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Therapeutic efficacy of *Artemisia absinthium* against *Hymenolepis nana*: *in vitro* and *in vivo* studies in comparison with the anthelmintic praziquantel

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Abstract

Hymenolepis nana is a common intestinal tapeworm that affects humans. Drugs are available for the treatment of this infection, including praziquantel (PZQ), nitazoxanide and niclosamide. Although the drug of choice is praziquantel, due to its high cure rates, indicators of the development of PZQ resistance by different parasites have begun to appear over recent decades. Therefore, this study was a trial to find an alternative to PZQ by assessing the activity of the crude aqueous extract of the medicinal herb *Artemisia absinthium* against *H. nana*. *In vitro*, the extract was used against adult worms at concentrations of 1 and 5 mg/ml, in comparison with 1 mg/ml of PZQ. The times of worm paralysis and death were determined. Ultrastructural morphological changes were studied using transmission electron microscopy (TEM). For the *in vivo* study, infected mice were divided into untreated, PZQ-treated and *A. absinthium*-treated groups (400 mg/kg and 800 mg/kg). Pre- and post-treatment egg counts per gram of faeces (EPG) were performed; then, the reduction percentages of the EPG and worm burden were calculated. The best results were obtained with praziquantel. *Artemisia absinthium* induced worm paralysis, death and ultrastructural alterations, such as tegumental damage, lipid accumulation, and destruction of the nephridial canal and the intrauterine eggs, in a dose-dependent manner. Additionally, significant reductions in the EPG and worm burden were recorded in *A. absinthium*-treated mice. Although the results obtained with *A. absinthium* were promising and comparable to PZQ, further studies using different extracts, active ingredients and concentrations against different parasites should be conducted.

Introduction

Intestinal parasitic infections are among the main causes of human morbidity and mortality, especially in developing countries (Kiani *et al*., 2016). According to World Health Organization (WHO) estimates, more than 2 billion people are chronically infected with at least one gastrointestinal helminth parasite. These infections have been categorized as 'Neglected Tropical Diseases' because they affect the poorest and most deprived communities (World Health Organization, 2005). One of these intestinal parasites is *Hymenolepis nana*, which is a common tapeworm that affects humans (Thompson, 2015). Generally, *H. nana* infection has a worldwide distribution, but the highest prevalence is found in the temperate regions of developing countries, where poor sanitation and bad hygiene favour its direct transmission, especially among children (Abdel Hamid *et al*., 2015).

Some drugs are available for the treatment of *H. nana* infection, including praziquantel, nitazoxanide and

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niclosamide. The drug of choice is praziquantel, due to its high cure rate, which may reach 100% (Campos *et al*., 1984). Conversely, the efficacies of nitazoxanide and niclosamide are variable. For instance, the cure rate with nitazoxanide was 84% even after three treatments in one clinical trial (Diaz *et al*., 2003). Additionally, in a comparative study, the anticestodal drug niclosamide was found to be less effective against *H. nana* than praziquantel (Gupta & Katiyar, 1983).

Hymenolepis nana resistance against praziquantel has not been reported to date. However, there is a growing fear of developing this resistance as a logical consequence of drug abuse, dependence on a narrow range of drugs (Hoste & Torres-Acosta, 2011) and poor patient compliance, especially in endemic areas where poverty and ignorance are dominant. Unfortunately, indicators of the development of praziquantel resistance by different parasites have begun to appear during recent decades. For instance, *Schistosoma mansoni* field isolates with praziquantel resistance have been reported in Senegal (Fallon *et al*., 1995) and Egypt (Ismail *et al*., 1996). Additionally, the failure of praziquantel treatment was documented among *Taenia saginata*-infected patients (Lateef *et al*., 2008). Therefore, there is an urgent need to search for new antiparasitic agents to overcome this problem. Although the resistance mechanisms mentioned above may operate with any new drug, the presence of several alternatives to a single drug of choice should delay the time, or decrease the chance, of emergence of resistance.

Approximately 80% of people living in endemic areas for parasitic diseases rely on medicinal plants to cover their primary healthcare needs (World Health Organization, 2000). Additionally, almost 80% of the drugs used globally either originated from natural plants or were inspired by their natural derivatives (Li & Vederas, 2009). Therefore, the WHO traditional medicine strategy (World Health Organization, 2013) recently encouraged research studies inspired by traditional medicine to evaluate both the safety and effectiveness of widely used remedies worldwide.

One well-known medicinal herb is *Artemisia absinthium*. This herb is commonly known as 'wormwood' due to its anthelmintic properties, which have been recognized since the time of the ancient Egyptians (Padosch *et al*., 2006). In traditional medicine, the dried whole plant and its essential oil have been used as anthelmintic, antiseptic, antispasmodic and sedative remedies (Deans & Kennedy, 2002). During the past century, its use had declined due to fear of absinthism, which is a syndrome characterized by addiction, hyperexcitability, epileptic fits and hallucinations. This syndrome was attributed to thujone (a monoterpene ketone often present in the essential oil of wormwood). However, recent studies have shown that the concentrations of this substance in *A. absinthium* extracts are not sufficient to exceed the toxic thresholds. Additionally, thujone is less soluble in water than in ethanol. Therefore, the fears about absinthium have decreased, and in recent medical studies wormwood has started to gain attention as an alternative to conventional drugs (Lachenmeier, 2010).

Few experimental reports have investigated the use of *A. absinthium* as an antiparasitic agent. For example, *A. absinthium* has been used against gastrointestinal ovine nematodes (Tariq *et al*., 2009), *Trichinella spiralis* (Caner *et al*., 2008), *Toxocara catti* (Yıldız *et al*., 2011), *S. mansoni*, *Fasciola hepatica* (Ferreira *et al*., 2011), *Trypanosoma congolense* (Kifleyohannes *et al*., 2014), *Trypanosoma cruzi* and *Trichomonas vaginalis* (Martínez-Díaz *et al*., 2015). However, to the best of our knowledge, no previous studies have evaluated the use of *A. absinthium* against cestodes in general and *H. nana* in particular.

