

In vitro* evaluation of the antiparasitic activity of *Syzygium aromaticum* against adult and larval stages of *Trichinella spiralis

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Abstract. Benzimidazole is the most commonly used therapeutic drug for trichinellosis clinical treatment, but it has many drawbacks. The quest for alternative natural compounds, such as essential oils, is, therefore, a target for researchers. The present work is intended to test the *in vitro* anthelmintic effect of clove oil (*Syzygium aromaticum*) against adult and muscle larva of *Trichinella spiralis*. Adult forms and muscle larvae of *Trichinella spiralis* were incubated with clove oil at concentrations ranging from 5 to 500 µg/ml to analyze the lethal effective concentrations on the parasite and to track the changes occurred on the cuticle by scanning electron microscopy. At 50 µg/ml, a 100% death rate on the adult worms of *T. spiralis* was observed only at 24 hours. However, at concentrations of 100 and 500 µg/ml, the lethal effect started at 16 and 8 hours respectively. Clove oil killed the total larvae at the concentrations of 100 and 500 µg/ml at 24 and 16 hours of *in vitro* incubation respectively. Adult worms and muscle larvae of *T. spiralis* incubated with 100 µg/ml of clove oil exhibiting marked morphological changes, multiple vesicles, and blebs, sloughing of some areas of the cuticle with fissures, loss of normal annulation, and destruction of the cuticle. Our results suggested that clove oil has the potential as a therapeutic agent and an alternative drug against adults and larvae stages of *Trichinella spiralis*.

Keywords: Trichinellosis; Clove oil; Anthelmintic activity; Electron microscopy.

Evaluarea *in vitro* a activităţii antiparazitare a *Syzygium aromaticum* faţă de stadiile larvare şi adulte a lui *Trichinella spiralis*

Rezumat. Benzimidazolul este medicamentul cel mai frecvent folosit în tratamentul clinic al trichinelozei, dar cu multiple dezavantaje. Căutarea compușilor naturali alternativi, cum ar fi uleiurile esențiale, este, prin urmare, un scop important pentru cercetători. Lucrarea de față este menită să testeze efectul antihelmintic *in vitro* al uleiului de cuișoare (*Syzygium aromaticum*) împotriva adulților și larvelor musculare de *Trichinella spiralis*. Formele adulte și larvele musculare ale *Trichinellei spiralis* au fost incubate cu ulei de cuișoare la concentrații cuprinse între 5 și 500 µg/ml, pentru a analiza concentrațiile letale eficiente și pentru a urmări modificările survenite în

cuticule, prin microscopie electronică cu baleiaj. La 50 µg/ml, s-a observat o rată de mortalitate de 100% la adulții de *T. spiralis*, dar acest efect a apărut doar la 24 de ore post expunere. Cu toate acestea, la concentrații de 100 și 500 µg/ml, efectul letal a început la 16 și respectiv 8 ore. Uleiul de cuișoare a omorât toate larvele la concentrații de 100 și 500 µg/ml la 16, respectiv 24 ore de incubare *in vitro*. Paraziții adulți și larvele musculare de *T. spiralis* incubate cu 100 µg/ml de ulei de cuișoare au prezentat modificări morfologice marcate, vezicule multiple și hemoragii, slăbirea unor zone ale cuticulei cu fisuri, pierderea anulației normale și distrugerea cuticulei. Rezultatele noastre au sugerat că uleiul de cuișoare are potențial terapeutic și poate fi folosit ca un medicament alternativ împotriva adulților și a stadiilor de larvare ale *Trichinellei spiralis*.

Cuvinte cheie: Trichineloză; Ulei de cuișoare; Activitate antihelmintică; Microscopie electronică.

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Introduction

Trichinellosis is a zoonotic parasitic disease caused by *Trichinella spiralis* (*T. spiralis*), a nematode of the genus *Trichinella* (Bai et al., 2017). These parasites have the capacity of infecting a wide range of mammals by eating improperly cooked or raw meat containing the infective larvae of *Trichinella* (Abou Rayia et al., 2017). It infects around 11 million individuals around the world (Luis Muñoz-Carrillo et al., 2019). In Egypt, it was diagnosed in a man (Abdel-Hafeez et al., 2015) and pigs slaughtered in Cairo Abattoirs (Dyab, 2019). All phases of development of *T. spiralis*, adult, migratory, and encysted stages are found in the same host, so, this parasite has been normally utilized as a trial model to assess the effectiveness of numerous anthelmintic agents (Yadav and Temjenmongla, 2012).

