

In vitro Effects of *Punica granatum* Ellagitannins on Adult Worms of *Schistosoma mansoni*

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Abstract: Schistosomiasis ranks second behind malaria in terms of overall morbidity and mortality. We evaluated the lethal effect of *Punica granatum* ellagitannins, extracted from the fruit rind, placenta and barks of the root and stem, on adult worms of *Schistosoma mansoni* (*S. mansoni*). All four ellagitannins were lethal to *S. mansoni* adult worms. However, while the rind ellagitannins were the most potent, placental ellagitannins were the least. Rind ellagitannins were capable of killing 40% of adult worms at a concentration of 25 µg/mL after 5 days. The killing of 100% of the worms was achievable by rind ellagitannins at a concentration of 50 µg/mL after 5 days. The LD₅₀s of the rind ellagitannins after 96h and 120h were 41.25 µg/mL and 28.73 respectively. Ellagitannins-treated worms suffered from erosions, wrinkles, swellings and losses, degenerations of the surface tubercles and tegument. In addition, ellagitannins induced deformation and degradation of oral and ventral suckers and degenerations in the muscles of worms. Ellagitannins also caused a separation of coupled worms and reduction of their motility. Data obtained suggest that ellagitannins of pomegranate could be considered as a cheap candidate for the treatment of schistosomiasis.

Keywords: *Punica granatum*, ellagitannins, *Schistosoma mansoni*, tegument, praziquantel

Introduction

Human schistosomiasis is one of the neglected tropical diseases caused by an intravascular blood-dwelling fluke.¹ Most human infections are caused by *S. mansoni*, *S. haematobium* and *S. japonicum*, which are endemic in African, Asian and South American countries.¹

It was estimated that more than 230 to 250 million people are infected annually with schistosoma and 779 million are at risk of getting an infection. On the other hand, about 280,000 annually die as a result of schistosomiasis worldwide.²

In the Middle East and North Africa region alone about 12.7 million individuals are infected with *Schistosoma*.³ *S. mansoni*, causes an annual infection of 54 million and 393 million are at risk of infection.⁴

Schistosomiasis has been greatly reduced in Middle East countries like Saudi Arabia, Morocco and Egypt. In Saudi Arabia the control of schistosomiasis has reduced the incidence of infection to 0.1% in 2003 and 0.02% in 2010. Areas of risk are restricted to Asir in the south-western region of Saudi Arabia.³ In 2012, the incidence of schistosomiasis was reduced to <3% in most villages.³

Praziquantel is a safe, effective, and cheap drug, which was released in 1979. In 2002, the World Health Organization sponsored its use during pregnancy and lactation.⁵ Praziquantel causes Ca²⁺ influx leading to spastic paralysis of adult

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worms. In addition, it causes rapid vacuolization of the worm surface.⁶ Praziquantel has been used in integrated control programmes of massive treatment of people in high-risk areas. It is given as a single oral dose for the treatment of all human *Schistosoma* spp. According to the world Health Organization, at least 290.8 million people required preventive treatment for schistosomiasis in 2018, out of which more than 97.2 million people were treated.⁷

The long-term worldwide application of praziquantel has led to the appearance of praziquantel-tolerant schistosomes in Senegal.⁸ On the other hand, in Egypt, where praziquantel has been extensively used, 1–2.4% of the treated villagers were not cured even after repeated administration of high doses of the drug.⁹ Schistosomes obtained from those individuals were tolerant to 3 times higher doses than the reference control isolates.¹⁰ More other reports have been published indicating failures of praziquantel treatment.^{11,12}

The emerging of *S. mansoni* with reduced susceptibility to praziquantel in different infected human populations would have negative implications on the control programmes of schistosomiasis. Therefore, there has been an emphasis on the importance of finding alternatives to praziquantel.^{12,13}

