

Cucurbita pepo Seed Oil Induces Microsatellite Instability and Tegumental Damage to *Schistosoma mansoni* Immature and Adult Worms In vitro

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Amany Ibrahim Ammar¹ 
Amira Fathy Afifi¹
Abdallah Essa^{2,3} 
Asmaa Galal-Khallaaf⁴ 
Mostafa Mohamed Mokhtar⁵
Somaia Shehab-Eldeen^{2,3} 
Amany Ahmed Rady¹

¹Department of Medical Parasitology, Faculty of Medicine, Menoufia University, Shebin El Kom, Menoufia, Egypt; ²Tropical Medicine Department, Faculty of Medicine, Menoufia University, Shebin El Kom, Menoufia, Egypt; ³Internal Medicine Department, College of Medicine, King Faisal University, Al-Ahsaa, Saudi Arabia; ⁴Molecular Biology and Biotechnology Laboratory, Department of Zoology, Faculty of Science, Menoufia University, Shebin El- Kom, Menoufia, Egypt; ⁵Department of Zoology and Entomology, Faculty of Science, Al-Azhar University, Nasr City, Egypt

Background: Schistosomiasis is a tropical parasitic disease treated exclusively with praziquantel (PZQ). However, PZQ has low efficacy against schistosomula and juveniles. In addition, the emergence of PZQ resistance has prompted the search for new drugs.

Methods: This study investigated the effects of pumpkin (*Cucurbita pepo*)-seed oil (PSO) on *Schistosoma mansoni* adults, juveniles, and newly formed schistosomula in vitro by exposing the parasites to increasing concentrations of PSO (20, 40, 60, 80, and 100 µL/mL) with variable incubation periods (24, 48, and 72 hours). Dose-response effects of PSO on mortality rate, worm activity, and tegumental changes were studied. Also, effect on DNA were assessed with microsatellite analysis.

Results: All tested stages of *S. mansoni* were susceptible to PSO, which was more effective than PZQ on juvenile worms and schistosomula. Juveniles and schistosomula *S. mansoni* were more sensitive to the antischistosomal activity of PSO than adult worms. PSO showed evident changes in the integuments of adults, juveniles, and schistosomula. These changes were more evident with increased concentrations. At the genomic level, PSO induced clear qualitative and quantitative changes in the microsatellite loci R95529 and SMD57 of *S. mansoni* adults and schistosomula. This microsatellite instability is being reported through the current study for *S. mansoni* in response to PSO for the first time.

Conclusion: This study suggested that PSO possesses effective antischistosomal activity against various stages of *S. mansoni*. Further investigations are needed to figure out the mechanism of action of PSO on this parasite.

Keywords: *Schistosoma mansoni*, *Cucurbita pepo*, praziquantel, tegumental alteration, microsatellite

Introduction

Schistosomiasis is a tropical parasitic disease caused by blood flukes of the genus *Schistosoma*.¹ More than 700 million individuals are in danger of contracting schistosomiasis worldwide and more than 200 million are currently infected, making this an urgent global health concern.² There is no available vaccine against schistosomiasis, and the infection is treated solely with praziquantel (PZQ), which is advised by the World Health Organization (WHO) for schistosomiasis treatment.³ Regrettably, repeated use of PZQ for long periods, especially in mass drug administration, has increased the potential for drug resistance.⁴ Low activity against immature schistosomula is another drawback with PZQ.⁵ It is thus an urgent priority to find a novel drug to substitute or potentiate PZQ.

Correspondence: Abdallah Essa
Faculty of Medicine, Menoufia University,
Yassen Abd Al Ghafar Street, Shebin
Elkom City 32511, Menoufia
Governorate, Egypt
Tel +20-10-2242-4341
Email abdallah.essa@med.menofia.edu.eg

Herbal extracts are an important source of new drugs, due to their variety of biomedical activities and chemical components.⁶ The Cucurbitaceae plant family, collectively known as cucurbits, comprises 130 genera and around 800 species.⁷ Pumpkin (*Cucurbita pepo*) seed oil (PSO) is a remarkably rich source of various bioactive components with functional properties. The seeds are used for an edible oil and as a nutraceutical product. In addition, PSO has been recognized to have therapeutic properties as an antibacterial, antioxidant and anti-inflammatory agent.^{8, 9} Traditional described medicinal uses of PS have prompted experimental studies on its anthelmintic properties.¹⁰ Studies on the effect of PS on heterophyiasis and taeniasis have shown promising results.¹¹ However, such studies are few in number, and they still leave many questions to be answered.

Microsatellite loci are highly variable DNA markers that are commonly used in schistosomiasis research and allow population-level analysis.¹² As such, it could be useful to use these microsatellites in assessing the variability occurring in DNA of the parasites when exposed to drugs. The present work was conducted to study the effect of PSO on *S. mansoni* adults, juveniles, and newly formed schistosomula in vitro.

Methods

Schistosoma mansoni Maintenance

Ten adult male CD1 Swiss albino mice 6–8 weeks of age weighing 20±2 g were purchased from the Schistosome Biological Supply Centre (SBSC), Theodor Bilharz Research Institute (TBRI), Egypt. They were maintained on a standard commercial pellet diet and kept in an air-conditioned animal house at 20°C–22°C for 1 week before infection, in accordance with university regulations. Each mouse was infected with 70±5 cercariae of an Egyptian *S. mansoni* strain by using the tail-immersion technique, as described by Oliver and Stirewalt.¹³ Cercariae had been freshly shed from experimentally infected *Biomphalaria alexandrina* and used within 1 hour.

Parasite Preparation

Juvenile worms of *S. mansoni* were obtained from hepatic veins at 21 days postinfection, while adult worms were collected from the portal system and mesenteric veins of infected mice by perfusion 7 weeks later.¹⁴ Juvenile and adult worms were washed with PBS two to three times and undamaged worms picked out. They were placed in RPMI 1640 medium (Sigma-Aldrich) and stored in an incubator

chamber at 37°C for 6 hours for adaptation before starting the experiment. Schistosomula were prepared in vitro according to the method of Colley and Wikel.¹⁵ The newly transformed schistosomula (NTS) were resuspended in preheated RPMI 1640 medium containing 10% heat-inactivated FBS, streptomycin (100 µg/mL), and penicillin (100 IU/mL) and incubated overnight in 5% CO₂ and 100% humidity at 37°C.

Praziquantel and Pumpkin-Seed Oil

PZQ (Discocide 600 mg; EIPICO, Egypt) and *Cucurbita pepo* seed oil (Now PSO 1,000 mg; Now Health Group, USA) was mixed in dimethyl sulfoxide (DMSO; Sigma-Aldrich). A stock solution of 1 mL PSO was dissolved in 1%DMSO and then diluted with RPMI 1640 to prepare 2 mL of working solution (100 µL/mL).

In vitro Assessment of Antischistosomal Effect of PSO

In vitro evaluation was done on different stages of *S. mansoni* schistosomula, juveniles, and adults.

Mortality Rate, Worm Activity, and Morphological Changes

As per Xiao et al,¹⁶ juvenile worms were collected, washed with RPMI 1640, counted, and relocated to 24-well culture plates (TPP, St Louis, MO, USA), with 100 juveniles in each well containing 2 mL RPMI 1640 medium enriched with 2 mM L-glutamine, 20% FBS (Gibco), and antibiotics (penicillin, gentamycin, and streptomycin: 300 IU/mL, 160 µg/mL, and 300 µg/mL, respectively). Then, plates were incubated in 5% CO₂ at 37°C. The same was done with adult worms, with equal representation of males and females in each well. Numbers of adults in each well for untreated, PZQ-treated, PSO (100), PSO (80), PSO (60), PSO (40), and PSO (20) samples were 12, 20, 28, 24, 30, 28, and 24, respectively. PSO was tested at concentrations of 20, 40, 60, 80, and 100 µL/mL. Experiments were conducted in triplicate and read at 24, 48, and 72 hours, then repeated at least twice. Two groups of control worms were used. The negative-control group was assayed in RPMI 1640 medium containing 1% DMSO without drugs, and the positive-control group with 10 µg/mL PZQ, the most effective schistosomicidal drug, as reference drug.

