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Phenotypic changes of *Trichinella spiralis* treated by *Commiphora molmol*, *Lepidium sativum*, and Albendazole: *in vitro* study

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Summary

Trichinellosis is a nematode-causing disease distinguished by its continuous transmission in the carnivores and omnivores. Despite effective eradication of the enteral forms, conventional drugs fail to eliminate the migrating and muscle ones. Over the past years, researchers intensified the work on herbal medicines as alternatives or aids to albendazole, the reference drug. This research hypothesizes that the therapeutic agent absorption route could be an evidence-based carrier molecule or auxiliary drug to albendazole. Accordingly, this *in vitro* study was designed to investigate mainly the phenotypic changes induced by a mono-treatment of albendazole, *Lepidium sativum* (garden cress), and *Commiphora molmol* (myrrh). Incredibly, no data were reported on the morphological alterations of *T. spiralis* larvae treated by any of these drugs. The experimental design tested various concentrations (25, 50, 100, and 200 µg/ml) of each herbal medicine for the lethal effects on the parasite forms for a day (1, 12, and 24h). The data showed that the highest significant mortality rate of the parasite forms was in favor of the concentration 200 µg/ml of both plant extracts in a time-dependent manner. Therefore, albendazole at 200 µg/ml dose was tested in parallel, and all experimental groups were compared to non-treated muscle larvae and worms. Albendazole-treated worms accounted for the least significant ($p < 0.001$) survival rate (2 %), followed by myrrh (5 %), and the adverse was valid for the survival rate of the muscle larvae at that time. None of the larvae/worms was alive after 24 hours of incubation with the 200 µg/ml of either treatment. The scanning electron microscope investigation of the experimental groups provided a shred of evidence for different routes of taking up the candidate drugs by the parasite. In conclusion, the results of the previous work *in vivo* and current *in vitro* study recommend myrrh over garden cress as a complementary agent of albendazole.

Keywords: *Trichinella spiralis*; Drug discovery; Complimentary drug; auxiliary drug; myrrh; albendazole; garden cress

Introduction

Trichinella spiralis is a zoonotic, foodborne parasite found in marine, urban, and sylvatic environments, ensuring its existence (Ohio State University, 2001) and transmission cycle in the eco-

system. The infection leads to serious health complications in patients and animals, causing economic crisis due to the costly control, inspection, and freezing of meat (Devleschauwer *et al.*, 2015). Despite the anti-helminthic drugs: Mebendazole, thiabendazole, albendazole, and prednisone, the elimination, prevention, and

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treatment of *T. spiralis* are controversial due to the resistance encountered by the muscle tissue-encysted larvae (Jones & Capello, 2004). The buried larvae are too difficult to destroy (Rong-Yu & Feng-qj, 2015). The migrating larvae possibly induce myocarditis, thromboembolic disease, and encephalitis in the acute phase (Gottstein *et al.*, 2009; Sun *et al.*, 2015).

Several studies have recently focused on finding a potential medicinal herbal therapy to be used solely or in combination with albendazole to overcome its poor solubility and absorption by the host cells (Yadav & Tejenmongla, 2012; Basyoni & Elsabaa, 2013; Garcia-Rodriguez *et al.*, 2015; Yr & Yf, 2015; Abdel-Rahman *et al.*, 2020; Abuelenain *et al.*, 2021). Researchers and pharmacists favor the traditional natural agents for their multi-target and -channel characteristics (Balunas & Kinghorn, 2005, Yuan *et al.*, 2016, Park *et al.*, 2021)

Commiphora molmol plant stem contains myrrh with various components such as volatile oil, resin, and gum. Myrrh extracts are commonly used in traditional medicine as a potential antibacterial and antifungal (Dolara *et al.*, 2000) with effective analgesic and anti-pyretic properties. Recently, the potent effectiveness of myrrh as an anti-helminthic agent has been studied (Basyoni & El-Sabaa, 2013; Attia *et al.*, 2015; Abdel-Rahman *et al.*, 2020; Abuelenain *et al.*, 2021)

Lepidium sativum (garden cress) is one of the well-known culinary herbs that grew and cultivated in ancient Egypt. During the Roman Empire, the plant seeds were shipped to Europe because the Romans believed in the medical benefits of garden cress. Vitamin C, minerals, and carotenes are abundant in the plant, which is also used as a food additive and in-home decoration in Africa, China, and many other countries (Al-Yahya *et al.*, 1994; Wealth of India, 1998). A few studies reported the therapeutic effectiveness of garden cress on asthma (Mali *et al.*, 2008), blood coagulation, oxidative stress, and some inflammatory disorders (Al-Yahya *et al.*, 1994). One analytical study proposed garden cress as a treatment for hypertension and kidney failure (Patel *et al.*, 2009).