Punica granatum (*P. granatum*) is traditionally used for a wide variety of diseases, such as kidney problems, diarrhea, dysentery, hyperacidity, piles and cough.¹⁴ *P. granatum* extracts also possess antioxidant, anticancer and anti-inflammatory properties.¹⁴ Experimental data suggest that extracts of different parts of pomegranate are useful in the treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction, bacterial infections, infertility, Alzheimer's disease, arthritis, and obesity.^{14,15}

P. granatum is rich in polyphenols like ellagitannins which are the major element of pomegranate extracts and they are responsible for its biological activities.¹⁶

P. granatum extracts were proved to be active against different parasites. For instance, *Entamoeba histolytica* is killed with IC₅₀ of < 30 µg/mL pomegranate extracts.¹⁷ Extracts of the peel of *P. granatum* were reported to have anti-coccidial properties in both mice and Japanese quail.^{18,19} In vitro and in vivo anthelmintic effect of pomegranate leaves and stem bark extracts were lethal to *S. mansoni* adult worms.¹⁹ The *P. granatum* extracts was significantly found to reduced the number and diameter of hepatic granulomas, decrease the number of schistosomal eggs in liver tissues, lower the liver inflammatory infiltration, and decrease the hepatic fibrosis in mice.²⁰

In previous studies, we demonstrated the lethal effect of *P. granatum* ellagitannins against both miracidia and cercariae of *S. mansoni*.^{21,22} In this study, we investigated the lethal effect of ellagitannins of *P. granatum* on adult worms of *S. mansoni*. Data obtained suggest that *P. granatum* ellagitannins affect the viability, morphology and histology of *S. mansoni* adult worms.

Methods

Ethical and Legal Approval

Ethical and legal approval was obtained from the Deanship of Scientific Research at Taif University prior to the commencement of the study. All experiments were performed following the regulations of Taif University and the Saudi national guidelines for scientific research.

Preparation of Pomegranate Parts for Extraction

Fruit rind and placenta, stem bark and root barks of *P. granatum* were collected from pomegranate fruits and small trees grown in Taif farms, Saudi Arabia. All parts were air-dried at room temperature in the laboratory. The dried parts of the plant were ground by an electric blender.

Extraction and Separation of Ellagitannins

The powdered rind, placenta, stem and root barks were percolated in water overnight in a shaking incubator. XAD-16 (Sigma, USA) resin washed with methanol was packaged into a glass column and equilibrated with water. Aqueous extracts were applied to the column. The resin was washed with water and the adsorbed ellagitannins were eluted with methanol. Methanol was evaporated at 50°C in a rotary vacuum evaporator and kept at –20°C until used.²³

Preparation of Cercariae

Cercariae of *S. mansoni* NMRI strain were prepared from infected *Biomphalaria alexandrina* snails purchased from Theodor Bilharz Research Institute (Giza, Egypt). The snails were exposed to artificial light at 25°C to release cercariae which were used directly after shedding to infect mice.²⁴

Mice Infection

Male WF1-albino mice aging 6–8 weeks and weighing 25–35g were obtained from King Fahd Specialist Medical Centre, Jeddah, KSA. They were kept in wire-mesh polycarbonate cages with autoclaved bedding. Mice had free access to food and water. All animal procedures were done in accordance

with the ethical standards of Taif University and were approved by the Animal Experimentation Ethics Committee.

Adult male mice were infected according to the method of Olivier and Stirewalt.²⁵ Briefly mice were individually placed in mouse-retaining chambers which allowed the tails to be immersed into tubes containing 100 cercariae in de-chlorinated tap water for 1h. The tails were removed and allowed to dry.

