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ORIGINAL ARTICLE

Anti-helminthic effect of *Punica granatum* peel extract on *Trichinella spiralis* worms and muscle larvae: in vitro and in vivo studies

Shaimaa H. El-Sayed[1] · Soheir S. Mahmoud[2] · Amal M. El-Shenawy[2] · Hebat-Allah S. A. Yousof[3]

Abstract

Trichinella spiralis (*T. spiralis*) is a prevalent foodborne intestinal parasite in many developing countries. Albendazole (ABZ) is the drug of choice for treating trichinosis despite its several drawbacks as its week effect against encapsulated larvae, low bioavailability, and emerging drug resistance. As a result, new anthelmintic agents are required. This study aims to investigate the in vivo and in vitro effects of *Punica granatum* peels extract (PGPE) on intestinal and muscle phases of *T. spiralis*. The adult worms and larvae were isolated and cultured with different concentrations of PGPE ranging from 6.75 to 100 µg/ml and measuring the survival rate was done after 1, 3, 18, 24 and 48 h of incubation, followed by scanning electron microscopic (SEM) examination of isolated parasites. For the in vivo experiment, the infected animals were divided into two main groups: intestinal phase group and muscular phase group, each group was subdivided into; infected not treated, infected treated with PGPE, ABZ and combined PGPE and ABZ (6 mice in each). The drug effect was assessed by adults and larvae load. A significant increase in the percentage of dead adult parasite and muscle larvae cultured with PGPE with severe destruction and deformity of the tegument were observed with SEM. Also, a significant reduction of adult parasite number in the intestine and muscle larva number in the diaphragm of infected treated mice in comparison to the control group. This study proved that PGPE has a potential activity against trichinosis, particularly when combined with ABZ, and this could serve as a new agent in trichinosis therapy.

Keywords Albendazole · In vitro · In vivo · Mice · *Punica granatum* peel extract · *Trichinella spiralis*

Introduction

Trichinosis is a worldwide foodborne helminth infection, notably prevalent in developing nations, with almost 10,000 individuals infected each year. *Trichinella spiralis* (*T. spiralis*) is the causative nematode species of this infection (Wang et al. 2018). *Trichinella spiralis* is thought to be the most common pathogenic species in humans. It has two stages in its lifecycle: intestinal and muscular stages. Infection occurs by ingestion of the infective larvae of *T. spiralis* in improperly cooked pork (Pozio 2019). Anthelmintic medications, particularly derivatives of benzimidazole as albendazole (ABZ) and mebendazole (MBZ), were applied to treat trichinosis in humans, but without efficiency in destroying the encysted larval stage (Attia et al. 2015). Moreover, drug resistance was revealed by some of these drugs and restricted bioavailability. Some of these medications are contraindicated in children under the age of three and in pregnant women, while others are thought to cause cancer (Yadav 2012). Consequently, instituting effective and safe anti-trichinosis agents is a necessary requirement. Plant-derived medication could be a good promising alternative.

Punica granatum (*P. granatum*), usually called pomegranate and is newly defined as a powerful fruit, which has been applied as folkloric prescription for treating different diseases (Abdel Moneim 2012). Pomegranate has numerous therapeutic properties such as antioxidant, anti-carcinogenic, anti-obesity, anti-diabetic, cardioprotective and anti-inflammatory properties. It also has therapeutic effects in many diseases like male infertility and Alzheimer's disease. Its seeds, fruits, juice, and peel all contain medicinal

✉ Shaimaa H. El-Sayed
Shaimaa.helmy@med.helwan.edu.eg

1   Medical Parasitology Department, Faculty of Medicine, Helwan University, Cairo, Egypt

2   Medical Parasitology Department, Theodor Bilharz Research Institute, Giza, Egypt

3   Medical Parasitology Department, Faculty of Medicine, Cairo University, Cairo, Egypt

and health-promoting components. (Jurenka 2008; Saeed et al. 2018).