The present study aimed to comprehend the effect of the herbal extracts and albendazole on the survival rate and morphology of both the intestinal and muscle forms of *T. spiralis in vitro*; in the absence of the host responses.

Materials and Methods

Parasites

T. spiralis strain was obtained from infected pork meat collected from Cairo abattoir and kept in the Animal House, Theodor Bilharz Research Institute (TBRI, Giza, Egypt) by consecutive passages on rats and six-eight-week-old male Swiss albino mice (25 – 30 g, each). The animals were housed in proper cages, following institutional and national guidelines, and were fed with commercial rodent chow and tap water ad libitum. Mice were infected with an inoculum of *T. spiralis* larvae (n=200), as described by Abou Rayia *et al.* (2017). Forty-eight hours post-infection (pi), one group of

mice was decapitated for collecting the adult worms. The small intestine of each mouse was processed and kept in phosphate-buffered saline for 4 hours of incubation at 37°C to recover *T. spiralis* adult worms. Another group of mice was sacrificed 35 days pi to obtain the muscle larvae. Muscles were digested in pepsin-HCL as described by (Jiang *et al.*, 2012).

Plants extract and preparation

Commiphora molmol (myrrh) ethanol extract and *Lepidium sativum* (garden cress) methanol extract were courteously prepared as described previously (Abuelenain *et al.*, 2021) and provided by Dr. Abdel-Wanes A. Abdel-Aziz, Department of Medicinal Chemistry at TBRI (Giza, Egypt). The herbal extracts were fractioned into various concentrations (25, 50, 100, and 200 µg/ml) in RPMI-1640 medium (ThermoFisher Scientific, Gibco™, Scotland; pH 7.2) containing 10 % fetal calf serum, 200U/ml penicillin, and 200 streptomycins before being applied to the muscle larvae and worms *in vitro*

In vitro experimental design

Male and female *T. spiralis* intestinal worms and muscle stage larvae were cultured in parallel and separately in sterilized 24-well ELISA plates containing RPMI-1640 medium (1ml/well) supplemented with 10 % fetal calf serum, 200U/ml penicillin, and 200 streptomycin (pH 7.2). The lethal effects of various doses of each plant extract against muscle larvae and adult worms (10/well) were determined at time intervals included 1, 12, and 24h. The mean number of parasites (larvae/worm) collected from three wells for each dose was calculated. The high concentration (200 µg/ml) of garden cress and myrrh was applied to the cultured parasite stages (muscle larvae and adult worms), in parallel to 200 µg/ml albendazole- (reference drug) and untreated parasite stages. This protocol was also performed in triplicate, and the average for each treatment was calculated. All experimental stages were incubated at 37°C and 5 % CO₂ for 24hrs. The survival of *T. spiralis* stages was detected by the trichinoscopy based on their mobility and the body changes (C-/linear shape). Then, the survival percentage was calculated using the following formula:

$$\text{Viability percentage} = \frac{\text{No. of viable parasite}}{\text{total number of parasites}} \times 100\%$$

Examination of parasite samples by SEM

Samples of incubated *T. spiralis* worms and larvae were processed for examination by scanning electron microscope. The adults or larvae of *T. spiralis* were fixed with a solution of 2.5 % glutaraldehyde and medium at 4°C for 1h. The specimens were then washed with sodium cacodylate buffer (0.1M; pH 7.2) for 5 minutes, followed by one-hour post-fixation in sodium cacodylate buffer (2 % osmium tetroxide, w/v). The post-fixed specimens were then dehydrated by ascending alcohol concentrations and dried at a critical point of carbon dioxide drying. The prepared parasites were then examined by scanning electron microscopy