Isolation and in vitro Treatment of Adult *S. mansoni* Worms with Ellagitannins

Adult worms were recovered and cultured in vitro as previously described,²⁶ with some modifications. Briefly, infected mice after being exposed to cercariae by 6 weeks were killed by cervical dislocation and dipped in 10% ethanol to minimize contamination. Portal and mesenteric vessels were perfused with sterile normal saline, and the adult worm pairs were removed aseptically from the mesenteric veins. Worms were washed with sterile phosphate buffer and cultured in 12 well plates containing RPMI 1640 medium (Sigma, USA) supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), 10% foetal calf serum (Gibco, USA), 2g/L glucose, 0.39g/L glutamate, and 20g/L NaHCO₃.

Tannins were dissolved in sterile distilled water and added at the desired concentrations to the cultured worms in a final volume of 2 mL. Worms cultured in RPMI 1640 medium without the addition of tannins served as positive control. The plates were incubated in 5% CO₂ atmosphere at 37°C. The worms were kept for 5 days and monitored for their motor activity (motility), pairing and viability every 24h under a dissecting microscope. Death of the worms was defined with the absence of movement for at least two minutes from the examination.

Scanning Electron Microscopy

Adult worms were fixed in 4% glutaraldehyde in cacodylate buffer, pH 7.4, for two hours and washed by the same buffer. The worms were then post-fixed in 2% Osmic acid and washed in cacodylate buffer. Finally, worms were dehydrated with increasing concentrations of ethanol. Specimens were mounted on stubs coated with gold and examined with a Joel JEM-1200 EXII electron microscope (Japan).²⁷

Histopathological Investigation of the Recovered Worms

Worms were mounted with 70% ethanol, fixed in Bouin's solution, and dehydrated by passage through increasing

concentrations of alcohol. The worms were cleared with xylene, embedded in paraffin wax and finally, thin sections were prepared by a microtome. The sections were rehydrated and stained with hematoxylin and Eosin. The stained sections were dehydrated, cleared with xylene and mounted under a coverslip with Canada balsam. The sections were examined under the light microscope using the oil immersion lens.²⁸

Statistical Analysis

All experiments were done in triplicates and values of mean $\pm$ standard deviation were compared using Students *t*-test. The statistical analysis of data was carried out using SPSS 16 statistical software programme.²⁹

Results

Effects of *P. granatum* Ellagitannins on Adult Worms of *S. mansoni*

S. mansoni adult worms were exposed to three concentrations of *P. granatum* ellagitannins prepared from rind, stem, root and placenta for 120h. Throughout the experiment, the control female and male worms were viable and actively moving under the microscope.

Ellagitannins, at all the tested concentrations, caused a separation of the coupled worms and a reduction in their motor activity (data not shown).

The killing of 100% of the adult worms of *S. mansoni* was achievable by all the four investigated ellagitannins at 100µg/mL after 5 days. However, generally speaking, the ellagitannins of the rind was more active than other ellagitannins (Figure 1).

While at 25µg/mL, ellagitannins of the stem and placenta failed to kill adult worms, rind and root bark were lethal to 40% of the adult worms after 5 days (Figure 1).

The rind and root ellagitannins killed 20% and 10% of the worms respectively after 3 days at a concentration of 50µg/mL. Only the rind ellagitannins were capable of killing 100% of the worms at a concentration of 50µg/mL after 5 days. However, the 100% mortality of worms was also achieved after only 3 days by rind ellagitannins at a concentration of 100µg/mL as shown in Figure 1.

Praziquantel was more active on schistosoma worms than ellagitannins. It killed 100% of the adult worms at all the tested concentrations (25, 50 and 100µg/mL) after 2 days.