Schistosomula obtained by mechanical transformation were carefully counted and adjusted to a concentration of 100 NTS/50 µL,¹⁷ then placed in RPMI 1640 medium, as

previously described for juvenile worms. The plates were incubated in 5% CO₂ at 37°C for a minimum of 12–24 hours to achieve complete transformation into schistosomula. Then, PSO was tested in the same concentrations tested in adult worms and read at the same time points and the procedure repeated twice. Control NTS were assayed in RPMI 1640 medium containing 1% DMSO without drugs as the negative-control group and with 10 µg/mL PZQ as the positive-control group.¹⁶

Assessment of the schistosomicidal effect of PSO was carried out by determining worm motor activity and mortality rate using inverted microscopy at 40× and 100× magnification (IX70; Olympus, Tokyo, Japan).¹⁸ Worms that did not show any sign of motility for 2 minutes were counted as dead. To quantify the degree of motility and morphological changes, values assigned were 3 = normal motility, no morphological changes; 2 = reduced motility and/or moderate damage to integument noted; 1 = severe reduction in motility and/or severe morphological/tegumental changes; 0 = death of all worms.¹⁹

The same experiment was repeated six times to determine lethal concentration

s (LC₅₀ and LC₉₀). Worm mortality was counted in each case, and LC₅₀ and LC₉₀ values of PSO were calculated using probit analysis and SPSS version 20. For each concentration, the worm-mortality rate was recorded by computing the number of dead worms divided by the total.^{20,21} Since each experiment was repeated three times, means and standard deviations were calculated for each concentration at different times.

Scanning Electron Microscopy

Morphological changes in the integument of NTS, juveniles, and male adults were observed by scanning electron microscopy (SEM) as per Hassan et al.²² SEM was performed on worms that had been exposed to PSO at 60 and 100 µL/mL for 72 hours. Briefly, worms were washed in normal saline several times, fixed with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.3) for 4 hours, then fixed with osmium tetroxide (OSO₄) for 2 hours. That was followed by three washes in 0.2 M sodium cacodylate buffer and dehydration in gradual concentrations of ethanol (10%–100%, 10 minutes for each concentration). Further dehydration was performed in liquid carbon dioxide. Mounting of worms was done on copper stubs with double-sided adhesive tape. They were gold-coated using an S150A sputter coater, then viewed by

SEM (JEOL JSM-5400) at the National Center for Research, Radiation, and Technology, Cairo, Egypt.

DNA-Microsatellite Analysis

DNA Extraction and PCR Reactions

Frozen pools of *Schistosoma* subjected to the different treatments were transferred to the Molecular Biology Laboratory, Zoology Department, Faculty of Science, Menoufia University. Each pool of *Schistosoma* specimens, placed in 1.5 µL Eppendorf tubes, was cleaned to remove traces of oils using the protocol applied by Galal-Khallaf et al.²³ and Ardura et al.²⁴ In brief, each pool was rinsed with a mixture of water, methanol, and chloroform (0.8:2:1) for 2 hours, followed by another wash in distilled water and a final one in PBS. Three pools of worms per treatment, 30 mg each, were each subjected to total genomic DNA extraction.

DNA extraction was performed using the protocol described by Wasko et al.²⁵ and modified by Mohammed-Geba et al.²⁶ Briefly, 2.4 U/mL proteinase K solution (Thermo Fisher Scientific) and 100 µL TNES-urea buffer (10 mM Tris HCl pH 8, 125 mM NaCl, 10 mM EDTA pH 8, 0.5% sodium dodecyl sulfate, and 4 M urea) was added to each sample. The mixture was incubated at 55°C for 60 minutes with occasional vortexing until completely lysed. Later, 5 M NaCl (27 µL) was added, with mixing of the tubes and centrifugation at 4,000 g for 10 minutes. The supernatant was placed into a clean 1.5 mL Eppendorf tube. DNA was precipitated by adding 100 µL freezer-cold isopropanol with shaking. Centrifugation of the tubes was done at 11,000 g for 10 minutes, with complete removal of the supernatant. Washing of DNA pellets was done in 70% ethanol (200 µL), followed by centrifugation at 11,000 g for 5 minutes, then after the ethanol had dried, 30 µL of Tris-EDTA buffer (10 mM Tris HCl pH 8, 2 mM EDTA pH 8) were added for pellet resuspension. To check the quality of the DNA, 5 µL extracted DNA with 1 µL of 6x DNA loading buffer (bromophenol blue 0.25% w/v, sucrose 40% w/v) was run on agarose gels (1%) stained with ethidium bromide (0.5 µg/mL). Purity and quantity of the DNA were detected by spectrophotometric measurement for light absorbance at 260 nm and 280 nm.

For detecting different microsatellite loci, the primers identified by Durand et al.²⁷ were used, namely SMD57, R95529, SCMSMOXII, SMU31768, and SMIMP25. Primer sequences used are shown in Table 1. Amplification reactions were set up in a total volume of 15 µL. The reaction mixture was composed of 0.5 µL

Table 1 Sequences of primers of *S. mansoni* applied in the current study (Durand et al 2000). GenBank accession numbers for these loci are also shown

Locus	Accession number	Primer sequences (5–3)
SMD57	AF202967	F: TCCTTGATTCCACTGTTG R: GCAGTAATCCGAAAGATTAG
SCMSMOXII	M85305	F: TTCTACAATAATACCATCAAC R: TTTTCTCTCACTCATATACAC
R95529	R95529	F: GTGATTGGGGTGATAAG R: CATGTTTCTTCAGTGTCC
SMU31768	U31768	F: TACAACCTCCATCACTTC R: CCATAAGAAAGAAACCAC
SMIMP25	X77211	F: CACTATACCTACTACTAATC R: TCGATATACATTGGGAAG

DNA template (100 ng), 0.25 μ M each primer, 1X MyTaq red master mix (Bioline), and 0.2 mg/mL BSA, and reached 15 μ L with PCR-grade water. PCR amplification was done in a TPersonal 48 thermal cycler (Biometra). Thermocycler conditions followed Durand et al: initial denaturation at 94°C for 4 minutes, followed by 35 cycles of 94°C for 30 seconds, annealing for 30 seconds, 72°C for 30 seconds, and an extension of 72°C for 10 minutes as a final step. Electrophoresis of the PCR products was performed in 2% agarose gel.

Ethical Considerations

The study was approved by the ethics committee of the Faculty of Medicine, Menoufia University, Egypt (approval protocol FWA00005112), and was in compliance with guidelines for the care and use of laboratory animals.²⁸ Experiments were carried out at SBSC/TBRI, while DNA extraction and PCR procedures were carried out at the Molecular Biology Laboratory, Zoology Department, Faculty of Science, Menoufia University.

Statistical Analysis

Data were analyzed with SPSS 23.0 (IBM, Armonk, NY, USA). Descriptive statistics are expressed as numbers and percentages for nonnumeric data. For analytical statistics, χ^2 was used to determine the association between qualitative variables and Z-tests to compare two proportions in two groups. Data were considered statistically significant at 0.05. These results were used to define LC₅₀ and LC₉₀ values of extract using probit analysis and SPSS.

Results

In vitro Antischistosomal Effects of PSO on Mortality Rate and Worm Activity Adult Worms

Adult worms exposed to PZQ 10 μ g/mL showed 100% mortality, with contraction and paralysis after 24 hours' exposure. On exposure to PSO, no worm mortality was observed at any concentration after 24 hours' exposure. The mortality rate increased with increased duration of exposure, and occurred in a dose-dependent manner. At 100 μ L/mL, the mortality rate was 100% after 48 and 72 hours' exposure. The lowest mortality rate was seen at 20 μ L/mL: 25% and 37.5% at 48 and 72 hours, respectively, whereas no mortality was noticed in worms of the negative-control group. There was a highly significant difference in mortality rate among the PSO concentrations (80, 60, 40, 20 μ L/mL) and PZQ (P2–5, all <0.001 ; Table 2). Concentrations of PSO required for LC₅₀ and LC₉₀ in adult worms in vitro after 72 hours were 36.94 μ L/mL and 66.73 μ L/mL, respectively.