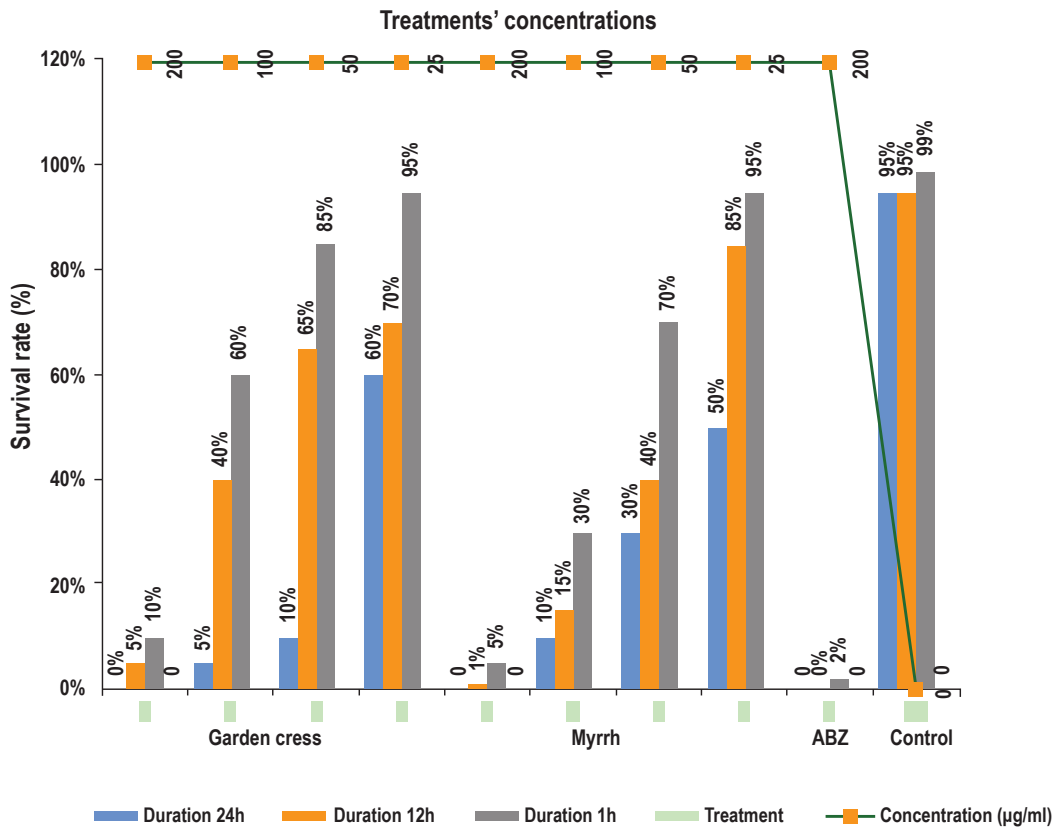


Fig. 1. Effects of various concentrations of myrrh and garden cress on the worm percentage survival for different durations. Albendazole (ABZ, 200 µg/ml) -treated and untreated control worms were counted within the exact durations: 1, 12, and 24 hours. The data is reproducible of triplicate experiments. The graph bars represent the durations relative to each specific treatment at the primary horizontal axis; the percent values above the bars provide the survival rate of worms. The secondary horizontal axis represents the doses of each treatment.

(Hitachi SU8040, Japan). Photos were recorded on electron image plates (Bolas-Fernandez, 2002; Bughdadi, 2010) to show if any differences in the lesions score detected across all groups.

Statistical analysis

The statistical software SPSS V. 20 was used to perform a Two-Way ANOVA test to identify the significant difference between the subgroups at different concentrations and time intervals. For a confidence level $p < 0.05$, the statistical difference between the subgroups was considered significant. A one-Way ANOVA test was also performed to compare the main groups at the most efficient 200 µg/ml dose after one hour with a confidence level $p < 0.05$. Both tests showed that the difference between the subgroups/groups was significant and is attributed to the treatment protocol.

Ethical Approval and/or Informed Consent

T. spiralis life cycle was managed in the Animal House of Theodore Bilharz Research Institute (TBRI, Giza, Egypt). All applicable national and institutional guidelines for the care and use of animals were followed.