Currently, benzimidazole derivatives are the main anthelmintic therapeutic drugs used for the clinical treatment of trichinellosis. However, they have many drawbacks (Huang et al., 2020), as none of these medications is compelling to kill encapsulated and newborn larvae (Nassef et al., 2018), due to their low bioavailability (Caner et al., 2008) and drug resistance (Shalaby et al., 2010). Likewise, most of them are contraindicated in pregnant women and children below two years of age. (Yadav and Temjenmongla, 2012). Therefore, finding a new, secure and efficient anthelmintic agent against *T. spiralis* is a target for scientific

researchers. The World Health Organization encourage researches on medicinal plants to produce new, easy-to-use anthelmintic compounds with lower side effects in the battle against diseases affecting people in underdeveloped countries. The wider acceptance of medicinal plants as treatments is due to the pharmacological activities attributable to their phytoconstituents, the lesser side effects and improved viability than their synthetic counterparts (Batiha et al., 2020).

Syzygium aromaticum (clove oil) is worldwide used as a food flavoring agent. It has been widely used in traditional medicine to treat a variety of diseases since ancient times; additionally, its major constituent, eugenol, showed a potential lethal efficacy against various parasites including *Giardia lamblia*, *Fasciola gigantica*, *Haemonchus contortus*, and *Schistosoma mansoni* (Machado et al., 2011; El-Kady et al., 2019). Clove oil has been traditionally utilized in inhibiting food-borne pathogens (Bhowmik et al., 2012), and has been recorded as a "Generally Regarded As Safe" substance by the United States Food and Drug Administration, and the World Health Organization (WHO) Expert Committee on Food Additives which had established the acceptable daily admission of clove oil at 2.5 mg/kg body weight for humans (Anderson et al., 1997). Considering the aforementioned reasons, an attempt has been made to assess the anthelmintic efficacy of clove oil (*Syzygium aromaticum*) against adult worms and muscle

larvae of *T. spiralis*. The study is also motivated by the lack of scientific data in the literature regarding *in vitro* activity of clove oil against this parasite.

Materials and methods

Parasite and animals

The strain of *T. spiralis* was obtained from infected pork meat collected from Cairo abattoir and kept in the lab of Parasitology, Theodor Bilharz Research Institute (Giza, Egypt) by consecutive passages on rats and mice. Male Swiss albino mice, 6–8 weeks old, weighing 25–30 g each were utilized. The animals were housed in proper cages and fed with commercial rodent chow and tap water *ad libitum*, as per the institutional and national guidelines. Mice have been orally infected with 200 *T. spiralis* larvae (Abou Rayia et al., 2017). After 48 hours (h), the animals were killed, the small intestine detached, cut into pieces, and kept in phosphate-buffered saline for 4 hours of incubation at 37°C to recover the adult worms of *T. spiralis*. To obtain the muscle larvae, the infected mice were sacrificed after 35 days of infection and the muscles digested in pepsin-HCL as indicated by the technique of Jiang et al. (2012).

In vitro experimental design

The collected *T. spiralis* adults and muscle larvae were added to a 48-well microtiter plate prepared with RPMI-1640 medium (containing 200 U/ml penicillin, 200 µg/ml streptomycin, and 20% fetal bovine serum). Clove oil was purchased from El-Captain Company in the local market, Cairo, Egypt, dissolved in dimethyl sulfoxide (DMSO) and diluted in RPMI-1640 medium. The final concentrations of clove oil against adults and muscle larvae ranged from 5 to 500 µg/ml at time intervals of 1, 2, 4, 8, 16 and 24 h. Every determination was performed in triplicate and the summation of wells for each concentration were calculated. The plates were incubated at 37°C and 5% CO₂ for 24 h, and the survival of *T. spiralis* stages was observed utilizing a microscope. Control parasites were incubated in RPMI-1640 medium having 1% DMSO. Worm viability rate (%) was calculated using the formula: number

of viable worms/total number of worms ×100. The viability of adult parasites was made by assessing their shape and mobility, the dead worms being characterized by C-shaped or linear body and lack of mobility. The same sequence was handled for muscle larvae. Samples of worms and larvae were taken after 24 h of incubation and handled for scanning electron microscopy.