After 72 hours' exposure to PSO, the drug had a deadlier effect on females than on males. At 80 and 100 μ L/mL, all female worms died, while in male worms mortality rates were 91.7% and 100%, respectively, with no significant difference between males and females (Figure 1). Adult *S. mansoni* motility was remarkably affected by rising concentrations of PSO. Significant reductions in motility and severe morphological changes (contraction and shrinkage) were observed after 48 and 72 hours' exposure with 60, 80, and 100 μ L/mL PSO (Figure 2). Bodies of adult worms were poorly flexible, and suckers became less contractile. With lower concentrations (20 and 40 μ L/mL), all worms showed normal motor activity at 24 hours' exposure, while reduced motility and minimal alteration in morphology, such as stretched head end, slight elongation, and focal swelling of the worm body, were seen at 48 and 72 hours' exposure. The positive-control group exposed to PZQ 10 μ g/mL showed significant reductions in worm motility and marked changes in morphology. All worms acquired a coiled appearance after 24 hours' exposure, along with spasmodic contractions and feeble movement of the tail portion and oral suckers and vesiculation along the surface of the worm. All *S. mansoni* adult worms in the negative-control group showed characteristic wavy and peristaltic movement along the body axis without morphological alterations (Table 3).

Immature Juvenile Worms

After incubation with PSO at different concentrations, the mortality rate of juvenile *S. mansoni* worms depended

Table 2 In vitro effects of PSO and PZQ on mortality of adult worms, juveniles and schistosomula of *S. mansoni* after different incubation periods

Period of incubation (hours)	Groups	Adult worms, n	Dead adults, n (%)	p-value	Comparison with PZQ	Juvenile worms, n	Dead juveniles, n (%)	p-value	Comparison with PZQ	Schistosomula, n	Dead schistosomula, n (%)	p-value	Comparison with PZQ
24	Untreated	12	0	<0.001	P1: <0.001 P2: <0.001 P3: <0.001 P4: <0.001 P5: <0.001	100	0	<0.001	P1: <0.001 P2: <0.001 P3: <0.001 P4: 0.155 P5: 0.155	100	0	<0.001	P1: <0.001 P2: <0.001 P3: <0.001 P4: NA P5: NA
	PZQ	20	20 (100)			100	2 (2)			100	0		
	PSO (100)	28	0			100	94 (94)			100	100 (100)		
	PSO (80)	24	0			100	78 (78)			100	70 (70)		
	PSO (60)	30	0			100	27 (27)			100	30 (30)		
	PSO (40)	28	0			100	0			100	0		
	PSO (20)	24	0			100	0			100	0		
48	Untreated	12	0	<0.001	P1: NA P2: 0.056 P3: 0.004 P4: <0.001 P5: <0.001	100	0	<0.001	P1: <0.001 P2: <0.001 P3: <0.001 P4: <0.001 P5: <0.001	100	0	<0.001	P1: <0.001 P2: <0.001 P3: <0.001 P4: <0.001 P5: <0.001
	PZQ	20	20 (100)			100	4 (4)			100	0		
	PSO (100)	28	28 (100)			100	100 (100)			100	100 (100)		
	PSO (80)	24	20 (93.3)			100	100 (100)			100	80 (80)		
	PSO (60)	30	20 (66.7)			100	71 (71)			100	70 (70)		
	PSO (40)	28	14 (50)			100	52 (52)			100	60 (60)		
	PSO (20)	24	6 (25)			100	48 (48)			100	50 (50)		
72	Untreated	12	0	<0.001	P1: NA P2: 0.356 P3: 0.033 P4: 0.003 P5: <0.001	100	0	<0.001	P1: <0.001 P2: <0.001 P3: <0.001 P4: <0.001 P5: <0.001	100	0	<0.001	P1: <0.001 P2: <0.001 P3: <0.001 P4: <0.001 P5: <0.001
	PZQ	20	20 (100)			100	10 (10)			100	0		
	PSO (100)	28	28 (100)			100	100 (100)			100	100 (100)		
	PSO (80)	24	23 (95.8)			100	100 (100)			100	100 (100)		
	PSO (60)	30	24 (80)			100	100 (100)			100	100 (100)		
	PSO (40)	28	18 (64.3)			100	90 (90)			100	100 (100)		
	PSO (20)	24	9 (37.5)			100	75 (75)			100	100 (100)		

Notes: p-values between PZQ-treated group and groups treated with PSO at different concentrations and different incubation times. χ^2 used to estimate p-values.

Abbreviations: NA, not applicable; P1, comparison between PZQ and PSO (100); P2, comparison between PZQ and PSO (80); P3, comparison between PZQ and PSO (60); P4, comparison between PZQ and PSO (40); P5, comparison between PZQ and PSO (20).

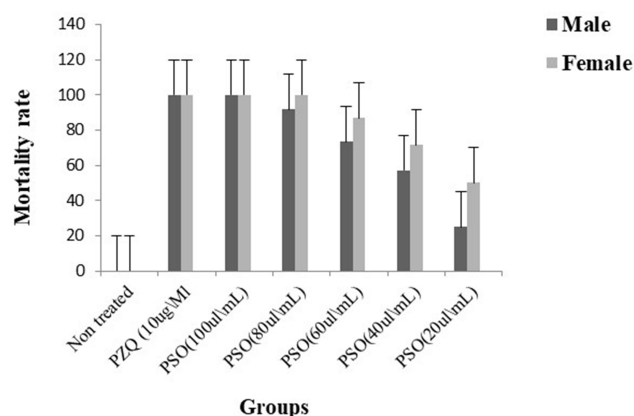


Figure 1 In vitro mortality rate of adult male and female *Schistosoma mansoni* of differently treated groups at 72 hours' exposure.

directly on the concentration evaluated and the incubation period. Concentrations of 80 and 100 $\mu\text{L/mL}$ of PSO had proved lethal to 100% of juvenile worms at 48 hours, while at 20, 40, and 60 $\mu\text{L/mL}$ the mortality rate in juvenile worms was 48%, 52%, and 71%, respectively after 48 hours. At the end of the experiment (72 hours), the mortality rate had increased to 100% at 60, 80, and 100 $\mu\text{L/mL}$ PSO. The positive-control group exposed to PZQ 10 $\mu\text{g/mL}$ showed slow motility and had a mortality rate of 4% and 10% after 24 and 72 hours, respectively, in juvenile worms (Table 2). These results were all highly significant ($p < 0.001$).

Regarding changes in worm activity, the motility of juvenile *S. mansoni* was remarkably affected by increasing concentrations of PSO. Significant reductions in motility and severe morphological changes in the form of abnormal body posture, elongation, and shrunken suckers were detected at higher concentrations of PSO (60, 80, and 100 $\mu\text{L/mL}$), followed by death of all worms after 72 hours' exposure ($p < 0.001$). At the lower concentration of 40 $\mu\text{L/mL}$, slight morphological changes and reduced motility were detected in 91% (score 2) after 24 hours' exposure. At the end of the experiment (72 hours), marked reductions in motility and morphological changes were seen even at lower concentrations ($p < 0.001$, Table 4).

Schistosomula

All schistosomula died at concentrations of 100 $\mu\text{L/mL}$ PSO, regardless of length of exposure. Worms in the control group remained viable until the end of the experiment. The lethal effect of PSO was dose-dependent and related to the duration of exposure. At lower concentrations (20 and 40 $\mu\text{L/mL}$), the viability of schistosomula

was not affected (zero mortality rate) after 24 hours while at 72 hours all schistosomula had died (100%), regardless of PSO concentration. No death was detected after treatment of schistosomula with PZQ at concentrations as high as 10 $\mu\text{g/mL}$ ($p < 0.001$, Table 2).