Results

Effects of myrrh, garden cress, and albendazole on worms and larvae

The data collected for the incubated worms demonstrated that the greater the dose and exposure time, the lower the percentage of worms in all treatment protocols, as indicated in Figure (1). It is noticeable from the histogram that all treated worms died with the highest concentration (200 µg/ml) after 24 hours of exposure, while the controls accounted only for 5 % death at that time (100 % – %survival). The most significant ($p < 0.001$) reduction (2 %) of worms' survival 1h post-incubation was attained by albendazole, which was comparable to its counterpart (5 %) of myrrh. The percent survival (10 %) of Garden cress-treated worms was significantly ($p < 0.001$) higher compared to its counterparts of albendazole- and myrrh-treated ones (Fig. 1).

The effectiveness on larvae survival has shown a highly significant ($p < 0.001$) reduction (2 %) with myrrh ethanol extract. Interestingly, the larvae percent (10 %) was five folds higher ($p < 0.001$) with albendazole and ten times greater with garden cress (20 %), as shown in the histogram of Figure 2.

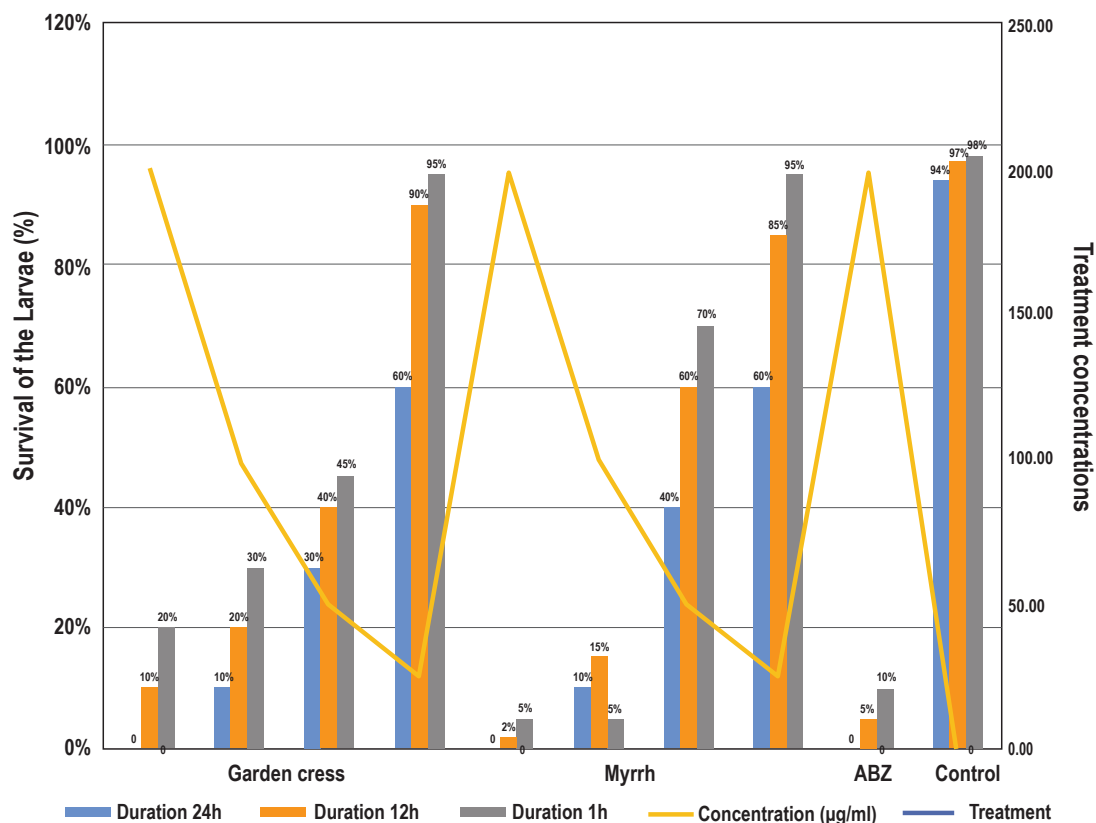


Fig. 2. Effects of various concentrations of myrrh and garden cress on the *Trichinella spiralis* muscle larvae survival percentage for different durations. Albendazole (ABZ, 200 µg/ml)-treated and untreated control larvae were counted at the exact time intervals (1, 12, and 24 hours). The data is reproducible of triplicate experiments. The primary vertical axis indicates the percent survival of muscle larvae, which is written above the bars. The secondary vertical axis provides the treatment concentrations. The graph bars represent the durations relative to each specific treatment at the primary horizontal axis.

