Efficacy of *Zingiber officinale* ethanol extract on the viability, embryogenesis and infectivity of *Toxocara canis* eggs

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Abstract This study evaluated the effect of *Zingiber officinale* (*Z. officinale*) ethanol extract on the viability, embryogenesis and infectivity *Toxocara canis* (*T. canis*) eggs. It was carried out both in vitro and in vivo. In the in vitro experiment, unembryonated *T. canis* eggs were incubated with 25, 50 and 100 mg/mL *Z. officinale* extract at 25 °C for 6, 12, and 24 h to assess the effect of *Z. officinale* on their viability and for two weeks to assess the effect of *Z. officinale* on their embryogenesis. In vivo experiment was performed to assess the effect of *Z. officinale* on infectivity of *T. canis* eggs. Treated embryonated eggs by *Z. officinale* extract at concentrations of 25, 50 and 100 mg/mL for 24 h were inoculated into mice and their livers were examined for the presence of *T. canis* larvae on the 7th day after infection and for histopathological evaluation at 14th day post-infection. *Z. officinale* showed a significant ovicidal activity on *T. canis* eggs. The best effect was observed with 100 mg/mL concentration after 24 h with an efficacy of 98.2%. However, the treated eggs by 25, 50 mg/mL of *Z. officinale* extract after 24 h showed ovicidal activity by 59.22 and 82.5% respectively. Moreover, this extract effectively inhibited *T. canis* eggs embryogenesis by 99.64% and caused their degeneration at the concentration of 100 mg/mL after 2 weeks of treatment. However, the lower concentrations, 25 and 50 mg/mL inhibited embryogenesis by 51.19 and 78.57% respectively. The effect of *Z. officinale* on the infectivity *T. canis* eggs was proven by the reduction of larvae recovery

in the livers by 35.9, 62.8 and 89.5% in mice groups inoculated by *Z. officinale* treated eggs at concentrations of 25, 50 and 100 mg/mL respectively. Histopathologically, the liver tissues of mice infected with *Z. officinale* treated eggs at the concentration of 100 mg/mL appeared healthy with slight degenerative changes of hepatocytes, opposite to that recorded in the infected mice with treated eggs by the lower concentrations. In conclusion; *Z. officinale* extract possessed dose-dependent anti-*T. canis* activity on the viability, embryogenesis and infectivity of *T. canis* eggs.

Keywords *Toxocara canis* · Eggs · Viability · Embryogenesis · Infectivity · *Zingiber officinale*

Introduction

Toxocara canis (*T. canis*), is a common roundworm of dogs. *T. canis* eggs excreted into the environment with dogs feces are unembryonated and uninfected then, become embryonated and infective within two to five weeks (Magnaval et al. 2001). The infected eggs can survive in the external environment up to years owing to their highly resistant to environmental conditions. It was proven that *T. canis* eggs resist varied disinfectants; glutaraldehyde (2%), benzalkonium chloride (10%), sodium hypochlorite (7%), potassium permanganate (1%), ethyl alcohol (70%), potassium hydroxide (10%) and phenol solutions (3%) (Ayçiçek et al. 2001).

Human infection is caused by ingestion of the embryonated eggs usually from contaminated raw vegetables and water or by eating infected raw meat containing encapsulated larvae. Larvae hatch within the small intestine, penetrate the enteric wall and migrate, via the blood stream, to

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the liver, lungs, muscles, eye and brain. Throughout their migration, they cause an intense inflammatory response and eosinophilia resulting in three clinical forms in humans: visceral toxocariasis, ocular toxocariasis, and covert toxocariasis. Dog ownership and geophagia are related to *Toxocara* infection (Magnaval et al. 2001; El-Sayed and Ramadan 2017).