As seen in Table 5, schistosomula of control groups showed normal activity without any morphological changes till the end of the experiment (72 hours). Following incubation with 100 $\mu\text{L/mL}$ PSO, schistosomula died rapidly: no movement was noticed at 3–4 minutes after incubation. Morphological changes like dark granular, crescent-shaped bodies and reduction in body length were seen regardless of exposure length. At lower concentrations (20 and 40 $\mu\text{L/mL}$), slight morphological changes, such as shorter bodies and reduced activity were observed after 24 hours' incubation. At this time point, schistosomula incubated with 80 and 60 $\mu\text{L/mL}$ showed reduced activity, with morphological alteration in 30% and 70%, respectively, which become marked with increased incubation time. In groups exposed to PZQ, no effect was observed at 10 $\mu\text{L/mL}$ throughout the experiment. These results were statistically significant in comparison to PZQ as drug control ($p < 0.001$).

Ultrastructural Observations

All worms examined except those in the untreated control groups, showed changes of variable degrees. SEM of control adult male *S. mansoni* showed intact integuments with preserved surface architecture (Figure 2A and B). Worms exposed to PZQ for 72 hours revealed deformities of suckers, distorted tubercles with erosion, and peeling of the surface (Figure 2C–E). Adult worms exhibited destruction of integuments with loss of normal pattern and distortion of the gynecophoric canal after 72 hours' exposure to PSO 60 $\mu\text{L/mL}$ (Figure 2F, and G). At the higher concentration of 100 $\mu\text{g/mL}$, there was more damage to the integuments, with edema, lacerations, and formation of blebs (Figure 2H–K). Juveniles from control groups showed intact, well developed oral and ventral suckers with primitive ill-developed gynecophoric canals. The dorsal surface was covered with tegumental folds (Figure 3A). PSO-exposed worms (100 $\mu\text{L/mL}$ for 72 hours) showed destruction of the integument in the form of lacerations and peeling with appearance of multiple vesicles and blebs. Some worms were dorsally bended (Figure 3B–D). Schistosomula incubated in medium with 1% DMSO (control group) exhibited intact integuments with relatively flat surfaces and slight tegumentary ridges. There were apical constrictions between the head and the

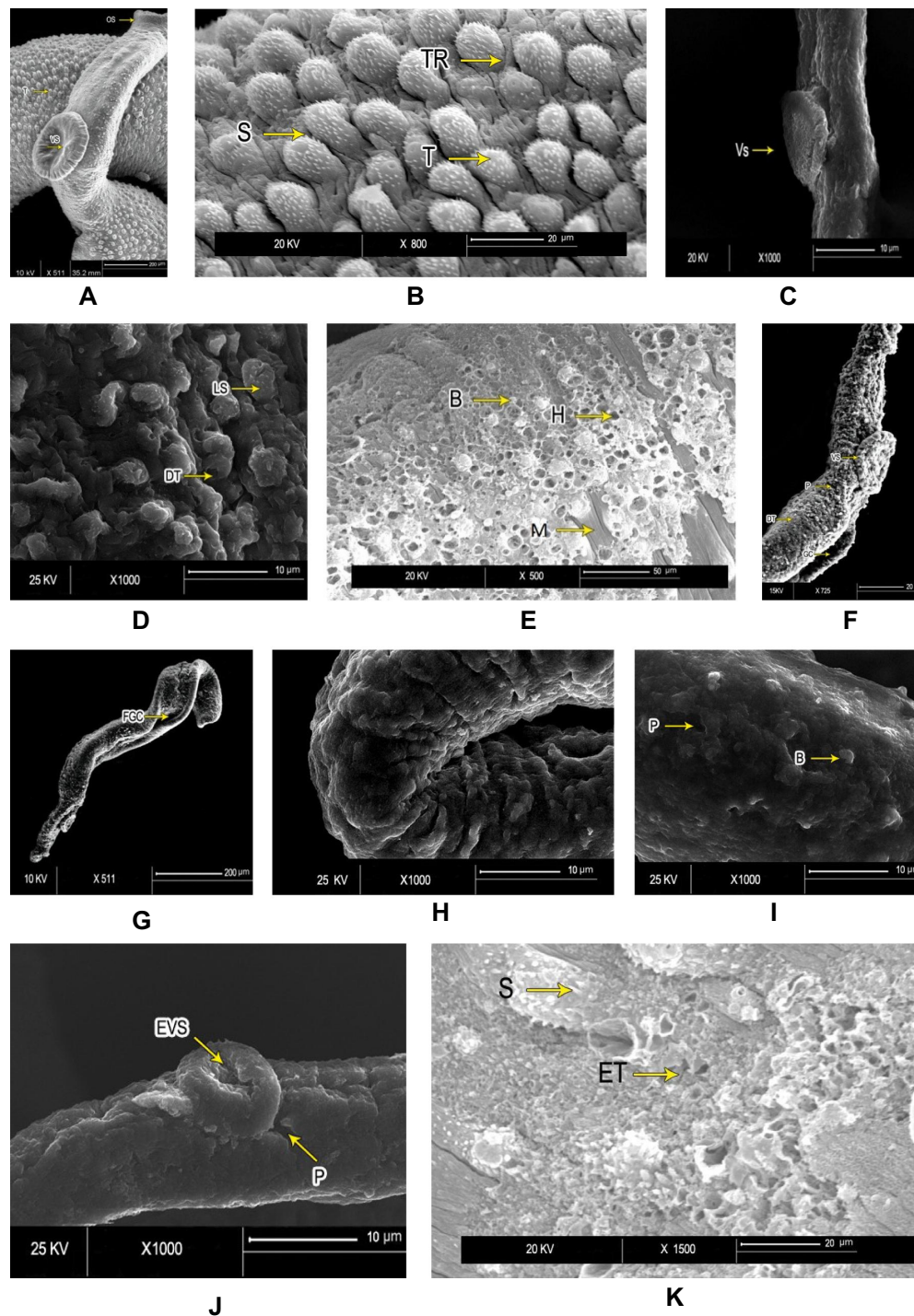


Figure 2 Scanning electron microscopy of *Schistosoma mansoni* adult male worm integuments. After in vitro incubation for 72 hours in medium with 1% DMSO (negative control group): **(A)** intact oral suckers (OS), ventral suckers (VS), and well-developed tubercles (T). Bar 200 μ m; **(B)** higher magnification showing well-developed tubercles (T), spines (S), and tegumental ridges (TR) on the dorsal surface (bar 20 μ m). PZQ-treated worms after 72 hours' incubation: **(C)** anterior ends exhibited deformity of ventral suckers (VS) (bar 10 μ m); **(D)** distorted tubercles (DT) with loss of spines (LS) and areas of surface peeling (bar 10 μ m); **(E)** and severe erosion of tubercles with multiple holes (H), bleb formation (B), and exposed muscle layer (M) in some (bar 50 μ m). Worms exposed to PSO for 72 hours at 60 μ g/mL concentration: **(F)** destruction of integument (DT) and loss of normal tubercular pattern, with peeling areas (P) and deformity of ventral suckers (VS) (bar 20 μ m); **(G)** and distortion and flattening of gynecophoric canal (FGC). bar 200 μ m). At higher PSO concentration (100 μ g/mL): **(H)** worms showed edematous integuments with more damage (bar 10 μ m); **(I)** deformity of tubercles, loss of spines, and tegumental ridges, with formation of blebs (B) and peeling (P; bar 10 μ m); **(J)** deformity and edematous ventral suckers (EVS) with tegumental peeling (P), laceration, complete loss of spines, and destruction of the integument (bar 10 μ m); and **(K)** erosion, marked laceration, loss of spines (S), and extermination of tubercles (ET) with bleb formation (bar 20 μ m).