The LD_{50s} of the rind were more significantly ($P < 0.005$) lower than the LD_{50s} of the other tested

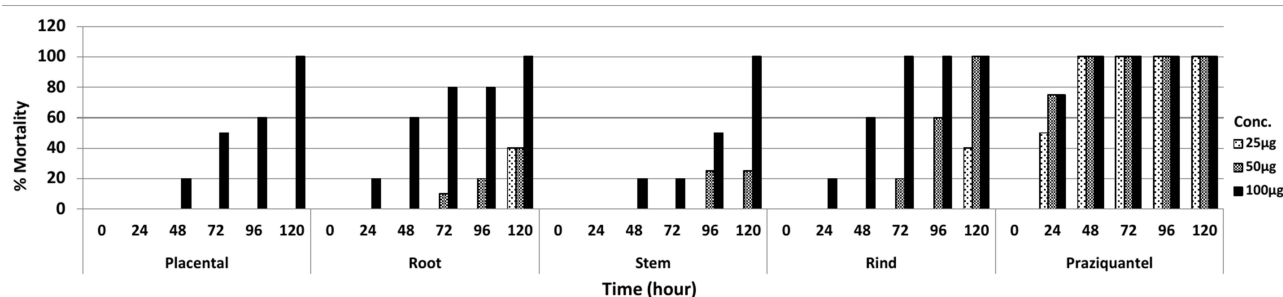


Figure 1 Lethal effect of *P. granatum* ellagitannins on *Schistosoma mansoni* adult worms after different time intervals.

ellagitannins which ranged between 75.31 to 99.72 µg/mL after 96h and 40.25 to 65.19 µg/mL after 120h (Table 1).

Scanning Electron Microscopy

Adult worms exposed to rind ellagitannins of *P. granatum* were examined by scanning electron microscope. Deformations and topographical changes of the tegument of the worms were observed compared to untreated worms as shown in Figure 2.

Worms treated with 50 µg/mL rind ellagitannins for 2 days suffered from alterations in the demography of the surface of the worms (Figure 2B). The tubercles were deformed and detached from the tegument and their remnants surrounded the treated worm (Figure 2B). The edges of the male gynaecophoric canal were fused at some sites as demonstrated in (Figure 2D and F). The surface of the deformed head of the worm was covered by corrugations and both the oral and ventral suckers were deformed, swelled and their surfaces were corrugated and wrinkled (Figure 2F).

Histopathological Investigation of Worms

Histopathological examination of adult male worms which were not exposed to *P. granatum* rind ellagitannins had

Table 1 LD₅₀ of Different Ellagitannins After 96 and 120 Hours

Ellagitannin	Time	LD ₅₀ (µg mL ⁻¹)
Rind	96h	41.25
	120h	28.73
Root	96h	75.31
	120h	40.25
Placenta	96h	87.12
	120h	60.52
Stem	96h	99.72
	120h	65.19

Abbreviations: h, hour; LD, lethal dose; µg, microgram.