The drugs available for visceral toxocariasis treatment are limited to albendazole, thiabendazole, tinidazole and diethylcarbamazine (El-Sayed and Ramadan 2017). One of the drawbacks of albendazole is its low water solubility, which can cause a low and/or variable bioavailability after oral administration (Torrado et al. 1996). Therefore, albendazole should be taken with a fatty meal (Dayan 2003). Although, the drug has been found to be safe and relatively well tolerated, it has some side effects, as well as nausea, dizziness, constipation, abdominal pain, headache, pruritus, alopecia and infrequently abnormal liver function tests and evoked acute and granulomatous hepatitis (Choi et al. 2008; Tas et al. 2012; Marin Zuluaga et al. 2013). Therefore, the investigation of plants employed by traditional medicine could be a strategy for finding the alternative treatments. In vitro and in vivo studies have proven that several medicinal plants extracts and their constituents exert significant antiparasitic activities (El-Sayed et al. 2012; El-Sayed and Safar 2014; El-Sayed and El-Saka 2015). These extracts act by interfering with parasites membrane integrity, DNA intercalation /alkylation, microtubules and neuronal signal transduction (El-Sayed 2009; El-Sayed et al. 2012; Wink 2012). Medicinal plants like *Lafoensia pacari* (Rogerio et al. 2008), *Chenopodium ambrosioides* (Reis et al. 2010) and *Nigella sativa* (Musa et al. 2011) were proven to have an anthelmintic effect against *T. canis*. Adedapo et al. (2007) also reported that aqueous crude extract of *Vernonia amygdalina* considerably reduced the fecal egg count of *T. canis*.

Zingiber officinale (ginger) belonging to the family *Zingiberaceae* is a perennial herb characterized by its versatile medicinal actions. Many experimental and clinical trials have established ginger for its therapeutic activities such as antiemetic, stomachic, expectorant, anti-inflammatory, antibacterial, antidiabetic, hypolipidemic and hepatoprotective activities (Imtiyaz et al. 2013). El-Sayed and El-Saka (2015) reviewed the antiparasitic activity of *Z. officinale*; it has a significant anthelmintic activity against *Schistosoma mansoni*, *Angiostrongylus cantonensis*, *Anisakis simplex*, *Dirofilaria immitis*, *Hymenolepis nana* and hydatid cysts either in vivo or in vitro. Also, it has a significant effect against protozoa like *Giardia lamblia*, *Toxoplasma gondii*, *Trypanosoma brucei* and *Blastocystis* species. Additionally, it has insecticidal, molluscicidal and anti-leech effects but, its

potential therapeutic effect against *T. canis* has not been tested. From this point of view, this study evaluated the effect of *Z. officinale* ethanol extract on the viability, embryogenesis and infectivity *T. canis* eggs.

Materials and methods

Z. officinale extract preparation

Fresh rhizomes of *Z. officinale* were obtained from a local market (Giza-Egypt), identified and authenticated at Medicinal and Aromatic Plants Department; Horticulture Research Institute, Giza-Egypt. Fine powder of air-dried rhizomes (200 g) was mixed with 1000 mL ethanol (95%) and left at room temperature for 72 h. with frequently shaking. The mixture was filtered and concentrated by evaporation and lyophilized. Tween-20 (10%) was used to dissolve the extract at concentrations of 25, 50, 100 mg/mL (Bardi et al. 2013).

Toxocara canis eggs

Unembryonated *T. canis* eggs were obtained from the uteri of adult female worms that were collected from the intestines of naturally infected stray dogs. The obtained eggs were suspended in distilled water and purified by straining through a sieve with 0.5 mm pores and washed with saline several times by centrifugation at 500 g for 3 min. Then, distilled water was added to the sediment and the number of eggs was determined using the hemocytometer to obtain a concentration of 1000 eggs/mL.

Embryonation of the eggs was induced by keeping unembryonated eggs in 0.5% formol-saline solution up to 4 weeks at 28–30 °C in Petri-dishes with aeration and shaking to prevent eggs' sticking. Then, embryonated eggs suspension was centrifuged and washed 3 times with distilled water to remove the formalin after that their number was adjusted to be 1000 eggs/mL.

The effect of *Z. officinale* extract on *T. canis* eggs was performed using three experiments

Experiment (I)

It was performed in a sterile 96-well microtitre plate. One hundred microliters of *T. canis* unembryonated eggs suspension was added to each well, then 100 µL of each concentration of *Z. officinale* extract were added into these wells. For the control, 200 µL of *T. canis* eggs suspension were used. Then, the plate was sealed and incubated at 25 °C for different incubation periods (6, 12 and 24 h).