Table 3 Activity of adult *Schistosoma mansoni* in PSO- and PZQ-treated groups after different incubation periods

Period of incubation (hours)	Groups	n	Worm activity				p-value
			Score 0, n (%)	Score 1, n (%)	Score 2, n (%)	Score 3, n (%)	
24	Untreated	12	0	0	0	12 (100)	<0.001
	PZQ	20	20 (100)	0	0	0	P1: 0.016
	PSO (100)	28	21 (75)	7 (25)	0	0	P2: <0.001
	PSO (80)	24	0	6 (25)	8 (33.3)	10 (41.7)	P3: <0.001
	PSO (60)	30	0	0	0	30 (100)	P4: <0.001
	PSO (40)	28	0	0	0	28 (100)	P5: < 0.001
	PSO (20)	24	0	0	0	24 (100)	
48	Untreated	12	0	0	0	12 (100)	<0.001
	PZQ	20	20 (100)	0	0	0	P1: NA
	PSO (100)	28	28 (100)	0	0	0	P2: 0.056
	PSO (80)	24	20 (93.3)	4 (6.7)	0	0	P3: 0.016
	PSO (60)	30	20 (66.7)	6 (20)	4 (13.3)	0	P4: <0.001
	PSO (40)	28	14 (50)	4 (14.3)	10 (41.7)	0	P5: < 0.001
	PSO (20)	24	6 (25)	0	18 (75)	0	
72	Untreated	12	0	0	0	12 (100)	<0.001
	PZQ	20	20 (100)	0	0	0	P1: NA
	PSO (100)	28	28 (100)	0	0	0	P2: 0.356
	PSO (80)	24	23 (95.8)	1 (4.2)	0	0	P3: 0.103
	PSO (60)	30	24 (80)	3 (10)	3 (10)	0	P4: 0.011
	PSO (40)	28	18 (64.3)	3 (10.7)	7 (25)	0	P5: <0.001
	PSO (20)	24	9 (37.5)	0	15 (62.5)	0	

Abbreviations: NA, not applicable; P1, comparison between Pzq and PSO (100); P2, comparison between Pzq and PSO (80); P3, comparison between Pzq and PSO (60); P4, comparison between Pzq and PSO (40); P5, comparison between Pzq and PSO (20).

body region. At the posterior end of the body, there were prominent tail-socket wounds left by breakage of the tail (Figure 4A and B). Schistosomula swelled with multiple blisters and blebs on the surface upon exposure to PSO for 72 hours (Figure 4C and D).

Microsatellite Analysis

All tested microsatellite loci showed positive bands in the agarose gel. However, only two showed significant variations among the treatments: R95529 and SMD57. For the R95529 locus, four alleles were found of size 250–1,000 bp (Figure 5A). For SMD57, three alleles were detected in the same size range (Figure 5B). For the R95529 locus, immersion of schistosomula in 100% PSO resulted in significant elevation of abundance in all four alleles found; however, the effect was more modest than in adult worms. For the adult worms, immersion in PSO and PZQ led to complete vanishing of the small allele (allele 4). Immersion in PZQ led to three- to fivefold increases in fluorescence levels of all three alleles that did not vanish. Allele 1 showed the same fluorescence level as the control in the group treated with 20% PSO.

However, both alleles 2 and 3 showed three- to fivefold increases. All these increases were significant compared the control upon treatment with 40% PSO. Upon treatment with 100% PSO, allele 1 dropped to about fivefold that of the control, while alleles 2 and 3 were only two- to threefold higher than the control (Figure 6). For SMD57, schistosomula response to PSO was less pronounced. However, a dose-dependent tendency in allele variations was found in all immersion groups. The large allele 1 vanished completely in response to PZQ and all PSO concentrations. Allele 2 was significantly higher (about 1.5-fold) than the control, but decreased with increasing PSO concentrations to below less than double its fluorescence level in the control group. Allele 3 showed slight but significant increases in the PZQ and 20% PSO groups, but a 1.5- to twofold drop in the 40% and 20% PSO groups (Figure 7).

Discussion

Praziquantel (PZQ) is effective against all *Schistosoma* spp., but shows relatively lower efficacy against immature stages of the parasite.²⁹ Continued use of PZQ as a sole

Table 4 Activity of immature juvenile *Schistosoma mansoni* in PSO- and PZQ-treated groups after different incubation periods (IPs)

IP (hours)	Groups	n	Worm activity				p-value
			Score 0, n (%)	Score 1, n (%)	Score 2, n (%)	Score 3, n (%)	
24	Untreated	100	0	0	0	100 (100)	<0.001 P1: <0.001 P2: <0.001 P3: <0.001 P4: <0.001 P5: 0.043
	PZQ	100	2 (2)	2 (2)	0	96 (96)	
	PSO (100)	100	94 (94)	4 (4)	2 (2)	0	
	PSO (80)	100	78 (78)	22 (22)	2 (2)	0	
	PSO (60)	100	27 (30)	65 (65)	6 (6)	2 (2)	
	PSO (40)	100	0	4 (4)	91 (91)	5 (5)	
	PSO (20)	100	0	0	0	100 (100)	
48	Untreated	100	0	0	0	100 (100)	<0.001 P1: <0.001 P2: <0.001 P3: <0.001 P4: <0.001 P5: < 0.001
	PZQ	100	4 (4)	4 (4)	0	92 (92)	
	PSO (100)	100	100 (100)	0	0	0	
	PSO (80)	100	100 (100)	0	0	0	
	PSO (60)	100	71 (71)	19 (19)	3 (3)	7 (7)	
	PSO (40)	100	52 (52)	30 (30)	14 (14)	0	
	PSO (20)	100	48 (48)	25 (25)	22 (22)	5 (5)	
72	Untreated	100	0	0	0	100 (100)	<0.001 P1: <0.001 P2: <0.001 P3: <0.001 P4: <0.001 P5: <0.001
	PZQ	100	10 (10)	0	0	90 (90)	
	PSO (100)	100	100 (100)	0	0	0	
	PSO (80)	100	100 (100)	0	0	0	
	PSO (60)	100	100 (100)	0	0	0	
	PSO (40)	100	90 (90)	7 (7)	3 (3)	0	
	PSO (20)	100	75 (75)	19 (19)	1 (1)	5 (5)	

Abbreviations: P1, comparison between Pzq and PSO (100); P2, comparison between Pzq and PSO (80); P3, comparison between Pzq and PSO (60); P4, comparison between Pzq and PSO (40); P5, comparison between Pzq and PSO (20).

drug can contribute to the development of drug resistance.³⁰ These pitfalls have driven researchers to seek novel and alternative antischistosomal compounds. The last decade has seen renewed interest in the use of natural compounds as a base for drug development.³¹ Among the natural plants studied as possible anthelmintic agents are the family Cucurbitaceae, which are administered in traditional medicine as antiparasitic agents and have been reported to have anthelmintic properties in both humans and animals.^{32,33}

The challenging part of new-drug discovery is to find a substance that can efficiently destroy the parasite without harming the host.³⁴ PSO is tolerable, safe, and has been found to play a significant role in guarding against alcohol-related hepatotoxicity and oxidative stress by modulating inflammation and decreasing oxidative stress.³⁵ Additionally, it has been found to have a defensive impact against sodium nitrate-induced oxidative damage.³⁶ This protective role could be attributed to phenolics, tocopherols, and sterols that play an antioxidant role.³⁷

In previous research by Beshay et al,³⁸ PSO was tested in vivo in *S. mansoni*-infected mice at 50 mg/kg/day for 4 weeks, starting in the fourth week postinfection. The results showed a significant reduction in worm burden and liver and intestinal egg load, with a significant increase in the percentage of dead eggs in the oogram pattern. However, to our knowledge, no previous study on the in vitro effect of PSO on larval stages (newly formed schistosomula and juvenile worms) has been conducted. To develop our comprehension of the effect of this compound against schistosomula, we studied the in vitro impact of different concentrations of PSO on various stages of *S. mansoni* and estimated its LC₅₀ and LC₉₀. In studies on schistosomula, several parameters are often assessed as indicators of biological activity and toxicity, such as mortality rate, motor function, and tegumental changes.³⁹ Here, besides the previous parameters, we assessed DNA changes through microsatellite analysis.