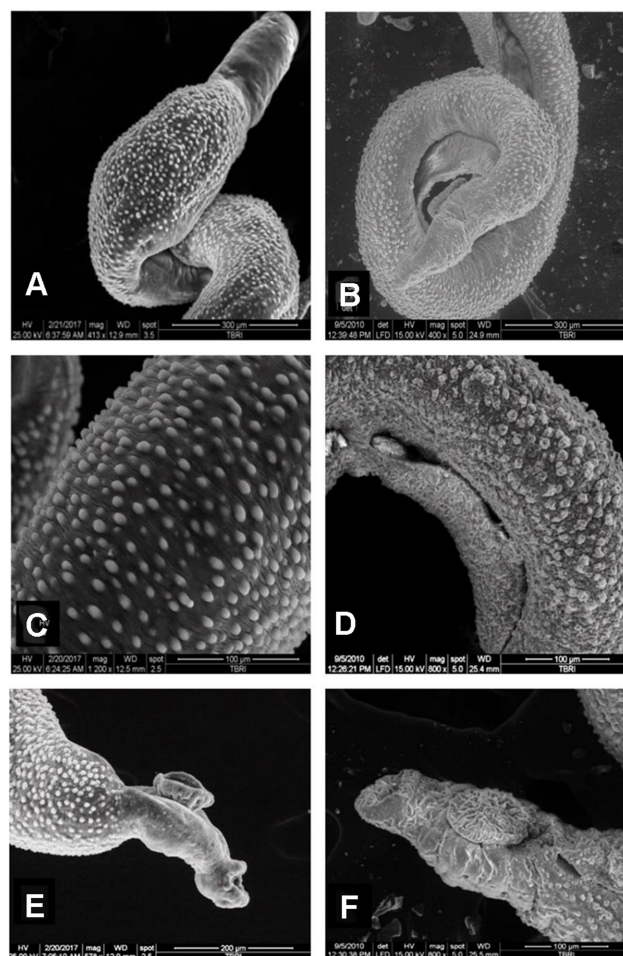


Figure 2 Scanning electron micrograph of adult male *S. mansoni*. (A) normal topography of the tegument; (B) *P. granatum* rind ellagitannins-treated adult male with degenerating tegumental changes; (C) normal tubercles of adult male *S. mansoni*; (D) distorted tubercles of adult male *S. mansoni* exposed to 50 µg/mL *P. granatum* rind ellagitannins for 72h; (E) normal appearance of oral and ventral suckers of untreated adult male *S. mansoni*; (F) oral and ventral suckers of adult male *S. mansoni* exposed to 50 µg/mL *P. granatum* rind ellagitannins for 72h. Arrows point to fusions of some edges of the gynaecophoric canal.

intact tegument with normal intact tubercles which covered the surface of the tegument (Figure 3A). Both the sub-tegumental longitudinal and circular muscles were intact (Figure 3A). On the contrary, worms exposed to

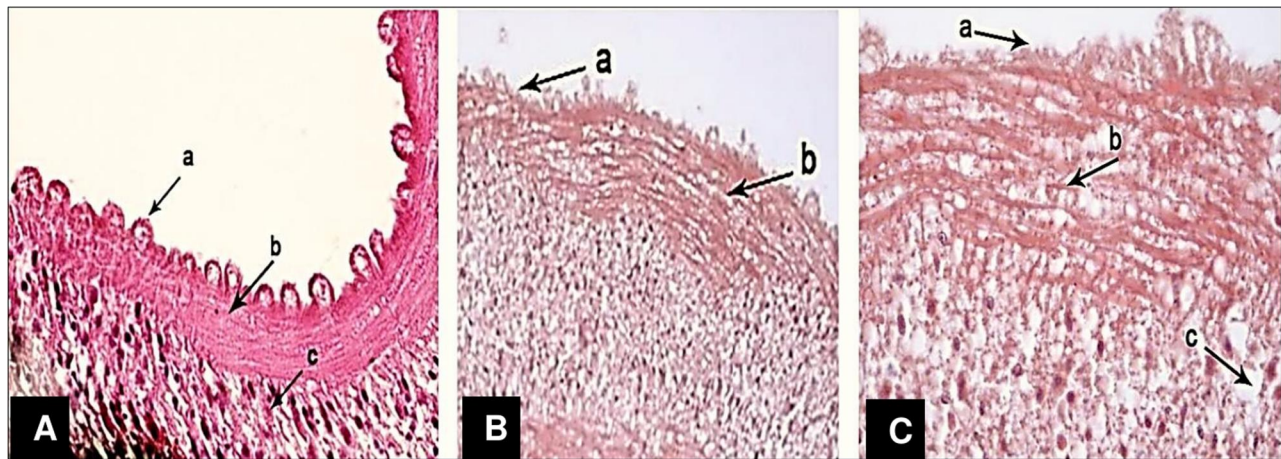


Figure 3 Histological sections of adult male worms of *S.mansoni*, stained by haematoxylin and eosin. The stained sections were examined using oil immersion lens of the light microscope. (A) untreated worms; (B) worm treated with 50µg/mL rind ellagitannins for 24 h; (C) worm treated with 50g/mL rind ellagitannins for 48h. Arrows point to tubercles (a), subtegumental circular musculature (b) and longitudinal musculature (c).