Therefore, the present study was designed to evaluate the therapeutic effects of the traditionally well-known medicinal herb, *A. absinthium*, against the common intestinal tapeworm *H. nana*. In addition to the medical importance of this parasite, *H. nana* is a good model that can be maintained and cultivated easily in the laboratory. In this study, *in vitro* and *in vivo* antiparasitic assays were conducted to compare the crude aqueous extract of *A. absinthium* to praziquantel, which is the drug of choice for the treatment of *H. nana* infection.

Materials and methods

Plant material and drugs

Plant material and extraction

Artemisia absinthium grows naturally on the open slopes of mountains in Saini, Egypt. The leaves of this erect plant are small, separated into three deeply lobed leaflets, and greyish-green with numerous small, pale yellow flowers. The leaves and flowers have a characteristic odour. For this study, fresh aerial parts were purchased from a local herb store in September of 2015. The plant was identified and authenticated by a botanist at the Botany Department, Faculty of Science, Menoufia University. The plant was thoroughly washed under running water and then air-dried in the shade. The dried plant was pulverized into a fine powder using an electric blender. The powdered plant material was stored in an airtight brown container at 4°C prior to extraction. In this study, the crude aqueous extract was chosen to decrease the possibility of the occurrence of systemic toxicity caused by thujone, which is more soluble in alcohol than in water. The extraction was performed according to the method of Kifleyohannes *et al*. (2014). Briefly, 100 g of the powdered plant was soaked in 500 ml of boiled distilled water in a flask and stirred intermittently for 72 h at room temperature. The material was filtered using a sterile Whatman No.1 filter paper into a clean flask. The filtration process was repeated three times. The pooled aqueous filtrate was freeze-dried and lyophilized to obtain a yield of 8.15 g. The extract was stored in a refrigerator at 4°C until needed.

Reference drug

Praziquantel (Discocide, 600 mg; Egyptian International Pharmaceutical Industries Company (EIPICO), Ramadan City, Egypt) was used as the reference drug in the anticestodal assays. This drug was dissolved in 0.1% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, St. Louis, Missouri, USA) prior to use.

Experimental animals

In the present study, Swiss albino male mice, 6–8 weeks of age and weighing an average of 20–25 g at the

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beginning of the experiment, were used. The mice were housed under controlled temperature and humidity conditions (25°C, 70%) and provided water and a balanced diet. An adaptation period of 2 weeks was allowed prior to commencement of the study.

Parasite maintenance

Fresh stool samples collected from patients attending Menoufia University hospitals were examined for the presence of *H. nana* eggs. The eggs were isolated from the stools using a saturated NaCl flotation method (Voge, 1970). The collected eggs were transferred into a clean 10-ml centrifuge tube and then washed with phosphate-buffered saline solution (PBS, pH 7.2) three times, to remove any trace of salt. The sediment containing the *H. nana* eggs was suspended in 0.1 ml of PBS (pH 7.2) to maintain their viability. To assess the egg viability, a sample of the isolated eggs was subjected to induction of hatching by addition of sodium hypochlorite solution together with a 0.01% trypan blue solution, as described by Wang *et al.* (1997). Hatching was observed under a microscope; the viable oncospheres were unstained. To maintain the strain in the laboratory, five mice were immunosuppressed by hydrocortisone acetate at a dose of 125 mg/kg per day orally for five consecutive days, starting on the same day as oral infection with the isolated *H. nana* eggs via gastric tube. The infective shell-free eggs were prepared by stirring the egg suspension with glass beads, as described by Matsuzawa *et al.* (1998). Each inoculum was adjusted to 200 eggs/0.1 ml of PBS, using a McMaster counting slide under a microscope.

Recovery of adult worms

Four weeks after infection, the mice were sacrificed and then dissected. The intestines were placed in Petri dishes with 0.9% NaCl to release adult worms from the intestinal mucus. The worms were washed several times with saline. The adult worms were examined individually under a microscope. Worms exhibiting any type of damage were isolated, and their gravid segments were homogenized in PBS to obtain the infective egg inoculum used in the *in vivo* study. The intact living adults were assigned to the *in vitro* study.

In vitro *study*

Living adult *H. nana* worms collected from freshly sacrificed mice were divided into four groups ($n = 5$) in separate Petri dishes with PBS, in an incubator at 37 ± 1°C. Group I served as the untreated control group. Group II was treated with the reference drug praziquantel at a concentration of 1 mg/ml. Groups III and IV were exposed to the *A. absinthium* crude extract at concentrations of 1 and 5 mg/ml, respectively. The extract and the reference drug were dissolved in a few drops of 0.1% DMSO, and the same amount of DMSO was administered to the control group.

The activity and survival of the adult worms were monitored under a microscope. Worm activity was determined by the presence of movement and contractions at the neck region. Worms not showing any physical movement upon gentle stimulation with a soft brush were picked up and transferred to warm PBS (40 ± 1°C). A total loss of noticeable worm movement during exposure to the warm solution was considered a sign of paralysis. Worm death was determined if stimulation by the warmer solution (~45°C) for more than 30 min did not stimulate any physical activity in the paralysed worm (Deori & Yadav, 2016). The experiments were repeated three times for each group.

Ultrastructure determination by transmission electron microscopic examination

Immediately after the induction of worm paralysis, the worm samples were fixed and prepared according to the method of Roy *et al.* (2008). Briefly, the control and treated worms were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2) at 4°C. Small pieces of the worm were post-fixed in 1% osmium tetroxide at 4°C for 2 h, dehydrated in an acetone series, and then embedded in Spurr's epoxy resin. Semi-thin sections were prepared and stained with 1% methylene blue in borax solution. For the transmission electron microscopy (TEM) analysis, ultrathin sections were placed on copper grids and double-stained with uranyl acetate and lead citrate. The examination was performed under the JEOL JEM 1230 transmission electron microscope (JEOL, Tokyo, Japan) operated at an accelerating voltage of 80 kV.