Pomegranate peel extract contains exceptional phytochemicals with nutritional and therapeutic value. Pomegranate peel contains over 48 phenolic components (polyphenols, flavonoids, ellagitannins, and proanthocyanidins). (Singh et al. 2002; Vučić et al. 2019).

Pomegranate peel extracts have biological functions such as normalising gastrointestinal movements, inhibiting growth of bacteria, and preventing atherosclerosis and obesity. As they have lipid lowering effect, acceleration of wound healing, prevention of hypertension and cardiovascular disease, having antidiabetic effects and protection against cerebral ischemia (Dos Santos et al. 2016; Arun et al. 2017). It also possesses other pharmacological properties, such as antiviral, anti-inflammatory, antimicrobial, antifungal and antidiarrheal properties (Bassiri-Jahromi et al. 2018; Zhao et al. 2018). Various research suggests that pomegranate peel extract is effective in suppressing intestinal cestodes, trematodes, and nematodes (Abdel-Ghaffar et al. 2011; Boonmasawai et al. 2017).

Consequently, the present study aimed to show the PGPE effect on adult and larval stages of experimental *T. spiralis* as well as its effect on in vitro cultured worms and larvae in comparison with ABZ.

Material and methods

T. spiralis isolate

The parasite isolate used in this study was initially taken from infected pork from Cairo slaughterhouse. *Trichinella* isolate was maintained in the animal house at Theodor Bilharz Research Institute (TBRI) via serial oral mice infection by muscle larvae. The Parasite isolate used in this study was identified as *T. spiralis* by the European Union Reference Laboratory for Parasites at the Superior Institute of Health in Rome, Italy.

Drugs and drug preparation

Punica granatum peels extract (PGPE)

The peels were used to prepare *P. granatum* extracts. It was obtained from a local market in Cairo, Egypt. It was kept in the TBRI's Medicinal Chemistry Department. The fruit was washed, the peels were manually peeled, sliced into little pieces, and dried before being processed to powder in the grinder and stored. In a Soxhlet extraction equipment, the plant sample powder was extracted with 85% Methanol for 24 h. The preceding procedure was repeated three times, and the PGPE was concentrated under vacuum with

a rotatory Buchi evaporator before being collected in desiccators. (Rowayshed et al. 2013).

Just before use, Dimethyl sulfoxide was used to dissolve PGPE, which was then diluted in RPMI-1640 medium to prepare the stock solution of 1 mg/ml.

Albendazole

ABZ was manufactured and purchased by Pharma Cure, Pharmaceutical Industries, Egypt in a suspension form.

Isolation of muscle larvae and adults for in vitro culturing

Muscle larvae

Artificial digestion was used to collect larvae from infected mouse carcasses thirty days after infection as stated by Ozkoc et al. (2009).

Adult parasite

Worms were extracted from the small intestines of mice infected with T. spiralis larvae a week earlier. After scarifying the mice, the small intestine was dissected, cut into two-centimeter sections, opened longitudinally, and placed for 3 h on gauze in a beaker containing 250 ml 0.8% saline at 37 °C. Afterward, the worms were collected and rinsed three times with phosphate buffered saline (PBS).

Trichinella spiralis adults and larvae were collected and placed in a 96-well microtiter plate (100 parasites/well) with RPMI-1640 media (containing 200 U/ml penicillin, 200 g/ml streptomycin, and 20% foetal bovine serum).

The final concentration of ABZ against adults and muscle larvae was 250 μg/ml and the final concentrations of PGPE against adults and muscle larvae were 6.75–100 μg/ml. Blank and DMSO controls were placed, and each determination was done in triplicate.

Assessing the survival of *T. spiralis* adults and larvae

The survival of *T. spiralis* adults and larvae was studied using a dissecting microscope in the first, third, 18th, 24th, and 48th hours after incubation with ABZ and PGPE for body motility and death incidence. Worms that did not move for sixty seconds after being observed were considered dead.