50µg/mL of rind ellagitannins for 2 days suffered from tremendous degeneration and loss of tubercles of the tegument (Figure 3B and C). In addition, after 2 days in the presence of 50µg/mL rind ellagitannins, extensive degenerations of both the circular and longitudinal muscle tissues were observed with the appearance of vacuoles in both muscles (Figure 3B and C).

Discussion

Schistosomiasis ranks the second human parasitic diseases behind malaria in terms of overall morbidity and mortality.^{1,30} Praziquantel has been the drug of choice for schistosomiasis and millions of doses have been administered in control programmes in different countries.⁷ Therefore, there is a concern about the possibility of the appearance of praziquantel-resistant schistosomes.³¹ Consequently, there is an urgent need for the discovery of cheap and safe alternatives for praziquantel. *P. granatum* is an edible plant and its tannins have been used safely for thousands of years in folk medicine.^{14,15} In this study, ellagitannins extracted from the fruit rind, placenta and the barks of the root and stem of *P. granatum* were tested for their lethal effect on *S. mansoni* adult worms. Both pomegranate rind extracts and its high contents of ellagitannins are quite safe. Human overweight volunteers took safely tableted 1.42g/day pomegranate fruit extract for 28days without adverse effects.³³ On the other hand, the oral LD₅₀ of pomegranate fruit extract standardized to 30% punicalagin, which is one of the major ellagitannins, was greater than 5g/kg body weight.³² In addition, the intraperitoneal LD₅₀ of the

extract for rats was 217mg/kg body weight and 187mg/kg for mice.³²

In this study, adult worms were exposed in vitro to different concentrations of ellagitannins extracts for 120h. No detrimental effects were observed during the duration of the experiments on untreated worms as revealed under the microscope. Some other researchers examined the schistosoma worms in vitro for 120h³⁴ and 168h³⁵ without reporting any detrimental effects.

All the tested ellagitannins in this study were lethal to *S. mansoni* worms, particularly the rind ellagitannins which killed 40% of the adult worms after 5 days at a concentration of 25µg/mL. The 100% killing of worms was achievable when the worms were exposed to 50 µg/mL and 100µg/mL for 120h and 72h respectively. The values of LD₅₀ of the ellagitannins were 41.25µg/mL and 28.73µg/mL after 96h and 120h respectively. Therefore, the previously observed lethal effect of crude *P. granatum* extracts³³ may be attributed, at least in part, to their contents of ellagitannins.

The tegument of schistosoma is a living anucleate and cytoplasmic structure. It is connected to the underlying nucleated cells that are located beneath the circular and longitudinal musculature. The tegument plays a critical role in the worm. It is essential for secretion and absorption of nutrient, protection from host immune response and it is an important target for anti-schistosomal drugs.³⁴

In this study, ellagitannins caused marked changes in the topography of the worm's tegument. The tegument of the worms treated with ellagitannins suffered from erosions, wrinkling, swellings, loss and degeneration of the surface of tegument and the tubercles. The severe damaging effect of

the ellagitannins was noticed on adult worms after 48–72h. Tegumental changes were previously observed in adult worms treated with crude alcoholic extracts of the stem bark, rind and leaf of pomegranate.^{20,33} Crude extracts of pomegranate leaf and stem bark caused separation of the worm couples and decreased worm's motility.²⁰ Our data suggest that these reported tegumental changes and the separation of worm couples are probably, at least in part, due to the ellagitannins of the pomegranate.