Preparation for scanning electron microscopic examination

The adults or larvae of *T. spiralis* were added to a fixed solution of 2.5% glutaraldehyde and incubated medium at 4°C. The parasites were washed in 0.1M sodium cacodylate buffer at pH 7.2 for 5 minutes, post-fixed in a 2% (w/v) osmium tetroxide in sodium cacodylate buffer for 1 hour. Post-fixed specimens were then dehydrated in ascending concentrations of alcohol and dried utilizing a critical point of carbon dioxide drying. The parasites thus prepared were examined by scanning electron microscopy (Hitachi SU8040, Japan). Photos were recorded on electron image plates.

Statistical analysis

Graph drawing and statistical analysis were performed using Excel Software 2013. The data were expressed as means ± SD, and the Student's *t*-test was used to determine the significance of differences between mean values.

Results

The activity of clove oil on adult worms and muscular larvae of T. spiralis

Clove oil markedly affected both adult worms and muscular larvae of *T. spiralis*. The lethal effect of clove oil on the adult worms is revealed in figure 1. At 50 µg/ml, a 100 % death rate on the adult worms of *T. spiralis* was observed only at 24h. However, in 10 up to 500 µg/ml concentrations, the clove oil significantly influenced the adult worms from the first hour of *in vitro* exposure at concentrations from 10 µg/ml ($p < 0.01$) to 500 µg/ml ($p < 0.001$) (table 1).

Clove oil only killed all larvae at concentrations of 100 and 500 µg/ml at 24 h of in vitro incubation, while the viability rates of larvae diminished with prolonged exposure at lower concentrations (figure 2). At concentrations of 50, 100 and 500 µg/ml, the larvicidal impact of clove oil was significantly higher beginning in the first hour of incubation (P<0.001) compared with controls (table 2). The worms and larvae treated with 100 µg/ml were chosen for scanning electron microscopy (SEM) examination due to the early lethal effect when compared with the other low concentrations.

SEM assessment of the adult worms revealed that culturing adult worms with clove oil (100 µg/ml) for 24 hours caused serious alterations. The cuticle of the indicated areas showed marked swellings, numerous large blebs, fissures and vesicles accompanied by loss of

the normal creases, ridges and annulations (figures 4, 5). The sloughing of certain territories of the cuticle was also noticed. The body collapsed, and there was a lot of carrions. While culturing adult worms in the incubation medium only saved the normal morphology of the cuticle with the characteristic annulations and ridges well-arranged as vertical lines between the stripes on the surface of the parasite (figure 3). Examining of *T. spiralis* larvae incubated in culture medium only by scanning electron microscope demonstrating normal cuticle with transverse creases and longitudinal ridges (figure 6). Incubation of *T. spiralis* Larvae at 100 µg/ml clove oil indicated marked morphological changes, multiple vesicles and blebs, sloughing of some areas of the cuticle with fissures, loss of normal annulation, destruction of the cuticle, and collapsing of the body of *Trichinella* larvae was seen (figures 7, 8).

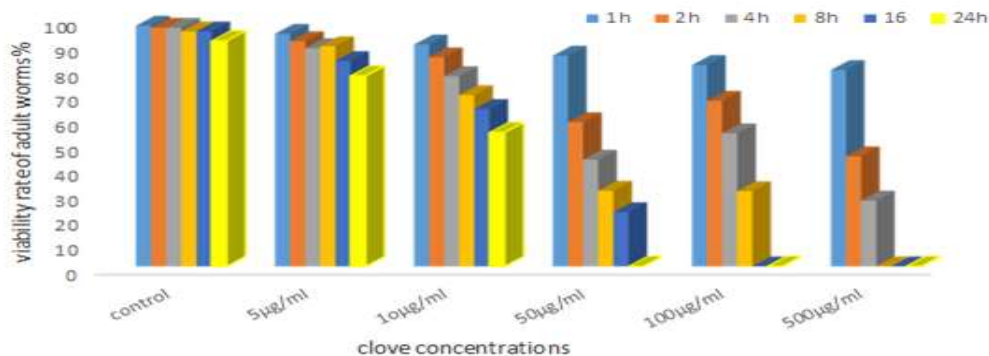


Figure 1. In vitro effect of *Syzygium aromaticum* on viability rates (%) of *T. spiralis* adult worms

Table 1. In vitro effect of *Syzygium aromaticum* (clove oil) on viability rates (%) of *T. spiralis* adult worms