The morphological changes

The electron microscopy examination of control incubated worms demonstrated the average rounded, annulated body with a short and thick male (Fig. 3C) and longer, thinner female (Fig. 3A). Both genders have tapered anterior and wider posterior (Fig. 3A, B & C). It is worth mentioning that the morphology of dead worms of each treatment protocol had similar alterations overall detected time intervals and concentrations. Utilizing a 200 µg/ml dose of albendazole resulted in a severe deterioration of the cuticle and tegument with internal opacity and loss of the identity of the *T. spiralis* adult worm ends (Fig. 3D and E). When incubated with myrrh ethanol extract, the worm's body architecture showed flattened cuticles with clumps of blebs, damaged ridge with fissures, and well-noticeable posterior and anterior regions (Fig. 3F and G). However, the garden cress methanol extract slimmed and destroyed the architecture of the posterior end of the worm, distorting some worms and retaining the others (Fig. 3H and I).

The morphology of normal control larvae showed well-annulated regions, defined ends, and a standard coiled body (Fig. 4A). The larvae lost their striations, revealing short longitudinal folds (Fig. 4B, C and D) when treated with albendazole (200 µg/ml). The body

architecture was partially (Fig. 4E) or entirely destroyed (Fig. 4F & G) by the exposure to myrrh ethanol extract. In addition to internal opacity (Fig. 4E), degeneration and shrinkage of the larvae were detected (Fig. 4F and G). The most significant changes of the larvae treated with garden cress methanol extract included opacity and deformity of the posterior region (Fig. 4H and I).

Discussion

Trichinella spiralis nematode parasite is one of the chronic health problems due to the closed system of transmission between animals and humans, eating undercooked meat, and consuming raw sausage in many areas of the world. Moreover, the clinical therapy is challenged by either poor solubility or encapsulated larvae resistance (Abu El Ezz, 2005; Caner *et al.*, 2008; Gottstein *et al.*, 2009). The risk of cardiovascular diseases and encephalitis can result in the death of some patients (Ohio State University, 2001). The development of a toxic-free, feasible, and affordable herbal medicine against *T. spiralis* is a quest of research to overcome the drawbacks of conventional drugs and intervention at late diagnosis (Shipata *et al.*, 2012). Understanding the mechanism of action of