On the other hand, the histopathological investigation of the treated worms revealed a severe degeneration in the musculature of the worm. The alterations of the schistosomal tegument by various natural and synthetic antischistosomal drugs have been previously reported. Examples of these natural and synthetic drugs are artemether,³⁵ astiban,³⁶ amoscanate,³⁷ hycanthone,³⁸ niridazole,³⁹ aspidine and flavaspidic acid purified from the rhizomes of *Dryopteris* species,⁴⁰ artesunate,⁴¹ mirazid purified from myrrh oleoresin⁴² and ginger aqueous extract,⁴³ oxamniquine⁴⁴ and praziquantel.¹⁰

In this study, ellagitannins led also to the release of tegumental blebs. This phenomenon was previously observed when schistosomes were exposed to oxamniquine⁴⁰ and ginger extracts.⁴³ However, a unique phenomenon which was noticed in this study was the fusion of the edges of the gynaecophoric canal at some sites. This might be behind the uncoupling of worms which was observed in this study and was also caused by crude pomegranate extracts in another study.²⁰

The consequences of the severe damages of the tegument which were noticeable as early as 48h, on the ability of the worm to survive in presence of ellagitannins are complex. The damage of the tegument, which was obvious as early as 48h, would presumably inhibit the ability of worms to disguise the immune system and the worm would become more vulnerable to the immune response of the host. Consequently, ellagitannins would likely facilitate the ability of host inflammatory cells to attack worms. Ellagitannins of pomegranate are known to enhance the innate immune system⁴⁵ as praziquantel does.⁴⁶ Therefore, ellagitannins of *P. granatum* would additionally help the host to eradicate the already vulnerable worms. On the other hand, the damage of the tegument would also render the worms more vulnerable to host oxidant challenges. Peroxidation of the membranes of the tegument would cause further damages and further losses of its integrity.⁴⁷ In schistosoma, glucose is mostly absorbed by the tegument and most of the ATPase activity is located in

the tegument.⁴⁸ Therefore, the damage of the tegument would be on the expense of the feeding of worms and the production of energy.⁴⁹

Schistosoma worms use tubercle spines, and suckers to hold to blood capillaries. The observed severe damages of tubercle spines and suckers by ellagitannins might cause the worm to be dislodged by the blood flow.⁵⁰

In this study, ellagitannins caused degeneration of the sub-tegumental layers of longitudinal and circular muscles. Similar observations were previously reported in *S. mansoni*, treated with praziquantel⁵¹ and artemether.³⁵ The observed damages of muscles might explain the observed decrease in the activity and motility of treated worms.

The exact mechanism of the degenerative effect of ellagitannins of pomegranate on the tegument of the adult worms of schistosoma needs further investigation. Generally speaking, different phytochemicals have been reported to affect gene expression.^{52–54} Ellagitannins like ellagic acid and punicalagin were reported to inhibit the promoter of the matrix metalloproteinase-9 (MMP-9) of the THP-1 cell line.⁵⁵ Also pomegranate fruit extracts which are rich in ellagitannins reduced the proliferation of the breast cancer MCF-7 cells by the up regulation of 505 genes and the down regulation of other 398 genes.⁵⁶ Therefore, the damaging and lethal effects of ellagitannins on the adult worms of schistosoma might be attributed at least in part to the down/upregulation of some vital genes for the parasite. On the other hand, pomegranate ellagitannins were reported to have inhibitory effect on some vital enzymes like rat intestinal α -glucosidase, porcine α -amylase⁵⁷ and the vertebrate squalene epoxidase.⁵⁸ Therefore, there is a possibility that pomegranate ellagitannins might inhibit some enzymes which are vital for the synthesis and/or integrity of the tegument.

Conclusion

It may be concluded that ellagitannins of *P. granatum* are lethal to *S. mansoni*. They caused severe damages to the tegument of the worms, affect their suckers, damaged their circular and longitudinal muscles and impair their motor activity. These effects are expected to cause schistosoma worms to be vulnerable to the immune system of the host. In a previous study, the oral administration of pomegranate crude peel extract, for three consecutive days, 45 days post-schistosomal infection, resulted in the death of 72.2% of adult worm.³² Therefore, we are currently evaluating the in vivo effect of purified ellagitannins on mice suffering from schistosomiasis.

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Disclosure

The authors declare that there is no conflicts of interest regarding the publication of this paper.

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