Ultrastructural studies using SEM

Ultrastructural morphological characteristics of adults and larvae samples were investigated and compared to the control groups to evaluate the impact of the assessed drugs. Adults and larvae were examined according to the method of

Glauert (1974) using Environmental SEM (Inspect S: FEI, Holland).

Experimental animal and infection

A batch of 64 outbred male CD-1 Swiss albino mice aged 6 weeks, with an average weight of 20 ± 2 g. All animal experiments were carried out in accordance with the National Institutes of Health's guidelines for the care and use of laboratory animals and its revisions, and were carried out at TBRI's animal house in Giza, Egypt. Each mouse was orally infected by *T. spiralis* larvae (N = 200) (Dunn and Wright 1985).

Experimental design

The mice were divided into two main groups which were intestinal phase group and muscular phase group. Each group was subdivided into four subgroups which were infected not treated, infected treated with 1000 mg/kg of PGPE, infected treated with 50 mg/kg of ABZ and infected treated with both PGPE and ABZ.

Each subgroup was consisted of 6 mice, and the sample size was calculated by G*Power (version 3.1.9.2; Germany).

Concerning the intestinal phase group, the treatment started 2 h post infection and continued daily for five successive days and animals were sacrificed on the sixth day post infection to detect adult parasite in the small intestine. Concerning the muscular phase group, the treatment was started on day 30 post infection and continued daily for five consecutive days and animals were sacrificed on 35th day to detect the encysted larvae in the muscles.

Counting of adult and larval stages of the parasite

Adult counting in the intestine

The intestine was cleansed and chopped into small two-centimeter sections before being incubated at 37 °C in ten ml saline for 2 h to allow the worms to exit the gut, after which the saline was withdrawn, and the intestine was washed multiple times. The fluid was collected in tubes and centrifuged at 1500 rpm for 5 min then the supernatant was decanted and was examined and counted microscopically at a magnification $\times 10$ (Issa et al. 1998).

Encysted larvae counting in the diaphragm

Each mouse from muscular phase group was longitudinally opened, each mouse's diaphragm was meticulously dissected and examined under a microscope at a magnification of $\times 4$ to count the larvae in the whole diaphragm (Basyoni and El-Sabaa 2013; Munoz-Carrillo et al. 2017).

Statistical analysis

The SPSS version 24.0 software was used to document, review, and enter all data (IBM, Chicago, USA). The results obtained were expressed as the mean standard deviation (SD) of six mice in each group. At 95% confidence level, all data were analyzed using an ANOVA test, followed by a student's t-test. p 0.05 values were considered statistically significant.

Ethical considerations

All procedures were carried out with the agreement of the Ethical Committee of the Faculty of Medicine, Helwan University, Cairo, Egypt, in accordance with the ethical regulations for animal experiments (Serial: 5-2023).

Results

In vitro Effect of PGPE on survival of cultured *T. spiralis* adult worms and larvae

Data obtained for the incubated worms showed that all treatment regimens had greater percentages of dead worms depending on dose and exposure period, as indicated in Table 1. After 24 h of exposure, it was evident that all treated worms died at the highest concentrations of PGPE (100 g/ml) and ABZ (250 g/ml), but the control only accounted for 20% of deaths at that time. In compared to the ABZ and control groups, the percentage of dead worms treated with PGPE (100 g/ml) after just 3 h post-incubation was 70%, which was significant (p 0.001). Also, it was discovered that, as compared to the control group, all of the PGPE concentrations employed were considerably effective 48 h after incubation.