Cloves oil dose (µg/ml)	Time intervals					
	1 hours	2 hours	4 hours	8 hours	16 hours	24 hours
Parasite control	97.67 ±0.67	96.83 ±0.40	96.83 ±1.08	95.33 ±0.76	95.33 ±1.02	91.50 ±0.76
5	94.33 ±1.71	91.33 ±2.50	88.50 ±3.60	89.33 ±3.53	83.33 ±5.38	77.33 ±6.88
10	90.00 ±1.93**	84.83 ±2.24***	77.17 ±4.85**	69.50 ±3.87***	63.83 ±1.74***	54.33 ±1.78***
50	85.50 ±1.36***	58.50 ±3.02***	43.33 ±2.72***	30.67 ±2.98***	22.00 ±2.98***	0.00 ±0.00***
100	81.67 ±1.67***	67.17 ±2.43***	54.00 ±2.18***	30.50 ±2.55***	0.00 ±0.00***	0.00 ±0.00***
500	79.67 ±1.74***	44.67 ±1.87***	26.67 ±1.86***	0.00 ±0.00***	0.00 ±0.00***	0.00 ±0.00***

Data were expressed as the mean ± SD **P<0.01 and ***p<0.001 compared with the corresponding parasite controls.

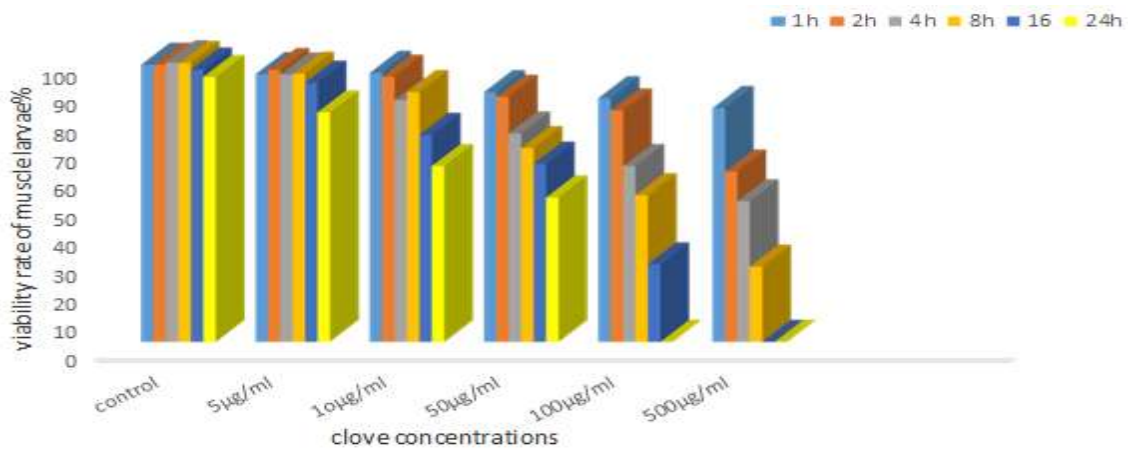


Figure 2. *In vitro* effect of *Syzygium aromaticum* on viability rates (%) of *T. spiralis* muscular larvae

Table 2. *In vitro* effect of *Syzygium aromaticum* (clove oil) on viability rates (%) of *T. spiralis* muscular larvae

Cloves oil dose (µg/ml)	Time intervals					
	1h	2h	4h	8h	16h	24h
Parasite control	98.17±0.79	98.17±0.60	99.00±0.37	98.83±0.48	96.50±0.50	93.83±1.11
5	94.83±1.64	96.33±0.71	94.67±2.48	95.00±1.73	91.83±2.20	81.33±5.74
10	95.33±1.56	94.00±1.83	85.67±2.92**	88.50±2.85**	73.33±2.79***	62.33±1.67***
50	88.50±1.38***	86.83±1.08***	73.83±1.22***	68.67±1.09***	63.17±2.02***	51.17±1.40***
100	86.33±1.93***	82.00±0.58***	62.33±1.56***	52.00±1.93***	27.50±1.91***	0.00±0.00***
500	82.83±1.49***	60.33±2.95***	49.83±2.63***	26.67±1.86***	0.00±0.00***	0.00±0.00***

Data were expressed as the mean ± SD **P<0.01 and ***p<0.001 compared with the corresponding parasite controls.