In vivo *study*

Experimental design

Forty Swiss albino mice were inoculated orally with 200 shell-free eggs/mouse in 0.1 ml of PBS. The mice were divided randomly into four groups, with ten animals per group. Group I served as the infected control group; each mouse was given 0.1 ml of distilled water with a few drops of 0.1% DSMO. Group II served as the praziquantel-treated group; PZQ was given at a dose of 25 mg/kg once, orally, according to Campos *et al.* (1984), on day 21 post infection (pi). Groups III and IV served as the *A. absinthium*-treated groups; the crude aqueous extract was given at doses of 400 and 800 mg/kg, respectively, once, orally for three consecutive days, commencing on day 21 pi. Assessment of efficacies relied on reductions in the percentages of the egg count per gram of faeces (EPG) before and after treatment, and the worm burden at necropsy. The pre-treatment EPG count was performed on days 18–20 pi, whereas the post-treatment EGF count was performed on days 24–26 pi. The experiment was terminated by sacrificing all animals on day 30 pi.

Calculation of the reduction percentages of eggs per gram of faeces (EPG) count and the worm burden

The reduction percentage of the EPG count was calculated according to Iqbal *et al.* (2004).

$$\text{EPG count reduction (\%)} = \frac{\text{Pre-treatment EPG count} - \text{post-treatment EPG count}}{\text{Pre-treatment EPG count}} \times 100.$$

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The worm burden reduction percentage was calculated according to Grzybek *et al*. (2016).

$$\text{Worm burden reduction (\%)} = \frac{a-b}{a} \text{ x } 100$$

where a = the mean number of worms recovered from the intestines of the infected and untreated (infection control group) mice, and b = the mean number of adult worms recovered from the treated mice.

Statistical analysis

All experimental data are presented as the mean ± standard deviation (SD) unless otherwise indicated. The data were analysed using the one-way analysis of variance (ANOVA) test followed by Tukey's *post-hoc* test to detect the statistical significance among the groups under study. Student's *t*-test was used to compare the pre-treatment and post-treatment data within each group. The results with $P < 0.05$ were considered significant.

Results

In vitro *study*

Worm paralysis and mortality

All tested concentrations of PZQ and the *A. absinthium* extract resulted in an initial spastic contraction of the adult worms within 10–30 s after exposure, followed by paralysis and then mortality of the treated worms at variable durations. Additionally, *A. absinthium* induced worm paralysis and death in a dose-dependent manner, and the difference between the *A. absinthium* concentrations used in groups III (1 mg/ml) and IV (5 mg/ml) was significant ($P < 0.001$) (table 1). Concerning the time of worm paralysis, a significant difference was found between both *A. absinthium* concentrations and PZQ. However, no significant difference was found in the time of worm death between *A. absinthium* at the 5 mg/ml concentration and PZQ (table 1).

Semi-thin and TEM ultrastructure morphological changes

The methylene blue-stained semi-thin sections of the control worms showed normal architecture of the tegumental area, with intact tegument, basal lamina and subtegumental tissues (fig. 1a). TEM revealed intact microtriches and basal lamina. Additionally, the subtegumental cells were rich in glycogen granules (fig. 1b). The tegument was rich in discoidal secretory bodies (fig. 1b, c). The muscular coat was formed with intact outer circular and inner longitudinal bundles (fig. 1b, d). The epithelial lining of the nephridial canal and its microvilli were intact (fig. 1e). Each intrauterine egg had an intact envelope and an intact oncosphere with hooks (fig. 1f).

Following exposure to PZQ, the semi-thin sections showed massive destruction and vacuolization of the tegument and the subtegumental area (fig. 2a, b). Blebs of different sizes were also observed on the tegument (fig. 2b). TEM revealed a complete loss of the normal architecture with vacuolization (fig. 2c), and wide areas were filled with cell debris (fig. 2d).

Conversely, semi-thin sections of the *H. nana* adults treated with *A. absinthium* showed areas of sloughing of the tegument with exposure and/or destruction of the underlying basal lamina. Intense vacuolization (fig. 3a) and the accumulation of variable-sized lipid droplets in the subtegumental area were observed (fig. 3a, b). TEM showed that exposure to *A. absinthium* at the 1 mg/ml concentration resulted in the appearance of membrane-bound vesicles over the microtriches (fig. 4a), whereas the 5 mg/ml concentration resulted in the loss of the microtriches, interruption of the circular muscle layer and shrinkage of the subtegumental cells (fig. 4b). Additionally, an accumulation of lipid droplets in the muscle bundles was observed in a dose-dependent manner (fig. 4c, d). The 5 mg/ml concentration also resulted in the destruction of the epithelial lining of the nephridial canal with the loss of its lining microvilli (fig. 4e), and degeneration of the intrauterine eggs (fig. 4f).

At the level of the cellular structure, exposure to *A. absinthium* at the 1 mg/ml concentration resulted in the appearance of heterochromatin islands in the nucleus (fig. 5a, b) and numerous secretory vacuoles in the cytoplasm (fig. 5a). The 5 mg/ml concentration resulted in swelling of the nucleus, the appearance of a big nucleolus, shrinkage of the cytoplasm and the formation of many double-membrane vacuoles containing inclusion bodies. These vacuoles were observed adjacent to the rough endoplasmic reticulum (fig. 5c).

In vivo *study*

Reduction in the EPG and worm burden percentages

All of the tested drugs resulted in significant reductions in the EPG counts and worm burden ($P < 0.001$) (tables 2 and 3). Additionally, a significant difference was found between the pre- and post-treatment EPG counts within each group (table 2). Dose-dependent significant differences in reductions in the EPG (table 2) and worm burden (table 3) were found between *A. absinthium*-treated groups III (400 mg/kg) and IV (800 mg/kg). The results obtained with *A. absinthium* at the 800 mg/kg dose were

Table 1. Times of paralysis and death of *H. nana* adult worms exposed to the tested drugs *in vitro*.