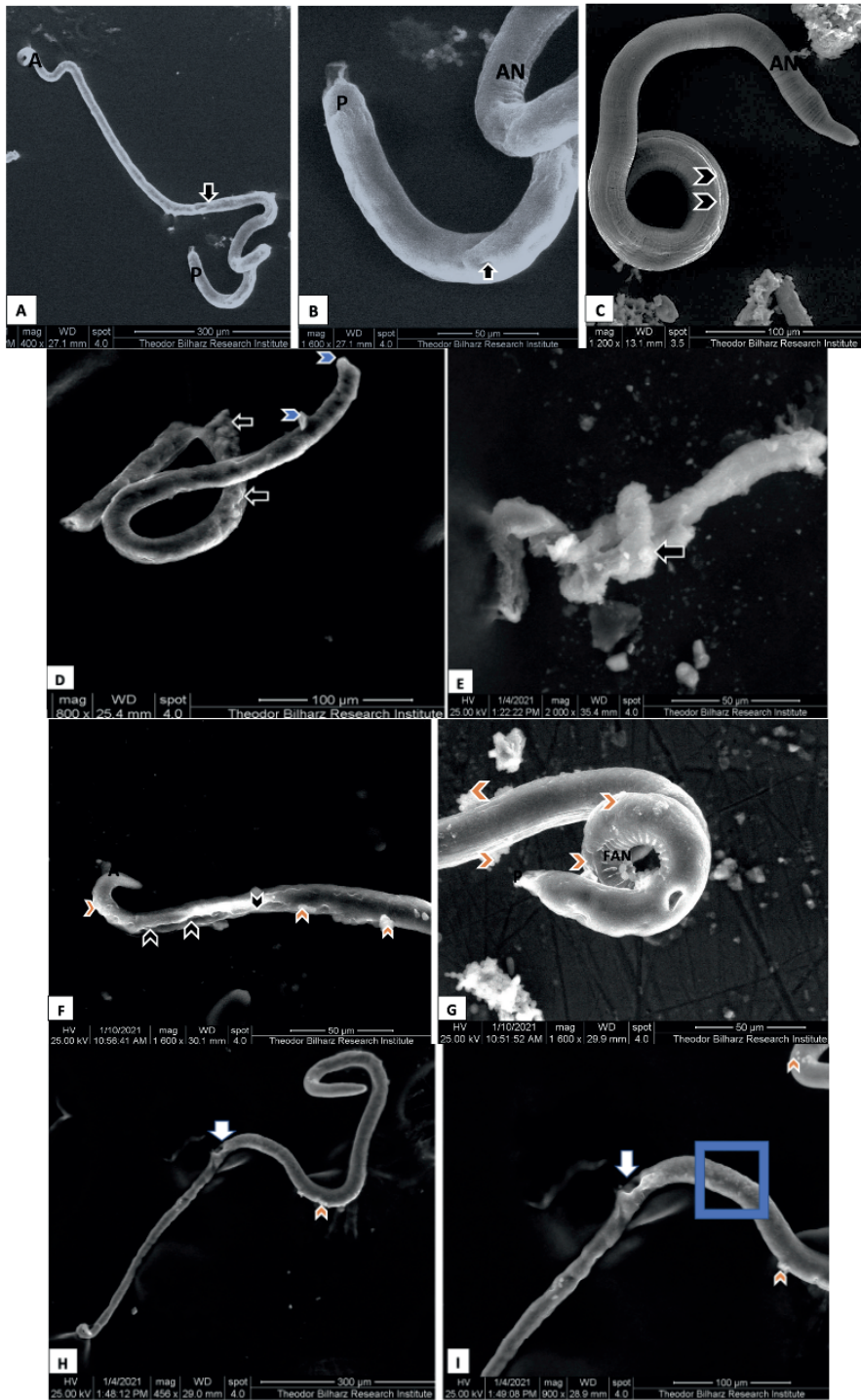


Fig. 3. *Trichinella spiralis* adult worm 1h post-incubation in the RPMI-1640 medium.

A-C, normal untreated worms showing the posterior (P) and anterior (A) regions, annulations (AN, lateral pores (black arrows), and a ridge (black arrowheads). D & E, worms treated with a single dose of albendazole (200µg/ml) showing blebs of the cuticle (blue arrowheads), carrions (black arrows), opaque body in (D), and partial or entirely sloughing cuticle in (D & E, respectively). F & G, worms treated with a single dose of *Commiphora molmol* (myrrh; 200µg/ml) showing clumps of blebs (orange arrowheads), a damaged ridge with fissures (black arrowheads), flattened annulations at the posterior region (FAN), and loss of transverse striations along the rest of the body. H & I, worms treated with a single dose of *Lepidium sativum* (Garden cress; 200µg/ml) showing a few blebs (orange arrowheads), destruction of the vulva (white arrows) with a post-region of sub-cuticle vesicles (blue square).