Fig. 1 *T. canis* eggs **a** unembryonated viable egg, **b** dead egg after exposure to *Z. officinale*, **c** embryonated egg, **d** inhibit embryogenesis after exposure to *Z. officinale*

After each incubation period, the efficacy of *Z. officinale* extract on the viability of *T. canis* eggs was assessed by counting the number of viable eggs using the hemocytometer and eosin (0.01%), viable eggs (Fig. 1a) remained clear whereas nonviable eggs (Fig. 1b) were stained. This experiment was done in triplicates.

Efficacy (%) = $100 \times (\text{mean viable eggs in controls} - \text{mean viable eggs in the treated eggs by } Z. \text{ officinale}) / \text{mean viable eggs in controls}$.

Experiment (II)

This experiment assessed in vitro effect of *Z. officinale* on the embryogenesis of *T. canis* eggs. It was performed as the experiment (I) except the microtitre plate was incubated at 25 °C for two weeks. The number of embryonated eggs was counted and the percentage inhibition of embryogenesis was calculated using this formula: Inhibitory percentage (%) = $\text{No. of embryonated eggs in controls} - \text{no. of embryonated eggs exposed to } Z. \text{ officinale} / \text{No. of embryonated eggs in controls} \times 100$.

Experiment (III)

This animal experiment was done to assess the effect of *Z. officinale* on the infectivity of *T. canis* embryonated eggs. It was conducted on 75 Swiss albino mice, 8 weeks old, weighing ~25 g, according to the institutional guidelines of feeding, drinking, lighting and temperature. Mice were proved to be free from any parasitic infections by examining their stool on three successive days using the formol-ether concentration methods. They were classified into five groups (GI-V); GI was normal control, GII was infected mice with 1000 embryonated *T. canis* eggs (Fig. 1c) via the

oral route (infected control), GIII-V were infected by 1000 embryonated eggs treated with *Z. officinale* at concentrations of 25, 50 and 100 mg/mL for 24 h, respectively, before mice inoculation. Seven days after infection, 10 mice from each group were sacrificed and their livers were examined for the presence of *T. canis* larvae. At the 14th day post-infection (p.i.), the remaining mice were sacrificed and their livers were examined histopathologically.

Assessment of mice infectivity with *T. canis* eggs treated with *Z. officinale*

T. canis larval counts in the liver

The sliced liver tissues (0.5 g) were digested in pepsin-HCl solution (0.5 g pepsin, 0.7 mL HCl, and 100 mL water). After incubation at 37 °C for 24 h, the digests were filtered through a sieve. The sediments were centrifuged and examined for the presence of *T. canis* larvae. Then, the larvae were counted using a light microscope at 10× magnification (Horiuchi et al. 2005). Efficacy of *Z. officinale* on the infectivity with *T. canis* eggs was determined by the count reduction of recovered larvae, using this formula: Efficacy (%) = $100 \times (\text{mean larvae count in infected controls} - \text{mean larvae count in infected mice by the treated eggs}) / \text{mean larvae count in infected controls}$.

Histopathological investigation

Liver tissues were fixed separately in 10% buffered formalin, dehydrated in different concentrations of alcohol, cleared with xylol, and embedded in paraffin blocks. Tissue sections of 5 µm thicknesses were stained with hematoxylin and eosin (H&E) and examined under the light microscope at 10×, 40×, 100× magnifications for the presence of any pathological changes resulting from migration of *T. canis* larvae.

Statistical analysis

Data were presented as a percentage of inhibition/efficacy and mean ± standard deviation (M ± SD). The means were analyzed by one-way ANOVA followed by the Student's *t*-test (*t*). *P* value < 0.05 was considered statistically significant. All statistical procedures were carried out using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Ethical considerations

Animals' experiment was performed according to EU Directive on the Protection of Animals used for Scientific Purposes (2010/63/EU) and this study was approved by

Research Committee, Research Institute of Ophthalmology, Giza-Egypt.

Results

Effect of *Z. officinale* ethanol extract on the viability of unembryonated *T. canis* eggs

The results showed that the ethanol extract of *Z. officinale* in the tested concentrations (25, 50, 100 mg/mL), showed ovicidal activity on unembryonated *T. canis* eggs (Fig. 1b). The best effect was observed with 100 mg/mL concentration after 24 h, where the efficacy was 98.2%. However, the treated eggs by 25, 50 mg/mL of *Z. officinale* extract after 24 h showed ovicidal activity by 59.22 and 82.5% respectively. The effects of all concentrations showed statistically significant differences compared to the controls and these differences were concentration and time-dependent (Table 1).