Our results showed that all tested stages of *S. mansoni* were susceptible to PSO. PZQ gave better results compared to PSO in adult worms, but PSO was more effective

Table 5 Activity of *Schistosoma mansoni* schistosomula in PSO- and PZQ treated groups after different incubation periods (IPs)

IP (hours)	Groups	n	Worm activity				p-value
			Score 0, n (%)	Score 1, n(%)	Score 2, n (%)	Score 3, n (%)	
24	Untreated	100	0	0	0	100 (100)	<0.001
	PZQ	100	0	0	0	100 (100)	P1: <0.001
	PSO (100)	100	100 (100)	0	0	0	P2: <0.001
	PSO (80)	100	70 (70)	30 (30)	0	0	P3: <0.001
	PSO (60)	100	30 (30)	70 (70)	0	0	P4: <0.001
	PSO (40)	100	0	0	100 (100)	0	P5: <0.001
	PSO (20)	100	0	0	100 (100)	0	
48	Untreated	100	0	0	0	100 (100)	<0.001
	PZQ	100	0	0	0	100 (100)	P1: <0.001
	PSO (100)	100	100 (100)	0	0	0	P2: <0.001
	PSO (80)	100	80 (80)	20 (20)	0	0	P3: <0.001
	PSO (60)	100	70 (70)	30 (30)	0	0	P4: <0.001
	PSO (40)	100	60 (60)	20 (20)	0	0	P5: <0.001
	PSO (20)	100	50 (50)	50 (50)	0	0	
72	Untreated	100	0	0	0	100 (100)	<0.001
	PZQ	100	0	0	0	100 (100)	P1: <0.001
	PSO (100)	100	100 (100)	0	0	0	P2: <0.001
	PSO (80)	100	100 (100)	0	0	0	P3: <0.001
	PSO (60)	100	100 (100)	0	0	0	P4: <0.001
	PSO (40)	100	100 (100)	0	0	0	P5: <0.001
	PSO (20)	100	100 (100)	0	0	0	

Abbreviations: P1, comparison between Pzq and PSO (100); P2, comparison between Pzq and PSO (80); P3, comparison between Pzq and PSO (60); P4, comparison between Pzq and PSO (40); P5, comparison between Pzq and PSO (20).

than PZQ in juvenile worms and schistosomula. Juvenile worms and schistosomula appeared to be more sensitive to the schistosomicidal activity of PSO than adult ones. It was observed that on exposure to PSO, mortality rates of adults, juveniles, and schistosomula increased with duration of exposure and occurred in a dose-dependent manner. PSO had caused 100% mortality in adults, juveniles and schistosomula at 100 µl/mL after 72 hours' incubation, and it affected females more than males. All schistosomula died at concentrations of 100 µl/mL PSO, regardless of the duration of exposure. It seems that the stage most affected by PSO was schistosomula, followed by juveniles and lastly adults. Being more susceptible than the adults, the juveniles and schistosomula also seen after exposure to mefloquine⁴⁰ and to artemisinin derivatives.⁴¹ This point needs to be researched further.

As previously mentioned by Utzinger et al⁴² and Xiao et al,⁴⁰ schistosomula and juveniles are not susceptible to PZQ. We assessed the effect of PZQ on schistosomula and juveniles in vitro, but observed no death at concentrations as high as 10 µg/mL. In another in vitro study by de Moraes

et al,³⁴ which studied the impact of PZQ on schistosomula of different ages (3 hours and 1, 3, 5, and 7 days old), no death was observed after treatment at concentrations as high as 20 µM. It was observed that changes in *S. mansoni* motility at different stages varied according to PSO concentration and incubation period. Motility and morphological changes started after 48 hours' incubation, especially at 60, 80, and 100 µl/mL concentrations. De Oliveira et al³⁹ suggested that effects on adult *S. mansoni* motility exposed to treatment are associated with alterations in neurotransmitters or the parasite-neuromodulator system, since parasite motility is related to important neurotransmitters or neuromodulators like serotonin, dopamine, epinephrine, glutamate, acetylcholine, and neuropeptides.

The positive control group exposed to PZQ, at a concentration of 10 µg/mL, demonstrated significant reduction in worm motility and marked morphological changes. After 24h of exposure, adult worms showed spasmodic contractions with weak movement of the oral sucker and tail portion vesiculation along the surface of the worm. All worms acquired a coiled appearance. Conversely, no