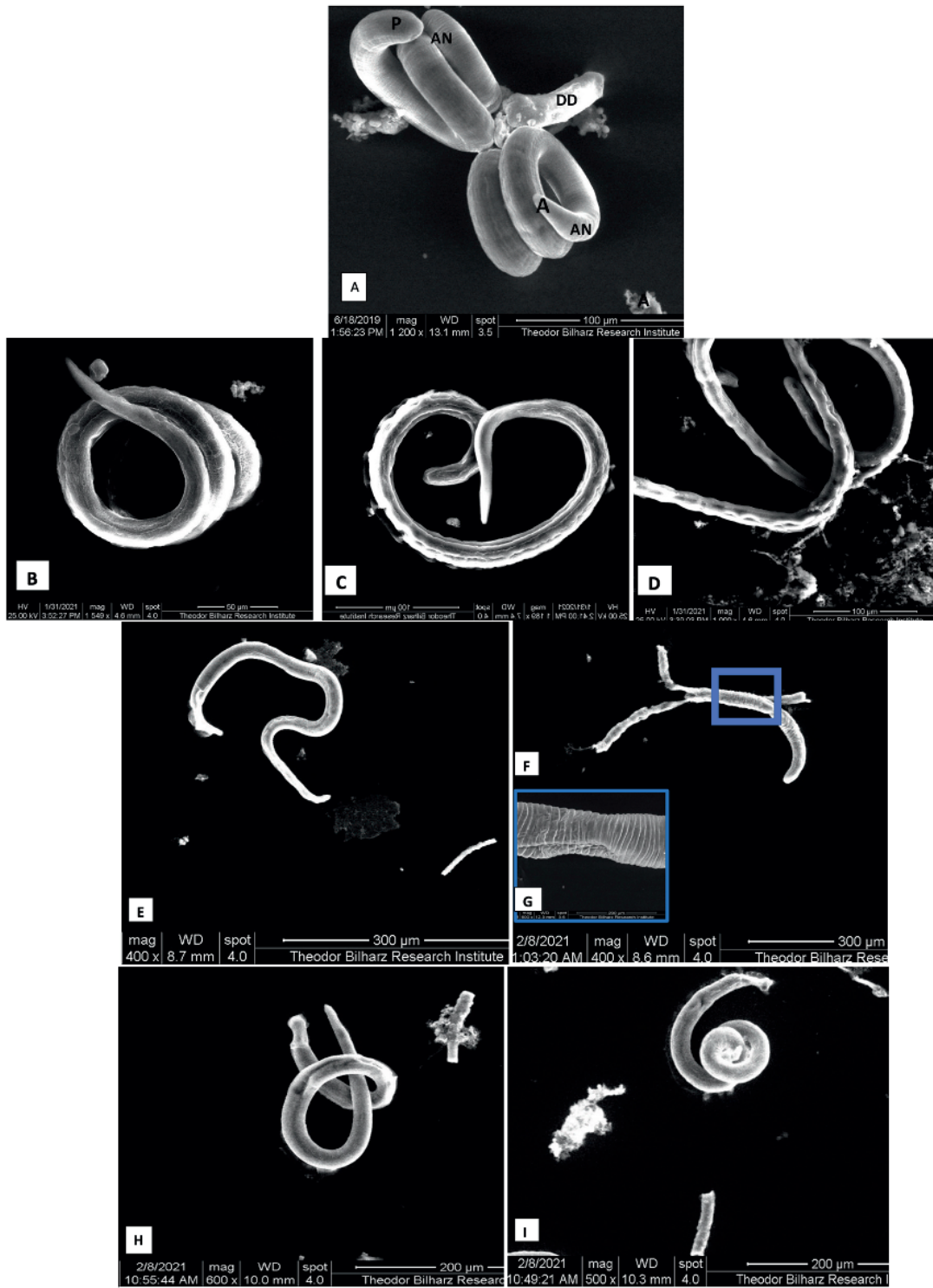


Fig. 4. *Trichinella spiralis* larvae 1h post-incubation in the RPMI-1640 medium.

A) Normal larvae with coiled body and well-defined annulated regions and ends. B-D) Larvae treated with a single dose of albendazole (200µg/ml) showing a few blebs, flattened transverse folds (B; alive), damaged ridges along the body, and internal opacity (C & D; dead). E-G) Larvae treated with a single dose of *Commiphora molmol* (myrrh; 200µg/ml), revealing flattened transverse folds and obscure corrugated cuticle. H & I) Larvae treated with a single dose of *Lepidium sativum* (garden cress, 200 µg/ml), showing normal coiled larvae without patent ridges and with mild blebs.

the natural medications is vital to fractionate them down to the most effective ingredient and dose.

This study investigated the effect of traditional natural medicines, myrrh, and garden cress on the *T. spiralis* worms and larvae *in vitro* to better understand their mode of action in the absence of host responses. To avoid any possibility of molting or sex differentiation of the parasite stages, the incubation was detected for 24 hours only based on Berntzen's work (1965).

Among the various tested concentrations of myrrh and garden cress, the highest concentration (200 µg/ml) indicated the best results one hour after incubation. Therefore, the anti-parasitic impacts of this dose were compared to their counterparts caused by 200 µg/ml of the reference drug albendazole. The latter effectively killed *Ascaris galli* larvae *in vitro* (Lalthanpuii & Lalchandama, 2020), which reckoned the chosen dose in the current study. Based on recent literature and studies approached by the researchers, the *in vitro* culture of *T. spiralis* worms with myrrh and garden cress has not been done.