Table 1 Effect of time-monitored incubation with different concentrations of PGPE compared to ABZ on survival of cultured *T. spiralis* adult worms

Drugs/concentrations μg/ml	Percentage of dead worms at different incubation periods				
	1 h	3 h	18 h	24 h	48 h
Control	0	0	10	20	30
ABZ 250	0	40	80	100	100
PGPE 100	0	70	82	100	100
PGPE 50	0	20	55	75	100
PGPE 25	0	0	30	40	100
PGPE 12.5	0	0	10	30	100
PGPE 6.25	0	0	10	25	75

For the larval stage, it was also observed that all treatment regimens had higher percentages of dead larvae when the dose and exposure duration were increased, as indicated in Table 2. After 48 h of exposure, it was evident that all treated larvae died with the highest concentrations of PGPE (100 g/ml) and ABZ (250 g/ml), whereas the control accounted for 30% death at that time. After 24 h post-incubation, 60% of the PGPE-treated larvae (100 g/ml) were dead, which was significantly higher (p 0.001) than the control group. Also, it was discovered that, as compared to the control group, all of the PGPE concentrations employed were considerably effective 48 h after incubation (Table 2).

It was observed that blank and DMSO controls had no effect on the incubated warms and larvae.

In vivo Effect of PGPE on number of *T. spiralis* adult worms in intestine and encysted larvae in diaphragm of infected mice (Table 3)

The mean number of adult worms in the small intestine was significantly much lower after treatment with ABZ (6.71 ± 2.67) and PGPE (9.42 ± 4.54) when compared to the control infected untreated group (73.23 ± 13.6) ($p < 0.05$) with a percentage of reduction 90.8% and 87.2%, respectively when compared to the control infected untreated

group. When PGPE and ABZ were combined, the reduction percentage rose to 95.5%.

Additionally, the mean number of encysted larvae in the diaphragm was significantly lower after treatment with ABZ (6647 ± 685.51) and PGPE (8552.33 ± 586.84) when compared to the control infected untreated group ($49,253.67 \pm 5862.39$) ($p < 0.05$) with a percentage of reduction 86.5% and 82.6%, respectively when compared to the control infected untreated group. When PGPE and ABZ were combined, the reduction percentage rose to 91.2%.

Ultrastructural changes of the cultured *T. spiralis* adult worms and larvae

According to a SEM analysis of adult worms, culturing them with PGPE caused serious damage to the adult worms. The cuticle showed occurrence of blebs, large vesicles, erosions, sloughing and opacity along with damage of the normal creases, ridges, and annulations of the cuticle. ABZ had a milder impact on the cuticle than PGPE. Meanwhile, the culture of the adult worms in the incubation medium only, revealed preservation of the normal morphology with the characteristic transverse creases, ridges, and annulations of the cuticle (Fig. 1).

Regarding the effect of PGPE on cultured larvae, it was noticed that the occurrence of deformity of cuticle with emergence of huge vesicles, many blebs, notches, fissures along the cuticle surface and opacity. ABZ had a milder impact on the cuticle than PGPE. The larvae were cultured with just the incubation media, which showed that the characteristic coiled body and normal cuticle appearance with fine transverse creases and well-defined annular ends had been preserved (Fig. 2).

Table 2 Effect of time-monitored incubation with different concentrations of PGPE compared to ABZ on survival of cultured *T. spiralis* larvae

Drugs/concentrations μg/ml	Percentage of dead larvae at different incubation periods				
	1 h	3 h	18 h	24 h	48 h
Control	0	0	0	10	30
ABZ 250	0	0	10	50	100
PGPE 100	0	0	20	60	100
PGPE 50	0	0	5	40	85
PGPE 25	0	0	0	25	75
PGPE 12.5	0	0	0	15	70
PGPE 6.25	0	0	0	0	60