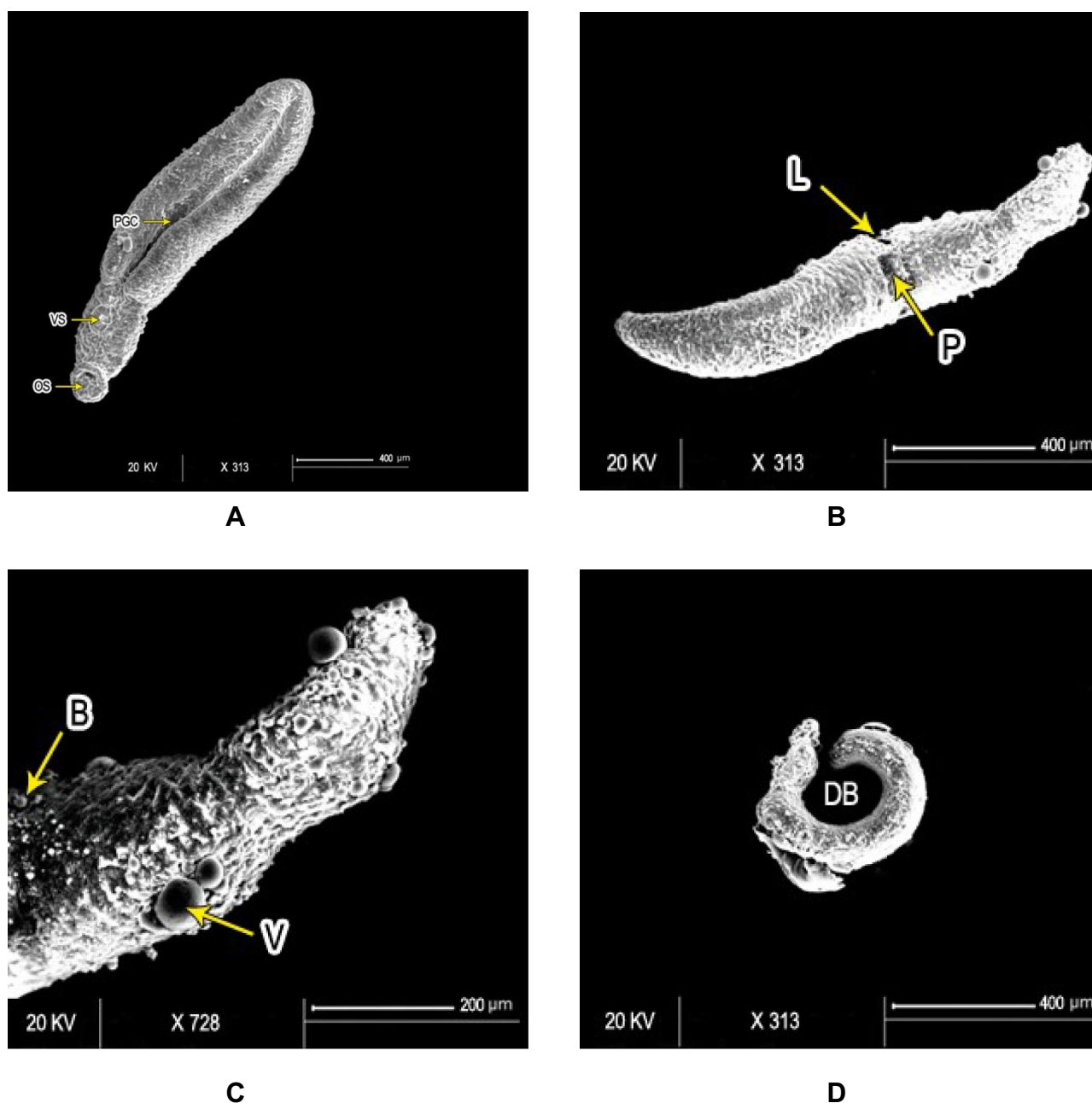


Figure 3 Scanning electron micrography of *Schistosoma mansoni* juvenile worms after in vitro incubation for 72 hours in medium with DMSO (negative-control group) showed (A) intact oral (OS) and ventral suckers (VS) and primitive ill-developed gynecophoric canal (PGC), whereas the dorsal surface was covered with rows of tegumental folds (TF; bar 400 μ m). At 72 hours after exposure to PSO at 100 μ g/mL concentration, worms exhibited (B) areas of laceration (L) and peeling (P), (C) multiple vesicles (V) and blebs (B) (bars 400 μ m and 200 μ m, respectively), (D) and some showed dorsal bending (DB) of the worm body (bar 400 μ m).

significant effect of PZQ on juveniles and schistosomula was observed. These findings were consistent with those reported by Pica-Mattoccia and Cioli⁴³ who demonstrated that PZQ, in adult *S. mansoni* worms triggered both contraction and paralysis with little effect on juvenile worms. On comparison of the two drugs used in the study, PSO showed greater effects on the developmental stages of *Schistosoma* worms than PZQ. It would be interesting to study the effect of

a combination of the two compounds on various stages of schistosomula. Beshay et al³⁸ performed an in vivo experiment on *S. mansoni*, and showed that the most significant results were obtained when combining PZQ and PSO.

The integument of schistosomula appears to be an important focus in the schistosomicidal action of the therapeutic compounds. SEM is a valuable approach for determining whether a tested substance can cause damage to

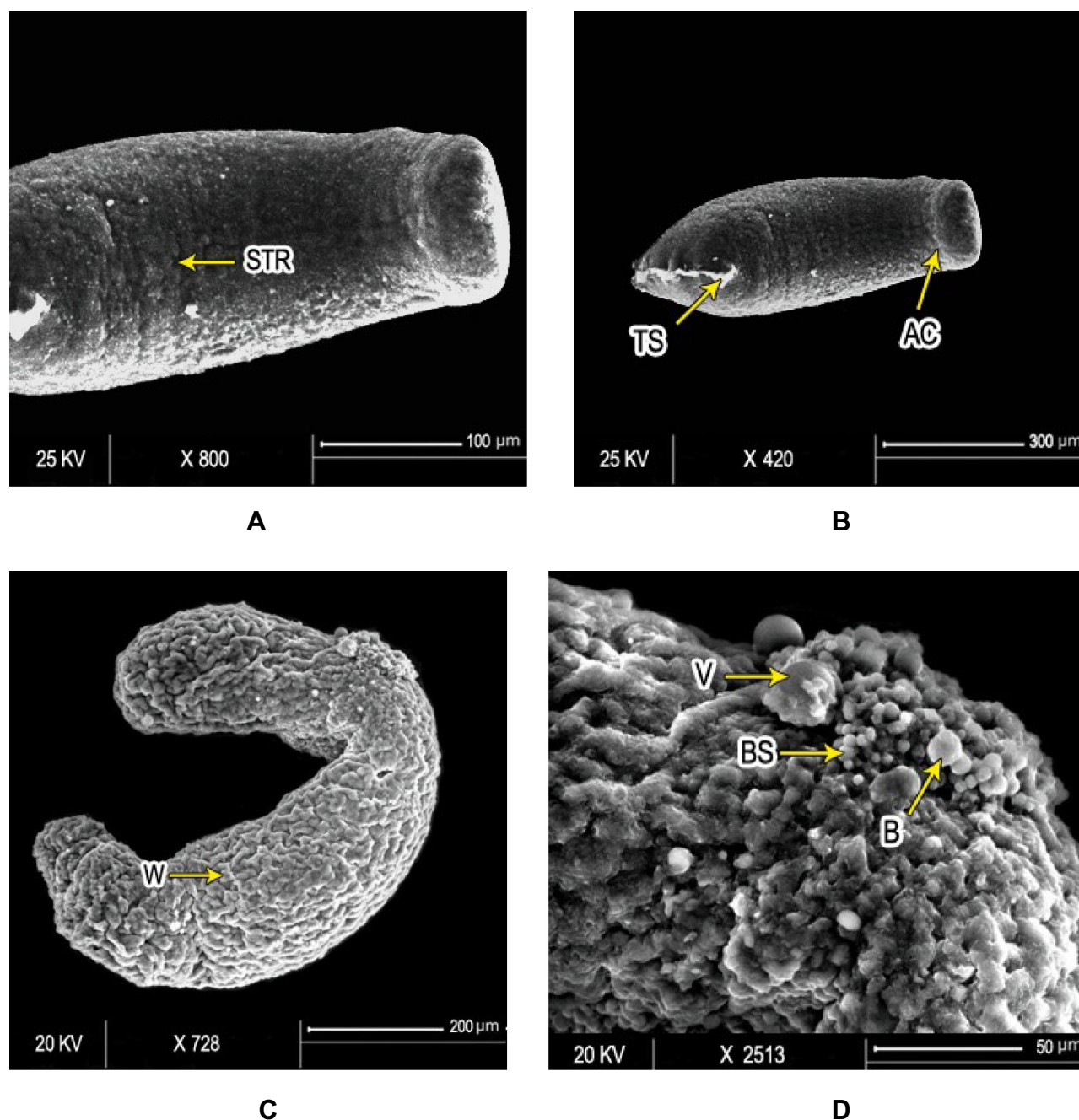


Figure 4 Scanning electron micrography of *S. mansoni* schistosomula. Ultrastructure of *Schistosomula* incubated in medium with 1% DMSO (control group) exhibited (A) intact integument with relatively flat surface and slight tegumentary ridges (STR; bar 100 μm) and (B) an apical constriction (AC) between the head and the body region. At the posterior end of the body, there was a prominent tail-socket (TS) wound left by the breakage of the tail (bar 300 μm). After 72 hours of exposure to PSO, *Schistosomula* showed (C) swelling of body with obvious wrinkling (W) and focal lesions (FL). There was an elongation of the worm, which may indicate flaccid paralysis (bar 200 μm). (D) Blisters (BL), blebs (B), and vesicles scattered on the surface (bar 50 μm) at high concentration (100 μL/mL) of PSO.

the integument of schistosomula. Disruption to the integument of the parasite exposes the worms to the immune system of the host and thus interferes with their survival.⁴⁴ Many schistosomulum medications currently in use, such as PZQ,⁴⁵ mefloquine,⁴⁶ and artemether,⁴⁷ cause damage to the schistosomal integument.