	Group I DSMO (0.1%)	Group II Praziquantel (1 mg/ml)	Group III *A. absinthium* (1 mg/ml)	Group IV *A. absinthium* (5 mg/ml)	ANOVA	*P* value
Time of paralysis (h)	–	0.38 ± 0.08	2.4 ± 0.16[b]	0.64 ± 0.11[bc]	$F = 410.75$	<0.001
Time of death (h)	43.8 ± 3.03	1.55 ± 0.22[a]	6.78 ± 0.19[ab]	3.2 ± 0.42[ac]	$F = 855.56$	<0.001

Time of death and time of paralysis were expressed as the mean ± SD; $n = 5$.
$P < 0.05$ was considered significant according to the *post-hoc* test: [a], significant difference vs. group I; [b], vs. group II and [c], vs. group III.

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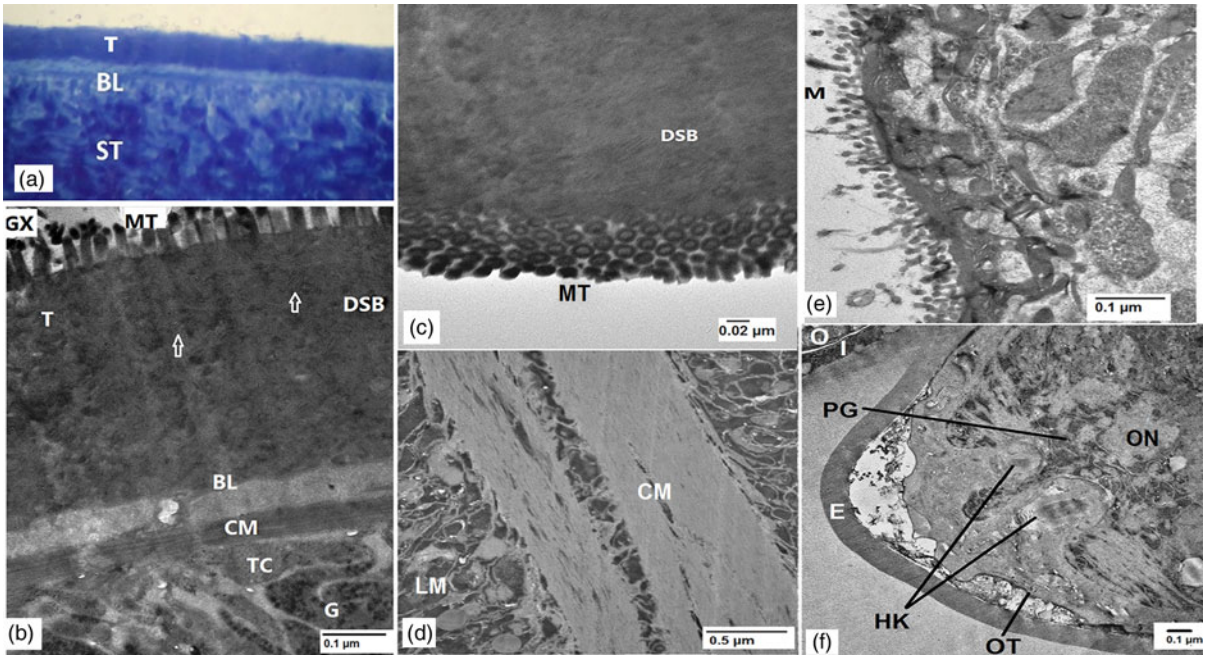

Fig. 1. (a) A semi-thin section of an untreated control *H. nana* adult segment, showing the intact tegument (T), basal lamina (BL) and normal subtegumental architecture (ST), after staining with 1% methylene blue, 100× magnification. (b–f) TEM ultrastructures of untreated *H. nana* adults. (b) Tegumental area showing the intact brush border of the microtriches (MT) with the glycocalyx (GX), tegument (T) rich in discoidal secretory bodies (DSB, white arrows), basal lamina (BL), transverse muscle bundles (CM), and tegumental cells (TC) rich in electron-dense glycogen granules (G), 20,000× magnification. (c) Cut sections in an intact brush border of microtriches (MT). The tegument is rich in discoidal secretory bodies (DSB), 40,000×. (d) Normal longitudinal muscles (LM) and transverse muscle bundles (CM), 6000×. (e) Nephridial canal showing an intact epithelium and microvilli (M), 20,000×. (f) Intact egg showing the outer (O) and inner (I) envelopes, embryophore (E), and oncosphere (ON) with penetration gland (PG), hooks (HK) and oncospheral tegument (OT), 8000× magnification.

comparable to the results obtained with PZQ, because the difference between groups II (PZQ) and IV (*A. absinthium*, 800 mg/kg) was not significant ($P > 0.05$) (tables 2 and 3).

Discussion

The present study aimed to evaluate the therapeutic effects of the crude aqueous extract of the traditionally well-known medicinal herb *A. absinthium* against the common intestinal tapeworm *H. nana*, in comparison with the drug of choice (praziquantel), in controlled *in vitro* and *in vivo* experiments. The results of this study revealed promising anticestodal effects of *A. absinthium* against adult *H. nana*, both *in vitro* and *in vivo*. These results were in accordance with previous studies utilizing *A. absinthium* herbal extracts against different parasitic diseases and recorded similar effects. For example, Caner *et al*. (2008) reported that the methanolic extract was effective against enteral and parenteral phases of experimental trichinellosis. Additionally, Ferreira *et al*. (2011) documented that the ethanolic extract, at a 2 mg/ml concentration, was effective against *S. mansoni* and *F. hepatica* adults *in vitro*. Tariq *et al*. (2009) reported that both the ethanolic and aqueous extracts of *A. absinthium* showed anthelmintic activity against *Haemonchus contortus* adult worms *in vivo* and *in vitro*. However, the authors found that the ethanolic extract was more efficient than the aqueous extract.