Discussion

Treatment of trichinosis has been confronted by the shortage of a totally successful drug of choice. ABZ, one of the derivatives of benzimidazoles, is the existing treatment of choice used for treating trichinosis. There are some limiting factors during the use of ABZ like its low
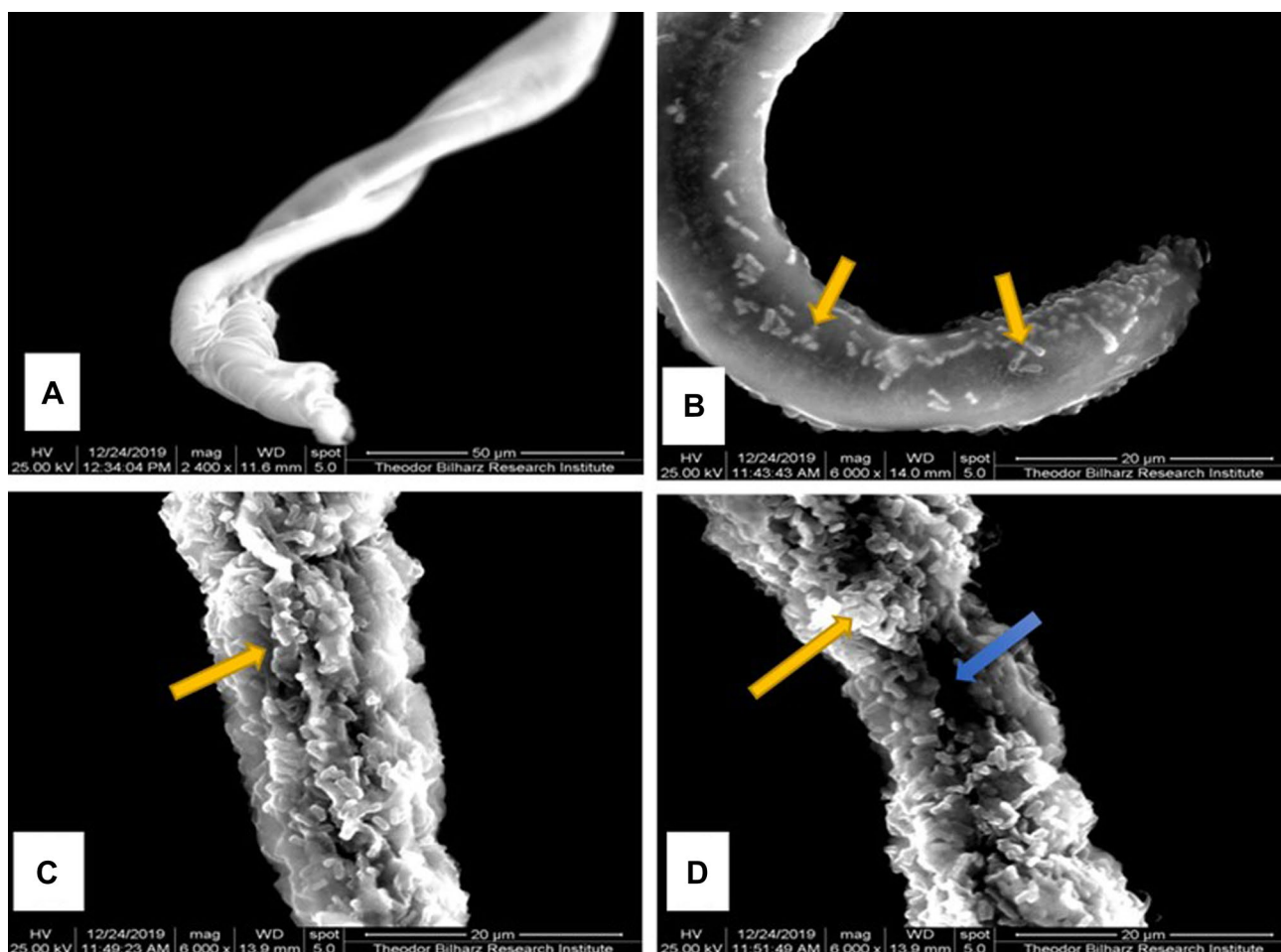
Table 3 Effect of PGPE compared to ABZ on number *of T. spiralis* adult worm in intestine and encysted larvae in diaphragm of infected mice