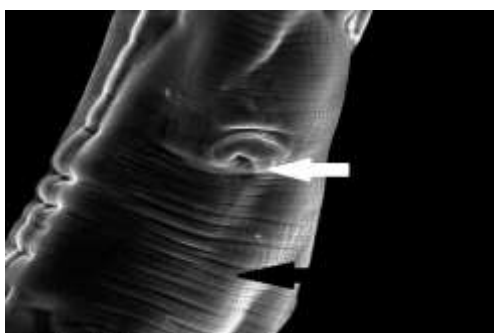


Figure 3. Higher magnification of *T. spiralis* adult worm showing the cuticle with intact annuli (black arrow) and hypodermal gland (white arrow). The cuticle is lined up in neat rows



Figure 4. Adult worm incubated with 100 µg/ml clove oil, large blebs (white arrows) and areas with small vesicles (black arrow) are seen

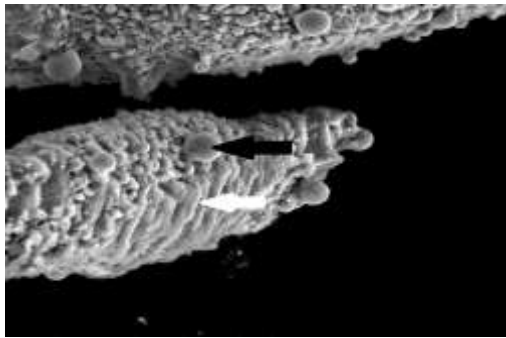


Figure 5. The cuticle of the adult worm after incubation with 100 µg/ml clove oil was severely damaged, and there was a large amount of carrion and blebs (black arrow). Intact annuli and the vertical lines disappeared and completely degenerated cuticle (white arrow).

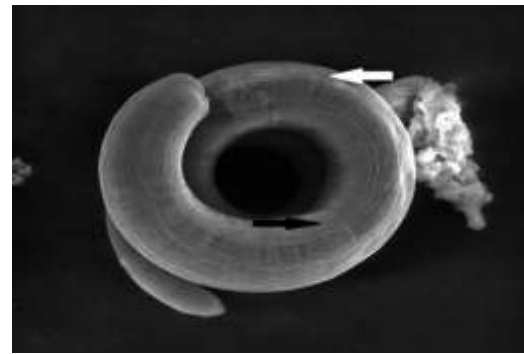


Figure 6. Isolated muscular infective larvae, demonstrating their typical coiled appearance, the cuticle is normal, annulated with transverse creases (black arrow) and longitudinal ridges (white arrow)

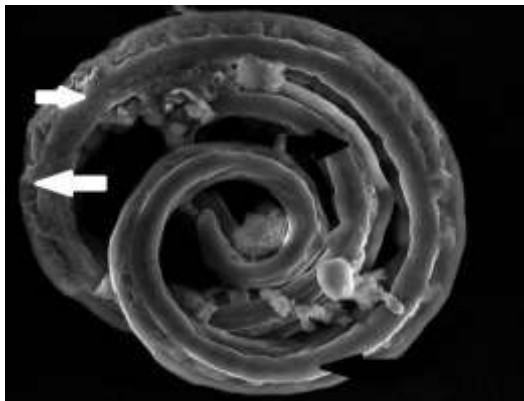


Figure 7. Muscle larva treated with clove oil (100 µg/ml) showing fissures of the cuticle (white arrow) and collapsing of the body (black arrow)

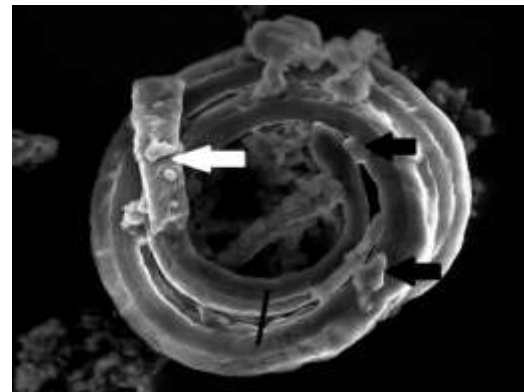


Figure 8. muscular larva incubated in 100 µg/ml clove oil showing sloughing of some areas of the cuticle (white arrow) with swelling and blebs (black arrows). Multiple fissures (headless arrow) and loss of the normal annulations

Discussion

The *in vitro* test with helminths is one of the useful tools to explore the anthelmintic properties of a certain agent and is also helpful to analyze its mode of action. *In vitro* tests are preferred to *in vivo* methods due to their low cost, simplicity, and rapid turnover. Our analysis has shown that clove oil has lethal action on adult stage and muscle larvae of *T. spiralis in vitro*. This remarkable effect was shown to occur in a dose- and time-dependent manner (figures 1, 2). Many experiments studied the *in vitro* effect of clove oil and other extracts against various nematodes such as *Haemonchus contortus* (Charitha et al., 2017), *Cotylophoron cotylophorum* (Manoj Dhanraj and Veerakumari, 2015), and *Meloidogyne incognita* (Meyer et al., 2008). As was shown in these experiments, the

lethal effect of clove oil for these parasites was expressed at various concentrations.