Effect of *Z. officinale* ethanol extract on the embryogenesis of *T. canis* eggs

Treatment of *T. canis* eggs by 100 mg/mL of *Z. officinale* extract inhibited embryogenesis by 99.64% and caused degeneration of these eggs after 2 weeks. However, the treated eggs by 25, 50 mg/mL of *Z. officinale* extract inhibited embryogenesis by 51.19 and 78.57% respectively and some of these eggs developed into larvae despite being damaged (Table 2, Fig. 1d). Comparing to the controls, there was a significant difference in relation to the tested extract, in which this difference was concentration-dependent.

Effect of *Z. officinale* ethanol extract on the infectivity of *T. canis* eggs

Estimation of *T. canis* larval counts in the liver

The data in Table 3 revealed the mean of larval counts in livers of the different experimental groups. In GII (mice

Table 1 In vitro effects of different concentrations of *Z. officinale* ethanol extract on the viability of unembryonated *T. canis* eggs (TcE) for different incubation periods

Experiment (I) (No = 3)	Duration of exposure (h)					
	6 h		12 h		24 h	
	Viable eggs M ± SD	Efficacy %	Viable eggs M ± SD	Efficacy %	Viable eggs M ± SD	Efficacy %
TcE only (control)	99.3 ± 0.57	–	93.6 ± 2.51	–	87.3 ± 3.51	–
TcE + <i>Z. officinale</i> 25 mg/mL	65 ± 6.55*	34.5	45.6 ± 4.50**	51.3	35.6 ± 2.51**	59.22
TcE + <i>Z. officinale</i> 50 mg/mL	41.3 ± 5.13**	58.4	25 ± 1.7**	73.3	15.3 ± 2.08**	82.5
TcE + <i>Z. officinale</i> 100 mg/mL	15.6 ± 2.51**	84.3	6.3 ± 1.52**	93.2	1.6 ± 0.57**	98.2

* Statistically significant difference in comparison to the controls ($p < 0.001$)

** Statistically significant difference in comparison to the controls ($p < 0.0001$)

Table 2 In vitro effects of different concentrations of *Z. officinale* ethanol extract on the embryogenesis of *T. canis* eggs (TcE) after two weeks

Experiment (II) (No=3)	Duration of exposure (2 weeks)		P value
	No. of embryonated eggs M ± SD	Inhibitory %	
TcE only (control)	84 ± 2.64	–	–
TcE + <i>Z. officinale</i> 25 mg/mL	41 ± 7.90	51.19	0.0009**
TcE + <i>Z. officinale</i> 50 mg/mL	18 ± 2.00	78.57	<0.0001**
TcE + <i>Z. officinale</i> 100 mg/mL	0.3 ± 0.57	99.64	<0.0001**

** Statistically significant difference in comparison to the controls

Table 3 The mean larval counts in the livers of mice infected with *Z. officinale* treated *T. canis* eggs at concentrations of 25, 50, 100 mg/mL for 24 h

Experiment (III) Mice groups (No = 10)	Larval counts in the livers		
	Mean $\pm$ SD	Efficacy %	<i>P</i> value
GI: (Non-infected)	0 $\pm$ 0	–	–
GII: (untreated <i>TcE</i>)	62.1 $\pm$ 10.72	–	–
GIII: (<i>TcE</i> + <i>Z. officinale</i> 25 mg/mL)	39.8 $\pm$ 6.92	35.9	<0.0001**
GIV: (<i>TcE</i> + <i>Z. officinale</i> 50 mg/mL)	23.1 $\pm$ 5.48	62.8	<0.0001**
GV: (<i>TcE</i> + <i>Z. officinale</i> 100 mg/mL)	6.5 $\pm$ 2.91	89.5	<0.0001**

** Statistically significant difference in comparison to the infected controls (GII)

were infected by 1000 embryonated *T. canis* eggs), the mean larvae count was 62.1 ± 10.72 . On giving treated eggs with *Z. officinale* extract, the larval counts reduced to be 39.8 ± 6.92 , 23.1 ± 5.48 and 6.5 ± 2.91 in GIII, GIV and GV respectively. These groups showed a highly significant decreased in the mean number of larvae in the livers ($P < 0.0001$) with an efficacy of 35.9% in GIII, 62.8% in GIV and 89.5% in GV.

