muscle specimens in propolis extract treated group that agreed with other studies[49-51]. Tian *et al*.[50] reported that propolis extract reduced apoptosis in muscle cells by increasing Bcl2 expression, decreasing BAX protein expression, and suppressing isoprenaline metabolism. Accordingly, it inhibited skeletal muscle proteolysis and played an essential role in anti-skeletal muscle aging. Alm-Eldeen *et al*.[51] also reported that propolis extract can stimulate hepatic antioxidant defense, reduce pro-apoptotic p53 and increases anti-apoptotic Bcl2 expressions in aflatoxin-treated mice. Induction of apoptosis during trichinosis could be associated with increased oxidative stress and increased lipid peroxidation that induce the calcium channels in the cellular membrane to open leading to calcium overload in the tissue cells and cell apoptosis. Moreover, the increase in NO production induces apoptosis by changing the expression of members of the Bcl-2 family[49]. In contrast, propolis extract may inhibit oxidative stress by increasing antioxidant enzymes activity such as superoxide dismutase, glutathione peroxidase, and glutathione reductase in the liver and brain tissues[52]. The antioxidant property is due to phenolic compounds that donate hydrogen ions to free radicals to protect cells from oxidation reactions[7]. Besides, propolis possesses anti-apoptotic activity against normal cells, it acts as pro-apoptotic to some cancer cell lines[53]. It is a complex product with a broad spectrum of activity that goes far beyond the simple sum of its isolated components[46].

In conclusion, ethanolic propolis extract has a promising *in vivo* and *in vitro* anthelmintic activity and can be an effective alternative drug against both adult worms and muscle larvae of *T. spiralis*. It is safe, commercially, and is a naturally available product. It can be used as prophylactic supplement or in combination with ALB to increase its effect as it showed anti-inflammatory, antifibrotic and antiapoptotic effects.

Authors contribution: Abd El-Samee NM performed the practical part, analyzed the data, and wrote the manuscript. Younis TA shared in choosing the study topic, designing the plan of work, and revising the manuscript. El Deeb HK conceived and designed the plan of work, analyzed the data, and revised the manuscript. Abdel Rahman AA shared in study topic, analyzing the data, writing and revising the manuscript. All authors accepted the authorship and the manuscript before publication.

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