In the present study, SEM demonstrated the detrimental effects of clove oil against the incubated adult worms and larvae of *T. spiralis*. These effects were described as swelling, sloughing and damage to the cuticle of the adult worms and muscle larvae of *T. spiralis* associated with the drug exposure. Previous studies have investigated the effects of clove oil on nematodes. (Charitha et al., 2017) proved that, *Syzygium aromaticum* is very effective in killing the *Haemonchus contortus* worms where 100% mortality was attained within minutes of exposure and attributed the wormicidal activity of clove to its strong corrosive action on cuticle and tegument of helminths. These results agreed with our results which characterized in table 1

and figures 4, 5 for *T. spiralis* worms also represented in table 2 and figures 7, 8 for muscle larvae. The difference in time of mortality was due to the variance in concentrations used in the two studies. Similarly, the study of Manoj Dhanraj and Veerakumari (2015) revealed anthelmintic property of *Syzygium aromaticum* ethanol extract against *Cotylophoron cotylophorum* worms, and the authors accredited this property to the disruption involved in the metabolic pathway of carbohydrates that may be fatal to the parasites.

Our research focused on exploring the impact of clove oil on the structure of the *T. spiralis* cuticle because tegumental and/or cuticular structures are the vital parasite-host interfaces that are both nutritional and defensive roles as well as retain their form (Roy et al., 2010). The lethal action of clove oil against adults and *T. spiralis* muscle larvae may be attributed to its deleterious effect on the parasite cuticle. This effect facilitating the diffusion of active oil constituents within the organism due to its lipid solubility, causing dramatic changes in the internal structural features of helminths (Roy et al., 2010). In addition, clove oil was reported to disrupting metabolism (Manke et al., 2015) and/or DNA synthesis or folic acid cycle of the parasite, triggering cellular damage. Such results were not surprising, as transcuticular passive diffusion is the key mechanism of drug entry into the helminths (Roy et al., 2010).

The observed *in vitro* anthelmintic activity of clove oil against the adult worm and larva stages of *T. spiralis* may be also attributable to the enrichment of clove oil with tannins (10-13%) (Mittal et al., 2014). Tannins have been reported to induce anthelmintic activities through binding to the free protein available in the wells for parasite nutrition; decreased supply of nutrients in the wells, resulting in malnutrition and death of *Trichinella* adults and larvae (Chandrashekhar et al., 2008). Tannins may also bind to the cuticle of the parasite, which is rich in glycoprotein, triggering its death. Clove oil contains also, monoterpenes and sesquiterpenes (Plata-Rueda et al., 2018), these constituents allows compounds to permeate the cell membrane that affects the metabolic pathways or organelles destroying the parasites. β -caryophyllene constitutes about 13% of clove oil and was identified for several important pharmacological

activities, including anti-inflammatory action (Sharma et al., 2016; Machado et al., 2018). As we know, pharmacotherapy used in trichinellosis includes the use of anti-parasitic drugs which are directed against the parasite (Gottstein et al., 2009) and steroidal anti-inflammatory drugs, whose purpose is to alleviate the signs and symptoms of the inflammatory response produced by *T. spiralis* infection (Shimoni et al., 2007). Therefore, β -caryophyllene may add another dimension for using clove oil as anti-parasitic plus anti-inflammatory in treating *T. spiralis*.

In conclusion, clove oil has a promising *in vitro* anthelmintic activity and can be considered an effective and safe alternative drug against adult worms and muscle larvae of *T. spiralis*. However, further *in vivo* testing will be imperative to investigate the main active components of clove oil in studies that will establish the doses, administration routes, bioavailability, and metabolic biotransformation. Studying combinations of clove oil with standard antitrichinellosis drugs may also be used to improve the treatment effect and to reduce the toxicity associated with these drugs and to suppress resistance.

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