Histopathological results

In the present study, the liver was chosen an indicator because in toxocariasis the liver is the first and most affected organ. Histological examination of GI mice livers for control group revealed the normal lobular pattern of liver cells with a radial arrangement of hepatocytes around the central veins and the hepatocytes and sinusoidal capillaries were clearly seen. However, the liver sections of mice infected with 1000 egg/mL (GII) showed necrosis of hepatocytes and focal hepatic hemorrhage. Other pathological findings were infiltration with variable inflammatory cells mainly of lymphocytes, eosinophils and few neutrophils around the central veins and portal blood vessels and numerous granulomas surrounded of *T. canis* larvae (Fig. 2). In GIII (infected mice with *Z. officinale* treated eggs at a concentration of 25 mg/mL), there was cytoplasmic vacuolization of hepatocytes and portal

infiltration with inflammatory infiltrate (Fig. 3a). In GIV (infected mice with *Z. officinale* treated eggs at a concentration of 50 mg/mL), the pathological features significantly reduced and appeared in the form of hydropic degeneration of hepatocytes and focal hepatic necrosis associated with inflammatory cells infiltration (Fig. 3b). In GV (infected mice with *Z. officinale* treated eggs at a concentration of 100 mg/mL), the liver tissues appeared healthy with slight degenerative changes of hepatocytes (Fig. 3c).

Discussion

Based on the significant role of *T. canis* eggs for the transmission of human toxocariasis, this study investigated the effect of *Z. officinale* ethanol extract on the viability, embryogenesis and infectivity *T. canis* eggs. *Z. officinale* showed a significant dose and time dependent effect on the viability of *T. canis* eggs. The best effect was observed at the concentration of 100 mg/mL after 24 h exposure where the inhibitory activity was 98.2%. This effect may due to the diffusion of the extract through the eggshell and destruction of the parasite by its potent proteolytic enzyme called zingibain (Khalil and El-Houseiny 2013). The obtained results harmonized with previous studies in which *Z. officinale* was used as anti-parasite. It was proven the

Fig. 2 Liver sections from *T. canis* infected control mice (GII) euthanized 2 weeks p.i. showed **a** *T. canis* larvae surrounded by granulomatous reaction (H&E X100), **b** necrosis of hepatocytes and focal hepatic hemorrhage (H&E X400)

Fig. 3 Liver sections from infected mice by *Z. officinale* treated eggs euthanized 2 weeks p.i. **a** section from GIII showed cytoplasmic vacuolization of hepatocytes and portal infiltration with inflammatory

infiltrate, **b** section from GIV showed hydropic degeneration of hepatocytes and Kupffer cells activation, **c** section from GV showed the normal structure of hepatic lobule (H&E X400)

ovicidal effect *Z. officinale* against *Fasciola hepatica* eggs by 100% at concentrations of 5 and 10 mg/mL after 48 and 24 h treatment time respectively (Moazeni and Khademolhoseini 2016). Dyab et al. (2016) found that *Z. officinale* extract at 50 mg/mL had a higher significant effect on *Giardia lamblia* cyst viability after 1 h treatment. Also, antimicrobial testing of the rhizome extract of *Z. officinale* at the concentration of 100 mg/mL demonstrated a potential activity against four pathogenic bacteria; *Escherichia coli*, *Staphylococcus aureus*, *Bacillus spp.* and *Salmonella spp.* (Saad et al. 2014). Ethanolic extract of *Z. officinale* at concentrations of 50, 100, 150 mg/mL exhibited high scolicidal activity against protoscolices of hydatid cyst with viability rates of 15.37, 20 and 0% after 1 h respectively (Baquer et al. 2014).

Also, the results indicated that *Z. officinale* effectively inhibited *T. canis* eggs embryogenesis and caused their degeneration at the concentration of 100 mg/mL. However, *T. canis* eggs treated with the lower concentrations developed into infective larvae despite being damaged. Consistent with this finding, Verocai et al. (2010) observed that *T. canis* eggs exposed to sodium hypochlorite were morphologically damaged, but still formed larvae and remained infective for 2 weeks. Additionally, ethanol extracts of *Allium cepa* and *Allium sativum* showed good anthelmintic activity against *T. canis* since they affected the survival of larvae (Orengo et al. 2016). Also, *Z. officinale* ethanol extract at the concentration of 800 µg/mL induced programmed death in *Trichomonas vaginalis* in vitro (Arbabi et al. 2016).