Generally, alcoholic extracts of medicinal plants show better anthelmintic properties than aqueous extracts (Eguale *et al*., 2007). However, use of the *A. absinthium* aqueous extract at the 5 mg/ml concentration *in vitro* and the 800 mg/kg dose for three consecutive days *in vivo* produced results close to those obtained with PZQ. Comparing these groups with PZQ revealed no significant differences ($P > 0.05$) regarding the time of worm death and the reduction percentages of EPG and the worm burden. In contrast to the present study, the ethanolic extract of *A. absinthium* was found to be less effective than the reference drugs against intestinal coccidiosis in goats (Iqbal *et al*., 2013). One explanation for this discrepancy could be that the antiparasitic activities of medicinal plants or their products depend not only on the nature of the extract (aqueous or alcoholic) but also on the plant composition, which varies from country to country (Orav *et al*., 2006), the targeted parasitic species and the concentrations or doses used (Ferreira *et al*., 2011). In the present study, the effectiveness of *A. absinthium* against *H. nana* was dose-dependent, which was in accordance with previous studies using different types of plant extracts against many parasites (Bashtar *et al*., 2011; Kundu *et al*., 2012; Deori & Yadav, 2016).

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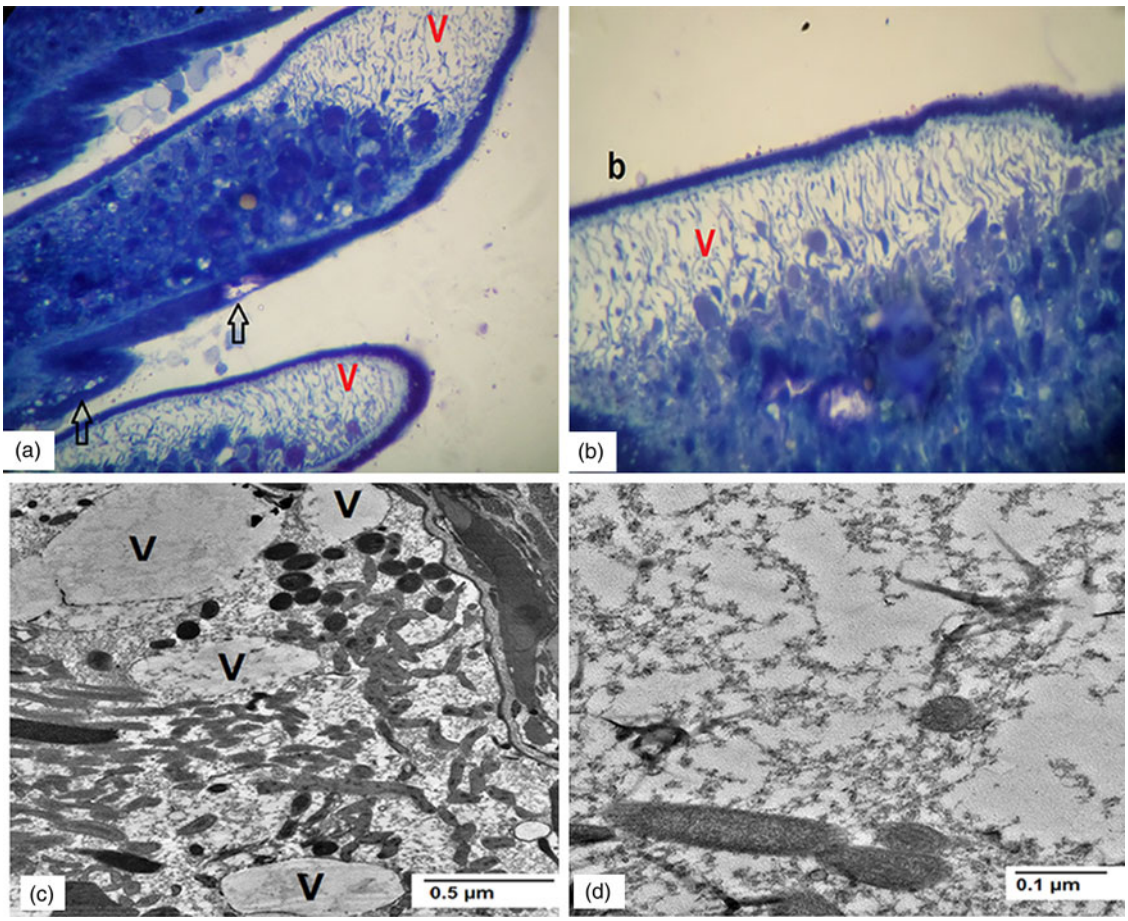

Fig. 2. Segments of *H. nana* adult treated with praziquantel (1 mg/ml). (a, b) Semi-thin sections, showing: (a) destruction of the tegument (black arrows) and vacuolization (V) of the subtegumental area; and (b) blebs on the tegument and vacuolization (V) after methylene blue staining; 100× magnification. TEM micrographs of praziquantel-treated *H. nana* adult, showing: (c) areas of vacuolization (V), 6000×; (d) degenerated cells and areas full of cell debris, 8000× magnification.

Generally, cestodes lack a digestive system. Therefore, all nutritive and gaseous exchanges must occur through their tegument and the microtriches, which are considered vital structures responsible for nutrition, immunoprotection and osmoregulation (Roy *et al*., 2008). With respect to the morphological changes induced by the tested drugs in the teguments of the treated worms, we observed an obvious loss of the normal architecture in the worms exposed to all the drugs tested *in vitro*.