The percent reduction of worms and larvae in this present study showed the same reduction pattern reported earlier (Abuelenain *et al.*, 2021) in a murine model. The data indicated that the mortality of worms due to the albendazole treatment protocol was the highest compared to myrrh and garden cress. The *in vitro* effect of albendazole on *T. spiralis* larvae concurs with the results recorded by Abdel-Rahman *et al.* (2020). However, the mortality detected in the current study was higher and faster, likely, because of using the RPMI-1640 (pH 7.2) as a solvent. This assumption is grounded on the data provided by Arias-Diaz *et al.* (2006), which revealed that albendazole's anti-nematode action was the best in an alkaline medium.

Interestingly, the larvae percent reduction due to myrrh was the highest compared to albendazole and garden cress. A work published by Abd-Elrahman *et al.* (2020) reported the superiority of albendazole in killing the larvae, *in vitro*, compared to myrrh. This contradiction may be mainly attributed to the difference in the solvents utilized.

On the one hand, the alterations of worm's morphology in albendazole protocol showed an entirely deteriorated cuticle and tegument. It is believed that the three-layered cuticle is non-cellular (Bruce, 1970), and the drug absorption is non-specific for being a non-polar hydrophobic agent (Lacey, 1990; Thompson and Geary, 1995; Lalchandama, 2010). Several studies demonstrated the anti-parasitic activity of albendazole alone or in combination with other drugs *in vitro* (Perez-Serrano *et al.*, 1994; Urrea-Paris *et al.*, 2000; Walker *et al.*, 2004; Elissondo *et al.*, 2006; Markoski *et al.*, 2006). The paralysis action of albendazole on the underlying tissue and muscles via the β -tubulin inhibiting activity was reported in several studies (Alvarez *et al.*, 2007; Rao *et al.*, 2009; Lalthanpuii & Lalchandama, 2020).

Unlike the sloughed and fissured cuticle and tegument of *T. spiralis* worms by albendazole, the dead larvae maintained the fully-to-semi coiled shape with occasional blebs over the corrugated cuti-

cle. This distinguished observation possibly reveals the challenge of combating albendazole's interaction with the muscle larvae stage, which have much more collagen content in the muscle cells than that measured in the adult worms (Bruce, 1970). The lack of bacillary band pores in the muscle larvae (Kozek, 2020) seems to limit the full access of albendazole to the hypodermis of the muscle larvae.

On the other hand, the morphological changes of worms and muscle larvae structure due to myrrh and garden cress propose an endogenous action mode. Some studies applied different drugs on nematodes and proposed the alimentary way for taking up those drugs (Mackenstedt *et al.*, 1993; Kopp *et al.*, 2008; Lalthanpuii & Lalchandama, 2020). The pernicious effect of the parasite forms seems to be a consequence of the extracts' metabolites in the alimentary parasite tract (larvae) and or vulva (worms). The metabolites of garden cress and myrrh are likely toxic enough to cause apoptosis to the hypodermal and epidermal cells but at a slower rate by garden cress. This belief is reinforced by the existence of ribosomes, mitochondria, lysosomal-like vesicles, endoplasmic reticulum, and lipoxidase activity in those cellular layers of the parasite forms (Bruce, 1970; Wright, 1987; Kozek, 2020). The cytotoxicity activity of both herbal extracts reported in many studies (Chen *et al.*, 2013; Abel-Aty *et al.*, 2019; Shan *et al.*, 2020; Chatoui *et al.*, 2020; Al Qahtani *et al.*, 2020) is due to the flavonoids, terpenoids, and inorganic constituents. The capabilities of myrrh to de-calcify the nurse cells for having manganese (Ahmad *et al.*, 2016), suppress the oxidative stress for inducing NO (Attia *et al.*, 2015), and flattening the muscle larvae by the anti-spasmodic action alongside its lethal effect make it a proposed auxiliary drug of albendazole.

In conclusion, the data collected from the previous *in vivo* work (Abuelenain *et al.*, 2021) and this subsequent one propose a combined clinical protocol of myrrh and albendazole in eradicating the larvae stage of trichinellosis.

Conflict of Interest

The research team declare that there is no interest of conflict with anyone, or entity and no fund was received for this work.

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