Experimental Groups	Number of Adult worms recovered from intestine. Mean ± SD	Number of Larvae recovered from diaphragm. Mean ± SD
Control	73.23 ± 13.6	$49,253.67 \pm 5862.39$
ABZ	6.71 ± 2.67* (90.8%)	6647 ± 685.51* (86.5%)
PGPE	9.42 ± 4.54* (87.2%)	8552.33 ± 586.84* (82.6%)
PGPE + ABZ	3.34 ± 1.13* (95.5%)	4313.33 ± 683.05* (91.2%)

Values are expressed as Mean ± SD and Reduction (%) from control, *Statistically significant compared to infection control group ($p < 0.05$)

Fig. 1 Scanning electron micrographs of *T. spiralis* adult worm 24 h post-incubation in the RPMI-1640 showing: **A** normal appearance with fine transverse creases and annulated cuticle with longitudinal ridges in normal control group, **B** loss of striation and transverse creases with multiple vesicles (yellow arrows) in treated group with ABZ (250 μg/ml), **C** degenerative changes of cuticle with occurrence of blebs, large vesicles (yellow arrow) and sloughing in treated group with PGPE (50 μg/ml), **D** complete destruction and deformity of cuticle with multiple large cauliflower vesicles (yellow arrow), clumps of blebs, erosions, sloughing and opacity (blue arrow) in treated group with PGPE (100 μg/ml) (Color figure online)

water solubility and consequently inadequate bioavailability (Hettiarachchi et al. 2016) as well as the emerging of drug resistance which is progressively reported worldwide (Dilks et al. 2020; Srivastava and Misra-Bhattacharya 2015).

Plants have long been the main competitors in the discovery of novel medications. Plant extracts are a valuable source of a wide range of biochemically active combinations that may have an influence on a wide range of therapeutic goals. (Yones et al. 2016; Anand et al. 2019; Ullah et al. 2020).

In the present work, we studied the therapeutic potential of a medicinal plant extract; PGPE in vitro on cultured *T. spiralis* adult worms and muscle larvae during different time-monitored incubation periods with different concentrations, then examining the ultrastructural changes using SEM. Also, in vivo testing of PGPE, throughout the intestinal and muscular phases of trichinosis in compared to or/and combined with ABZ.

Among the various tested in vitro concentrations of PGPE, it was revealed that all the used concentrations resulted in severe destruction and deformity of the tegument. The lethal effect of PGPE enhanced when the dose and treatment duration were increased as observed by SEM.