Anti-*T. canis* effect of *Z. officinale* extract may attribute to its phytochemical composition such as, flavonoids, alkaloids, tannins, saponins, glycosides that have been responsible for the varying inhibition against the microorganisms (Riaz et al. 2015). Tannins possess anthelmintic activity via the connection to the larval cuticle, rich in glycoproteins, causing death; or binding to free proteins resulting in decreasing the availability of nutrients and

larval death by starvation (Cala et al. 2012). Flavonoids inhibit the formation of DNA /RNA or inhibit the reproduction of the infectious agents (Jassim and Naji 2003). Saponins affect cell membrane permeability of the parasites and cause vacuolization and disintegration of the teguments (Wang et al. 2010). Also, alkaloids have the ability to interfere with the metabolism of aromatic amino acids or intercalate with DNA synthesis in the parasite (Shaibani et al. 2009). The mentioned effects may be involved in anti-*T. canis* activity of *Z. officinale* in this study.

The liver is the most common affected organ in visceral toxocarosis. Larvae reach the liver via the portal circulation inducing many pathological lesions along their migratory routes including necrosis, hemorrhage, diffuse eosinophilic infiltration and the larvae persisting in the liver cause granulomatous lesions (El-Sayed and Ramadan 2017). In this study, the effect of *Z. officinale* on the infectivity *T. canis* was proven by the reduction of larvae recovery in the liver within the first 7 days after infection. A considerable success was obtained statistically in all groups inoculated by *Z. officinale* treated eggs at concentrations of 25, 50 and 100 mg/mL compared with the infected controls. Anti-*T. canis* effect of *Z. officinale* might occur through a reduction in the infectivity of *T. canis* eggs by the direct damage of *Toxocara* larvae in embryonated *T. canis* eggs. This may be attributed to the bioactive compounds of ginger; [10]-shogaol, [6]-shogaol, [10]-gingerol, [6]-gingerol, and hexahydro curcumin. It was observed that the morphology of *Hymenolepis nana* adult worms damaged by these compounds, especially in scolox and proglottid segment (Lin et al. 2014). Lin et al. (2010) found that the ginger constituents have larvicidal activity against *Angiostrongylus cantonensis* larvae by the direct killing or reducing their spontaneous movement. Moreover, *Z. officinale* displayed some degree of anti-*Schistosoma* activity by decreasing *S. mansoni* worm burden and the egg load in the liver and intestine which synchronized with the

reduction in granuloma diameters size and also, by lowering some inflammatory mediators that play a critical role in schistosomal liver fibrosis and its complications (Aly and Mantawy 2013).

The effect of *Z. officinale* on the infectivity of *T. canis* eggs was also investigated by the histopathological study of liver sections at the 14th day p.i. In the infected controls, *T. canis* larvae caused mild to severe injuries in the liver due to the mechanical irritation caused by the migrating viable larvae and to host sensitization to the excretory and/or secretory products of the larvae as revealed by the presence of an eosinophilic granuloma around the larvae (Carvalho and Rocha 2011). However, the liver tissues of mice infected with *Z. officinale* treated eggs at the concentration of 100 mg/mL appeared healthy with slight degenerative changes of hepatocytes, opposite to that recorded in mice infected with treated eggs exposed to the lower concentrations. These minimal pathological changes may be due to the ability of the extract to penetrate the eggshell and destroy the larvae resulting in the reduction in the larval burden as well as the sluggish movements of the remaining live larvae in the liver tissues resulting in decrease the damage which caused by the mobile living larvae. Also, this amelioration may be attributed to the anti-inflammatory, hepatoprotective and antioxidant activities of this extract (Imtiyaz et al. 2013).

Based on the results of this study, it was clear that *Z. officinale* ethanol extract possessed dose-dependent activity on the viability, embryogenesis and infectivity of *T. canis* eggs and therefore, it can be considered strong ovicidal and larvicidal activities. More investigations will be needed to establish anti-*T. canis* potential of this extract and to determine its exact mechanism.

Compliance with ethical standards

Conflict of interest No conflict of interest is declared by the authors.

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