In this study, exposure to PZQ resulted in a complete loss of the normal architecture, with intense vacuolization and cellular degeneration. To explain how these changes were induced by PZQ, Prichard *et al*. (1982) suggested the occurrence of a sustained release of endogenous calcium with a subsequent increase in the calcium concentration in the cytoplasm, which might result in a spastic contraction of the PZQ-treated worms, followed by vacuolization of their teguments. The increase in the size of these vacuoles results in their protrusion above the surface, to become visible as blebs. These blebs finally burst, resulting in leakage of glucose and amino acids (Andrews *et al*., 1983). Continuous leakage would result in intense cellular destruction and worm death. Therefore, the results of the present study were in accordance with many studies that considered PZQ the anticestodal drug of choice (Kundu *et al*., 2012; Deori & Yadav, 2016; Giri & Roy, 2016).

Conversely, exposure to *A. absinthium* at a 1 mg/ml concentration resulted in the formation of membrane-bound vesicles over the microtriches and the accumulation of variably sized lipid droplets in the muscle bundles. At a 5 mg/ml concentration, *A. absinthium* resulted in a complete loss of the microtriches, interruption of the circular muscle, glycogen depletion, shrinkage of the subtegumental cells, accumulation of large lipid droplets, destruction of the epithelial lining of the nephridial canal and degeneration of the intrauterine eggs.

Alteration or loss of the microtriche brush border would affect the nutrient absorption process, and thus the worms would begin to consume their glycogen stores. This scenario could explain the occurrence of glycogen depletion upon exposure to the *A. absinthium* crude extract

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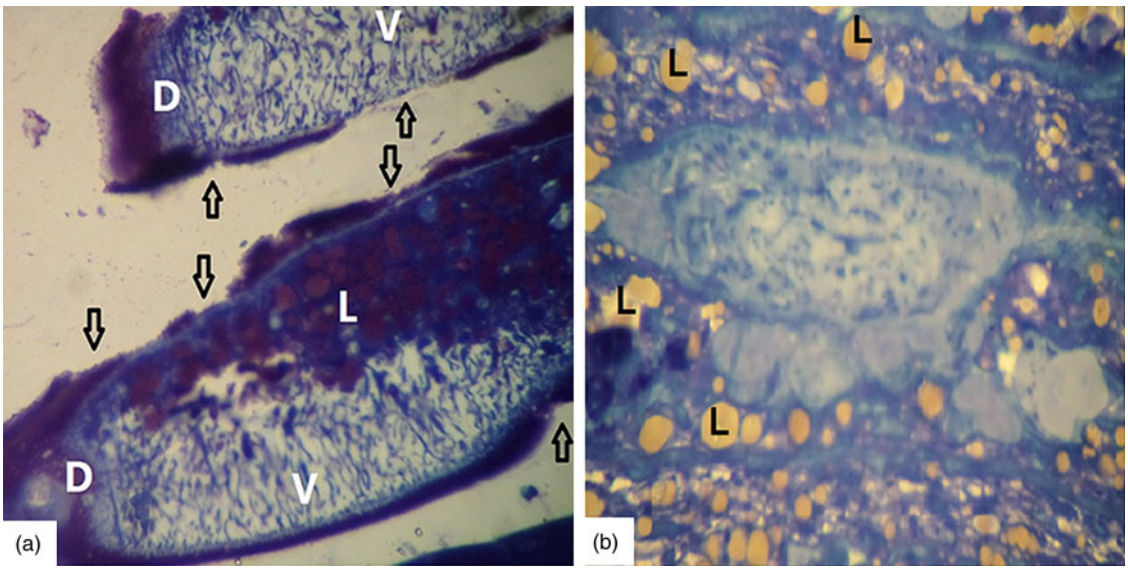

Fig. 3. Semi-thin sections of *H. nana* adult segments treated with *A. absinthium* at a 5 mg/ml concentration, showing: (a) sloughed areas of the tegument (arrows), lipid (L) accumulation in the subtegument, areas of degeneration and destruction of the basal lamina (D) and intense vacuolization (V) after methylene blue staining, 100× magnification; (b) subtegumental area with accumulation of variably sized, yellowish lipid droplets after methylene blue staining, 400× magnification.