The significant reduction of the adult worms' burden in the small intestine and encysted larvae in the muscles after treatment with PGPE may be attributable to its potent anti-inflammatory effect within the intestinal and muscle tissues. This may interfere with the parasite-induced immune response through various immunomodulatory mechanisms such as down regulation of CD4-T helper cells which play a critical adaptive immune response during infection with *T. spiralis* (Wang et al. 2020) and induction of
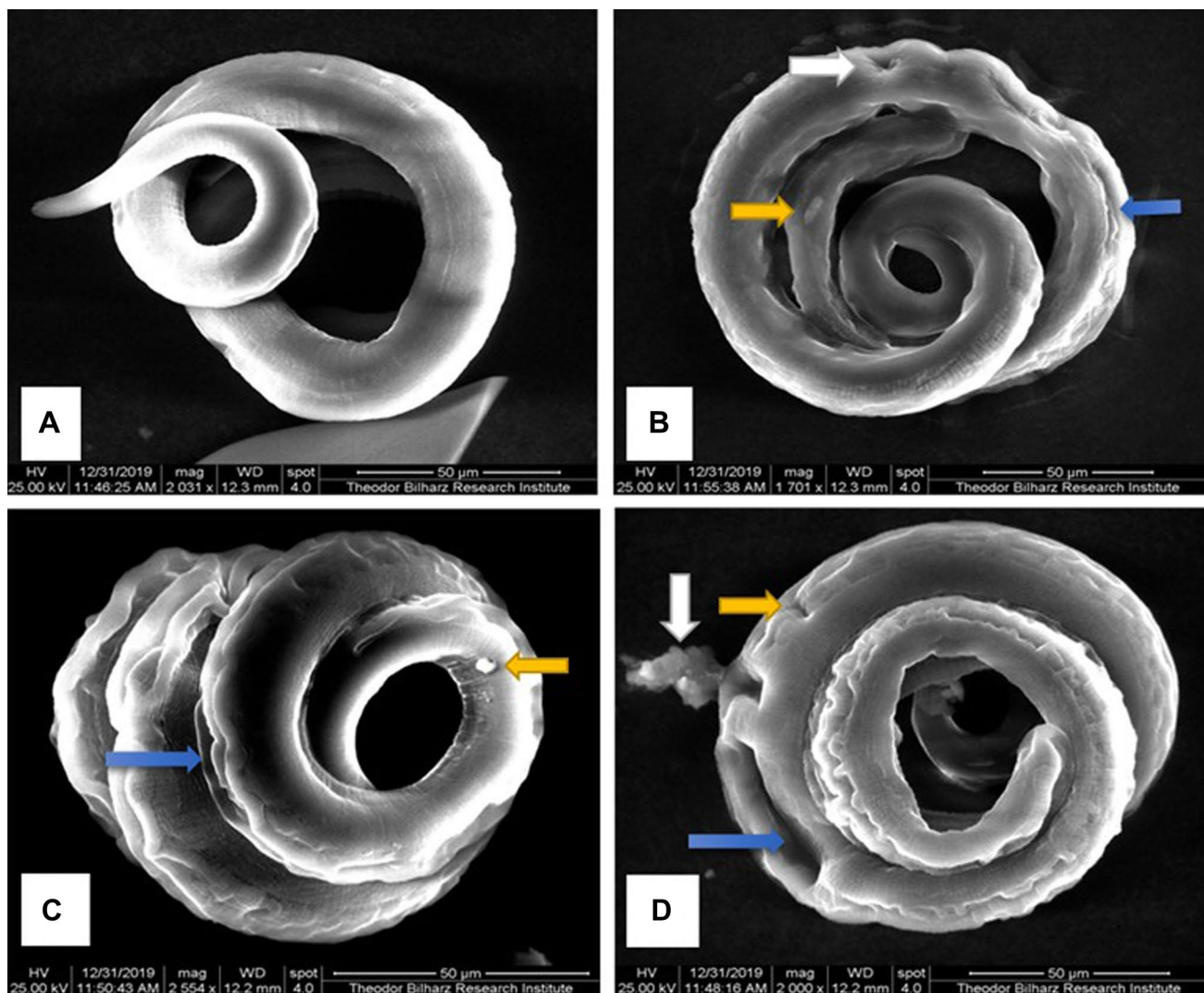
Fig. 2 Scanning electron micrographs of *T. spiralis* larvae 24 h post-incubation in the RPMI-1640 showing: **A** typical coiled body, normal cuticle appearance with fine transverse creases and well-defined annulated ends in non-treated control group, **B** few blebs (yellow arrow), notches (white arrow) and small fissure in the cuticle (blue arrow) in treated group with ABZ (250 µg/ml), **C** degenerative changes of cuticle with occurrence of blebs (yellow arrow), notches and fissures along the cuticle surface (blue arrow) in treated group with PGPE (50 µg/ml) **D** deformity of cuticle with appearance of large cauliflower mass (white arrow), multiple blebs, notches (yellow arrow), fissures along the cuticle surface and opacity (blue arrow) in treated group with PGPE (100 µg/ml) (Color figure online)

mastocytosis with degranulation of mast cells affecting the intestinal endothelial junctions which in turn enhancing intestinal fluid secretion. Furthermore, down regulation of CD4-T cells increases the contractility of the intestinal muscles triggering expulsion of the adult worm (Steel et al. 2019).