PSO showed evident changes in the integuments of adults, juveniles, and schistosomula in the present study. Changes in adults included destruction of the integument with loss of normal patterns and distortion of the gynephoric canal. These changes increased in severity with increased concentrations and ended with extreme damage,

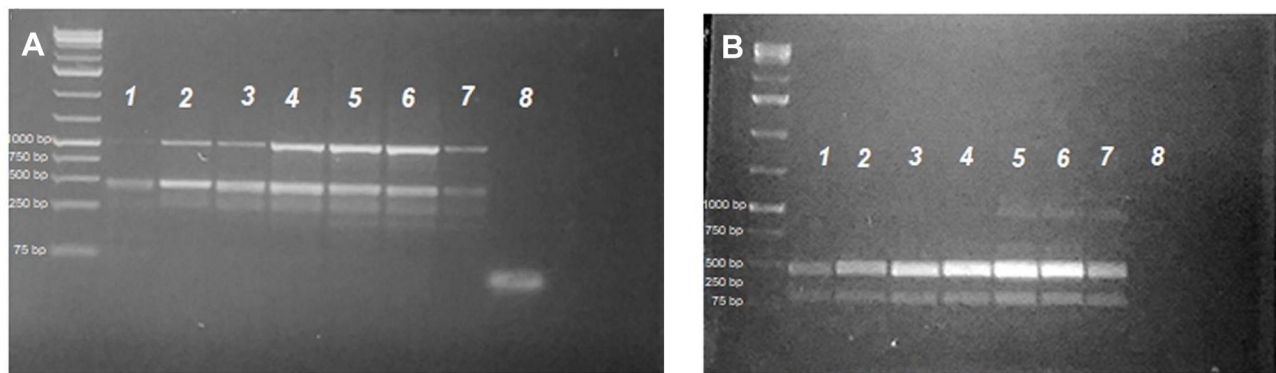


Figure 5 Lanes 2–7 after the 1 kb plus DNA-ladder PCR profiles for *S. mansoni* microsatellite loci R95529 (A) and SMD57 (B) in adult worms treated with 100 µL/mL PSO (lane 1), 40 µL/mL PSO (lane 2), 20 µL/mL PSO (lane 3), PZQ (lane 4), schistosomula treated with 100 µL/mL PSO (lane 5), schistosomula sham control (lane 6), and adult schistosomula sham control (lane 7). A negative PCR control was loaded in lane 8 in both gels.

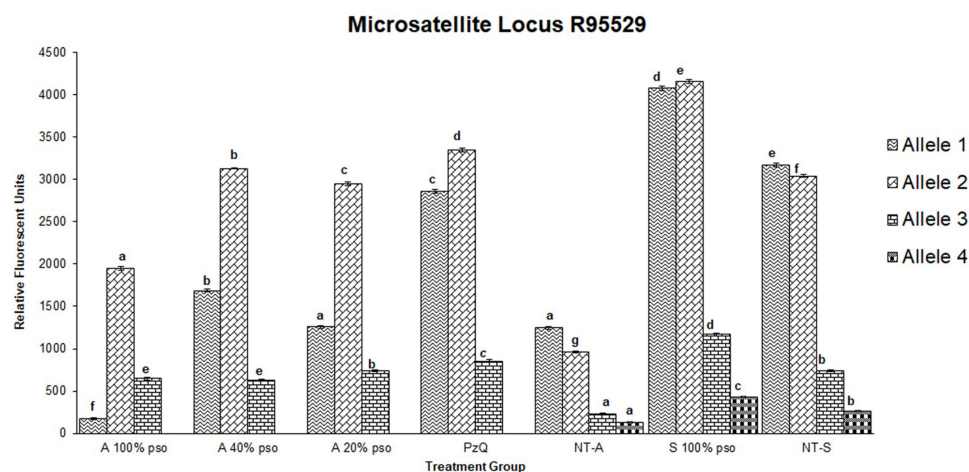


Figure 6 Densitometric measurements of alleles-band intensity for the microsatellite locus R95529 in adult worms treated with 100 µL/mL PSO (100% PSO), 40 µL/mL PSO (40% PSO), 20 µL/mL PSO (20% PSO), praziquantel (PZQ), untreated adult (NT-A) schistosomula treated with 100 µL/mL PSO (S100% PSO), and schistosomula untreated control (NT-S). Letters a–d refer to significant differences ($p < 0.05$) in comparison to the control of each tested maturation (adult schistosomula) group.

edema, lacerations, and blebs formation. Furthermore, PSO-exposed juveniles and schistosomula showed lacerations and peeling with vesicles and blebs. These findings were consistent with those found by Beshay et al,³⁸ who studied the in vivo effect of PSO on *S. mansoni* adults. Microsatellites (slight tegumental ridges) are hyper-variable repetitive sequences consisting of two to six bp of DNA that are broadly spread throughout the genome.⁴⁸ Microsatellite instability is distinguished by the extension, shortening, insertion, or deletion of microsatellites, which is the result of a defective DNA mismatch–repair system, which may result from exposure to some drugs or chemicals.⁴⁹

Immersion in PSO appeared to induce a state of microsatellite instability, which is to the best of authors' knowledge the first report of such a process in this species. Moreover, after their initial identification by Durand et al,²⁷ this is the

first study to report their variation in response to a natural product. The relative abundance of these microsatellite alleles seemed to follow the same tendency in adult and schistosomulum stages, yet they were more abundant in schistosomula than adults for the same initial DNA concentration. However, low and high concentrations of PSO seemed to induce over- or underreplication of such loci in a pattern that was found to be similar to that induced by PZQ. DNA-replication errors leading to instability of microsatellite markers have been identified as a main cause of cancers and aging.^{50,51} Assessment of genetic instability by comparing normal and affected individuals has been validated and applied at the level of human subjects, and proved to be an accompanying factor in different pathological cases that impact genomes.⁵⁰ However, in a wide spectrum of organisms, this phenomenon can be identified and directly related to mutations in genes responsible for postreplication DNA

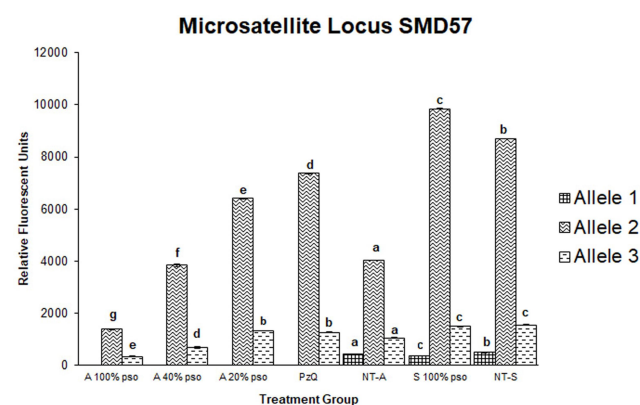


Figure 7 Densitometric measurements of alleles-bands intensities for the microsatellite locus SMD57 in adult worms treated with 100 μ L/mL PSO (100% group), 40 μ L/mL PSO (40% group), 20 μ L/mL PSO (20% PSO), praziquantel (PZQ), untreated adult (NT-A), schistosomula treated with 100 μ L/mL (S100% PSO), and schistosomula control (NT-S). Letters a–d refer to significant differences ($p < 0.05$) in comparison to the control of each tested maturation (adult schistosomula) group.

mismatch–repair genes.⁵² Mismatch-repair inefficiency results in either overproduction of nucleotide repeats or deletion of them.⁵³ Such microsatellite instability has been identified as a marker for mismatch-repair loss and an associated reduction in apoptosis in response to some mutations.⁵⁴ Mismatch repair is crucial in organisms at various taxonomic levels for maintenance of genomic stability and hence normal genetic and physiological performance. The antihelminthic activity of PSO may be due to specific secondary metabolites, such as cucurbitine, saponin sterols, berberine, and palmatine, present in *Cucurbita* spp. seeds.⁵⁵ Berberine and palmatine have been identified as antischistosomal agents.⁵⁶ In this regard, the microsatellite instability observed in this study as a result of different concentrations of PSO may induce genomic instability in adult worms and schistosomula. As a final remark, the resulting microsatellite instability and tegumental damage may lead to loss of the worm's infectious ability.

Conclusion

We have shown that PSO is an efficient compound against *S. mansoni* schistosomula, juveniles, and adults in vitro. The mortality rate, motility effect, morphological integument alternations, and PSO-induced DNA instability suggest that this compound is a promising schistosomicidal product. Further studies are needed to clarify the mechanisms of schistosomal PSO toxicity and to assess its adequacy for the development of new antihelminthic agents.

Disclosure

The authors have no competing interests to declare.

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