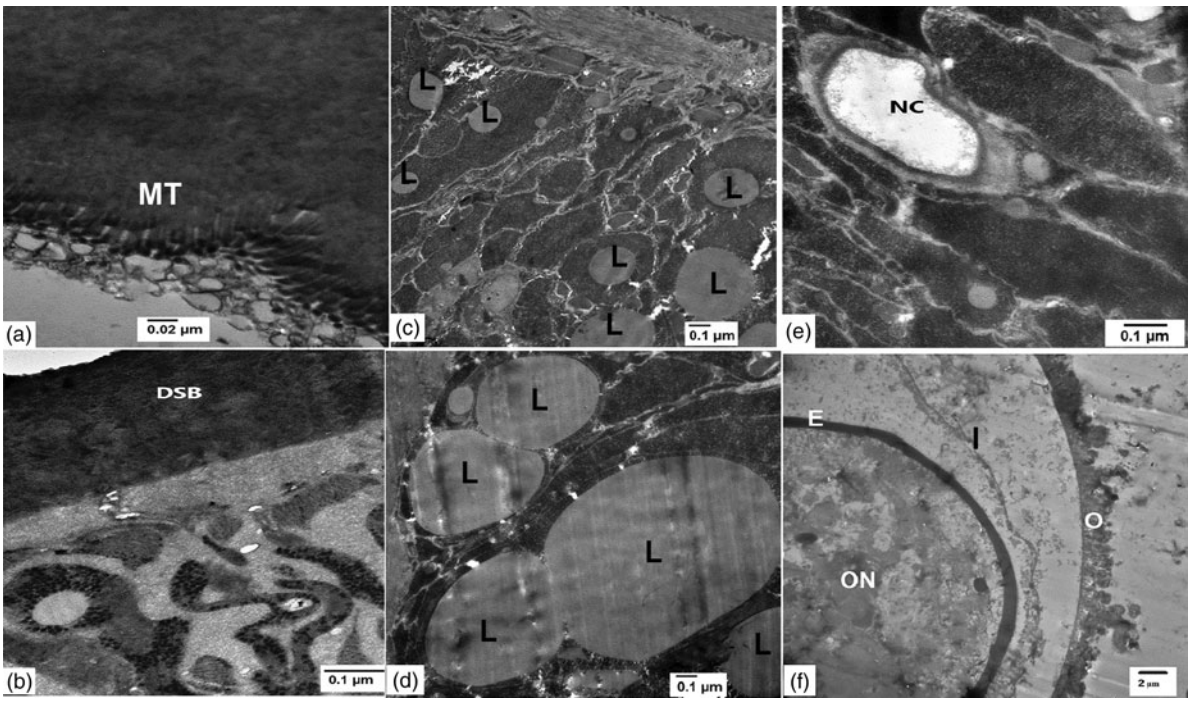

Fig. 4. TEM micrographs of *A. absinthium*-treated *H. nana* adults. (a) Tegumental area after treatment with 1 mg/ml showing microtriches (MT) with numerous membrane-bound vesicles, 40,000× magnification. (b) Tegumental area in an adult worm treated with 5 mg/ml showing loss of the microtriches (MT), with abundant dense secretory bodies (DSB), interruption of the circular muscle, shrunken subtegumental cells and widening of the areas occupied by connective tissue, 20,000×. (c) Accumulation of variably sized lipid droplets (L) in muscle bundles after treatment with 1 mg/ml of *A. absinthium*, 8000×. (d) Huge lipid droplet (L) accumulation in muscle bundles following treatment at a concentration of 5 mg/ml, 8000×. (e) Degeneration of the microvilli lining the nephridial canal (NC) following treatment with a concentration of 5 mg/ml, 12,000×. (f) Degenerated egg and the oncosphere (ON) lacking the hooks and penetration gland following treatment with a concentration of 5 mg/ml; O, outer envelope; I, inner envelope; E, embryophore; 6000× magnification.

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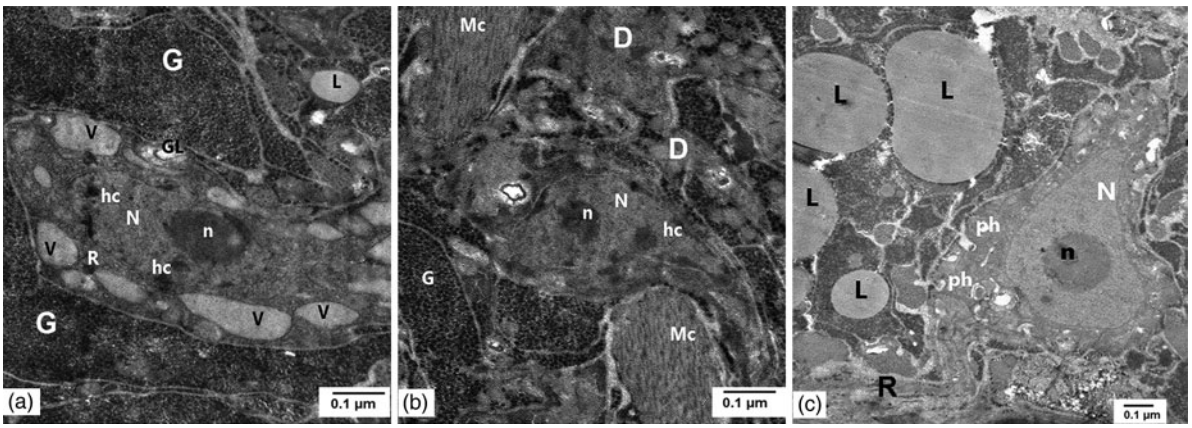

Fig. 5. (a, b) TEM micrographs of *A. absinthium*-treated (1 mg/ml) *H. nana* adults. (a) A subtegumental cell in between muscle bundles with glycogen granules (G) and a lipid droplet (L) starting to appear. The nucleus (N) contains a large electron-dense nucleolus (n) and heterochromatin islands (hc). The cytoplasm contains a rough endoplasmic reticulum (R), Golgi body (GL) and numerous secretory vacuoles (V). 20,000× magnification. (b) A cell showing shrunken cytoplasm and depletion of cellular organelles surrounded by intact muscle bundles (Mc) and areas rich in glycogen (G). Areas of glycogen depletion (D) were also observed. N, nucleus; n, nucleolus; hc, heterochromatin islands; 20,000× magnification. (c) Ultrastructure micrograph of a worm exposed to a 5 mg/ml concentration showing a myocyton with a swollen nucleus (N) with a big nucleolus (n). The cytoplasm contains a granular endoplasmic reticulum (R), many phagosomes (ph) containing inclusion bodies. The surrounding muscle bundles were infiltrated with variably sized lipid droplets (L), 10,000× magnification.

in this study. O'Neill *et al*. (2009) and Dasgupta *et al*. (2010) claimed that glycogen depletion together with mitochondrial dysfunction, which occurs as a result of stressful conditions, such as exposure to drugs (Pérez-Serrano *et al*., 1997), would result in impairment of the tegument. In the present study, not only was the nutrient uptake affected but the excretory system was also disturbed, which would result in an accumulation of waste products. Thus, the worms exposed to *A. absinthium* were most likely pushed into a vicious circle of depletion and damage. However, these worms would try to repair the damaged tegument by activation of cellular organelles to overcome the rapid turnover of the tegument (McCaigue *et al*., 1986). These trials to repair the damaged tegument could explain the formation of membrane-bound vesicles over the microtriches of the *A. absinthium*-treated worms, the appearance of an active Golgi apparatus and the numerous cytoplasmic secretory vacuoles (fig. 5a).