In muscle phase, the superior effect of PGPE on reduction of the count of muscle larvae could be explained by the anti-inflammatory properties of the extract. The inflammatory Th2-immune response elicited by the larvae enhancing their encapsulation and immune evasion. This was reversed by the down regulation of CD4-T cells elicited by the *P. granatum* peel ethanolic extract (Sun et al. 2019; Wang et al. 2020).

Amongst a variety of *P. granatum* peel extracts, the highest antioxidant potential has been observed for ethanolic extract which is capable of eliminating the free radicals and enhance immunity. Phenols, tannins, and flavonoids were detected as the most important extract components that have effective antioxidant, antimicrobial, and anthelminthic actions (Barathikannan et al. 2016; Castagna et al. 2020).

Phenols influence the oxidative phosphorylation decoupling which in turn leads to ATP synthesis and interference with energy production essential for parasite growth and viability (Salhan et al. 2011).

Tannins can increase the cell permeability which hinder absorption of nutrients, mobility, reproduction and

accordingly lead to parasites death (Botura et al. 2013). It was reported that *P. granatum* peel methanol extract decreases the motility of the larval stages (Jabeen et al. 2015) and reduces the hatching speed of some parasites' eggs (Ahmed et al. 2020). These findings were because of the secondary extract metabolites, mainly tannins.

Hafez et al. (2020) stated that PGPE had a promising therapeutic effect on muscular phase of trichinosis and recommended using it with radiation-attenuated vaccination as an adjuvant. Recently, Esmat et al. (2021) reported that PGPE-ABZ combination reduced load of muscle larvae and relieve myositis in experimental *T. spiralis* infection.

Various studies have studied the effect of PGPE on several intestinal helminths as well as extra-intestinal helminths. For example, Dkhil (2013) stated that methanol extract of *P. granatum* peel produced paralysis and killing of adult *Allolobophora caliginosa* in vitro. Also, Abdel Aziz et al. (2018) stated that *P. granatum* peel aqueous extract has a significant in vitro effect compared to fenbendazole on *Ascaridia galli.* Moreover, Castagna et al. (2020) found an in vitro ovicidal activity of *P. granatum* aqueous extract against sheep intestinal nematodes. Additionally, PGPE has an active role in treatment of *Hymenolepis nana* and *Echinococcus granulosus* infections through antihydatic scolicidal effect and immunomodulatory properties (Al-Megrin 2016; Labsi et al. 2016). *Punica granatum* leaf and peel and extracts have as well an in vivo and in vitro activity against larval and immature and mature stages of *Schistosoma mansoni* (Fahmy et al. 2009; Osman et al. 2013).

PGPE has antiprotozoal effects against *Trichomonas vaginalis, Blastocystis* species, *Giardia lamblia and* malaria in vitro (Abdel-Hafeez et al. 2016; Al-Megrin 2017). It is effective as an anticoccidial agent through decreasing the output of *Eimeria papillate* in vivo, improving the intestinal inflammation and vacuolation of jejunal epithelium without exhibiting any side effects (Dkhil 2013).

Furthermore, PGPE is effective in treatment of infection with *Cryptosporidium parvum*. After examination of different parameters such as diarrhea, shedding of oocysts, weight loss/gain and histopathological assessment of the ileal sections. Infected mice treated with PGPE revealed continuous weight gain, recovery of intestinal histopathology and decrease of oocysts shedding (Al-Mathal and Alsalem 2012; Oshiba et al. 2018). Similarly, Aboelsoued et al. (2019) reported that the efficacy of Nano-formula of PGPE against *Cryptosporidium parvum* oocysts and suggested the daily use of pomegranate peel in the animal diet and its use as a beverage for humans to protect against infection and to improve health.

It was assumed that PGPE could be a promising reliable natural medicinal agent against adult and encysted larval stages of *T. spiralis* parasite. Moreover, PGPE-ABZ combination offers a higher response than ABZ alone. Further research is recommended to explain the molecular basis of the PGPE action in trichinosis and isolate its active compounds so that the therapy can be significantly more successful while avoiding undesirable effects.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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