In addition to the stressful conditions, the mitochondria could also be affected by artemisinins, which were reported to cause inhibition of electron transfer and oxidative phosphorylation (Li *et al*., 2005), collapse of the mitochondrial membrane potential and triggering of cytochrome *c* release from the mitochondria into the cytoplasm, with final activation of caspase-3-mediated apoptosis (Jia *et al*., 2016). In the present study, TEM revealed some morphological signs of apoptosis in the *A. absinthium*-treated worms (fig. 5a, b), such as condensation and clumping of chromatin into heterochromatin islands, as described by Bashtar *et al*. (2011) and Roy *et al*. (2012) in parasites exposed to different plant extracts.

Accordingly, double-membrane phagocytic vacuoles containing inclusion bodies (most probably the destroyed organelles) were found upon exposure to the 5 mg/ml concentration of *A. absinthium* in this study (fig. 5c). These vacuoles were found in a close contact with the rough endoplasmic reticulum and thus were identified as autophagosomes according to Česen *et al*. (2012). The appearance of these autophagosomes is the morphological hallmark of autophagy (a self-eating process) (Eskelinen *et al*., 2011). This process usually occurs as a survival mechanism in cells exposed to stressful

Table 2. Pre- and post-treatment eggs per gram (EPG) and percentages of reduction among the different groups treated with the tested drugs.

	Group I DSMO (0.1%)	Group II Praziquantel (25 mg/kg)	Group III *A. absinthium* (400 mg/kg)	Group IV *A. absinthium* (800 mg/kg)	ANOVA	*P* value
Pre-treatment EPG	10521.6 ± 164.73	10639.8 ± 109.91	10615.4 ± 191.67	10553.2 ± 202.53	$F = 0.51$	0.682
Post-treatment EPG	10530.4 ± 122.97	0.0 ± 0.0^{a}*	4054 ± 107.47^{ab}*	122.2 ± 180.44^{ac}*	$F = 8246.74$	<0.001
Change or reduction (%)	1.052	−100%	−61.8%	−98.8%		

EPG values are expressed as the mean ± SD; $n = 10$.
$P < 0.05$ was considered significant, according to the *post-hoc* test: [a], significant difference vs. group I; [b], vs. group II and [c], vs. group III.
* Indicates a significant difference between the pre- and post-treatment EPG within each group according to Student's *t*-test.

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Table 3. Worm burden and its percentage reduction among the different groups treated with the tested drugs.

	Group I DSMO (0.1%)	Group II Praziquantel (25 mg/kg)	Group III *A. absinthium* (400 mg/kg)	Group IV *A. absinthium* (800 mg/kg)	ANOVA	*P* value
Worms/mouse	26.2 ± 1.92	0.0 ± 0.0[a]	11.4 ± 1.14[ab]	0.4 ± 0.55[ac]	$F = 574.28$	<0.001
Reduction percentage (%)	0%	100%	56.5%	98.5%		

Worm numbers per mouse were expressed as the mean ± SD; $n = 10$.
$P < 0.05$ was considered significant according to the *post-hoc* test: [a], significant difference vs. group I; [b], vs. group II and [c], vs. group III.

conditions such as starvation and pharmacological insults (Loos *et al*., 2014). Through autophagy, denatured proteins and damaged organelles are degraded into amino acids to be recycled in the synthesis of the essential proteins needed for the survival of these struggling cells (Xie & Klionsky, 2007). Thus, the appearance of these autophagosomes in the cells of the worms exposed to *A. absinthium* suggested an attempt to salvage any available nutrients to remain alive. This finding was in accordance with Loos *et al*. (2014), who reported the occurrence of autophagy in the larval stages of *Echinococcus granulosus* upon exposure to pharmacological stress.

In the present study, accumulation of lipid droplets in the muscle bundles was seen with administration of *A. absinthium* in a dose-dependent manner (Fig. 4c, d). McCaigue *et al*. (1986) also recorded lipid deposition in the immune-induced dark areas of *H. diminuta*. The latter authors explained this finding due to the lack of balance between the intake, use and excretion of lipids. They assumed that the normal lipid absorption pathway and its use in phospholipid synthesis would be stimulated and integrated into the cell membranes for tissue repair. Additionally, inhibition of lipid metabolism and excretion, which are energy-dependent processes, was expected to occur as a result of the impaired functions of the mitochondria and depletion of the glycogen store. Collectively, all of these factors would explain the occurrence of lipid accumulation in the muscle bundles of the *A. absinthium*-treated worms.

In the light of the above findings, the *A. absinthium* crude aqueous extract showed promising effects against adult *H. nana*, both *in vitro* and *in vivo*, in a dose-dependent manner. These results were comparable to the results induced by the reference drug praziquantel. *In vitro*, *A. absinthium* was able to induce worm paralysis and death. TEM examination of the treated worms revealed morphological changes, such as tegumental damage, lipid accumulation, and destruction of the nephridial canal and the intrauterine eggs. Additionally, signs of apoptosis and autophagy were observed, which could be ascribed to the impairment of nutrient uptake due to the tegumental damage caused by *A. absinthium*. Therefore, the damaged worms started to consume their glycogen stores. Lipid accumulation occurred due to inhibition of lipid metabolism and excretion, which are energy-dependent processes. The continuous exposure to stress and the accumulation of waste products most likely triggered the apoptosis process. However, these affected worms would not surrender to death without trying to remain alive through the autophagy (self-eating) process. Moreover, results of the *in vivo* study supported those obtained in the *in vitro* study, because *A. absinthium* induced significant reductions in EPG and the worm burden. Collectively, these results demonstrate the anticestodal efficacy of *A. absinthium* against *H. nana*.

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Conflict of interest

None.

Ethical standards

All of the experimental animal studies were conducted in accordance with the valid international guidelines after approval by the Scientific Research Ethical Committee, Faculty of Medicine, Menoufia University.

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