Tegumental alterations in adult Schistosoma Mansoni treated with ethanolic extracts of artemisia annua *in vitro*

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Abstract

Background: Schistosomasis is one of the most important parasitic diseases with high prevalence with Praziquantel is the only drug available for the treatment and control to which emerging reduced susceptibility in *S. mansoni* appeared.

Objectives: This work studied the tegmental alteration in adult *Schistosoma mansoni* in response to treatment with ethanolic extracts of *Artemisia annua*.

Materials and methods: About 50 g of the plant parts dry weight was used. After that 1 g of the prepared extract was dissolved in 50 ml of 0.9% saline. 100, 50, 25, 10 & 5 μg/mL different concentrations were used for *in vitro* antischistosomal efficacy. The experiments were conducted on three groups of adult *S. mansoni* worms. The first group was the negative control group where the adults were incubated in dimethylsulfoxide (DMSO); second group was the positive control group in which adult worms were incubated in 0.1 Praziquantel. The third group was the adult worms in Ethanolic extract of *Artemisia annua*. The stereomicroscope and scanning electron microscopywere used to assess survival and ultramorpphological changes respectively.

Results: Significant reduction in the parasites movements and the survival of the adult worms with different extract concentrations which was dose dependent. After 24 h of in vitro incubation of *S. mansoni* with different concentrations of ethanolic extracts of *A. annua* various ultramorphological changes were reported comparable to those caused by Praziquantel.

Conclusion: *A. annua* ethanolic extract provided lethal effects on the adult *Schistosoma mansoni* in vitro with faster and complete effect in the higher concentration

Keywords: Tegumental; *Schistosoma mansoni*; *Artemisia annua;* Ethanolic Extracts.

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Introduction

Schistosomasis, a helminthic disease caused by a blood fluke of the genus *Schistosoma*, is one of the most important parasitic diseases with high prevalence. The most common schistosome species that are able to infect humans are *Schistosoma japonicum*, *S. haematobium* and *S. mansoni.* Schistosomiasis is recognized by the world health organization as neglected tropical diseases (NTDs) that affect many countries in Africa. It is prevalent in 76 tropical and subtruoical countries, where 207 million infected (**Lammie et al., 2006; Steinmann et al., 2006; Mahmoud et al.,2014**).

Schistosomiasis is characterized by having high morbidity and disability rates with low mortality rates (**Engels et al., 2002; Finkelstein et al., 2008**). During chronic or advanced schistosomiasis, which can persist for decades in the absence of treatment, the gastrointestinal and urogenital tracts are affected, leading to hepatosplenic and pelvic organ diseases and other complications, including portal and pulmonary hypertension, abdominal ascites, upper gastrointestinal varices and hemorrhage, infertility, and increased risk of HIV-transmission (**Chofleet al., 2014**). Also, *S. haematobium* infection was classified as a group I biological carcinogen by the International Agency for Research in Cancer (**van der Werf et al., 2003**).

Praziquantel is the only drug available for the treatment and control of schistosomiasis for all human schistosome species (**Doenhoff et al., 2008; Black et al.,2009)** with a major disadvantage of lack of activity against immature stages of *Schistosoma* and a limited effect on already Schistosoma induced liver and spleen pathological lesions (**Doenhoff et al., 2009; Wang et al., 2012**). Although, praziquantel induces mild and transient side effects, severe reactions as neuro-psychiatric, cardiovascular, hepatic, dermatological manifestations in addition to bloody diarrhea were reported in the treatment in many countries as China and Zairein treatment of *S. japonicum* and *S. mansoni* respectively (**Minggang et al., 1983;Polderman et al., 1984**). The reduced susceptibility of the drug in *S. mansoni* was reported in many countries where schistosomiasis is endemic, as Egypt and Senegal (**Melman et al.,2009**).Therefore, new compounds with effective antischistosomal action were a must. The trend towards new, safe and effective drugs using natural plant extracts gave the alternative for many chemical therapeutics (**Magalha˜es et al., 2009; Parreira et al., 2011**).

The genus Artemisia have a very medicinal importance as a rich source of bioactive natural compounds - sesquiterpene lactones, which have medicinal effects, including anthelmintic effect in humans and animals (**Ferreira, 2009; Tariq et al., 2008; Valderrábano et al., 2010**). The leaves are the commercial source of artemisinin used to manufacture drugs, such as artemether, arteether, and artesunate, which are effective against chloroquine-resistant malaria (**Bhakuni et al., 2001**).*A. annua* has shown to be effective in treatment of many parasites as trypanosomiasis or ‘‘sleeping sickness’’ (**Mishina et al., 2007**),schistosomiasis (**Utzinger et al., 2001**), toxoplasmosis (**Oliverira et al., 2009**), leishmanaiasis (**Chawla and Madhubala, 2010**), cryptosporidiosis (**Arab et al., 2006**), coccidiosis(**Brisibe et al., 2008**).

The present study aimed to study tegumental alterations that occur in adults of *Schistosoma mansoni* in response to exposure to ethanolic extracts of *Artemisia Annua*.

Materials and Methods

This study was done in the Research Laboratory of Medical Parasitology

Department, Faculty of Medicine and the scanning electron microscope (SEM) Unit, South Valley University from April 2019 to October 2020.

Preparation of the ethanolic extract of *Artemisia annua*: About 50 g dry weight of the plant was used. Extracts were prepared according to (**El-Menshawi, 2003**). Whole plants or plant parts were dried in a solar oven at 40°C, ground and extracted with methanol at ambient temperature by percolation. Extracts were filtered and methanol was evaporated to dryness under reduced pressure and totally freed from water by freeze drying, and stored under freezing at −20°C till used. After that 1 g of the prepared fresh extract was dissolved in 50 ml of 0.9% saline. 100, 50, 25, 10 & 5 µg/mL different concentrations were used for *in vitro* antischistosomal effectivity (**Fahmy et al., 2009**).

Animals and parasites: Schistosome infected Swiss Albino Female Mice CD strain aging 3 weeks and weighing 35 g were obtained from the Schistosome Biological Supply Center at Theodor Bilharz Research Institute. Mice were maintained after the internationally valid guidelines regularly followed in the institute. Infection of Mice (80-100g) through percutaneous with 50–60 Schistosome cercariae and adult worms were cleared from the blood of maturely infected mice by perfusion technique using phosphate buffer through 20 µm mesh sieves and rapidly placed in culture medium Roswell Park Memorial Institute medium (RPMI) 1640 containing 300mg streptomycin, 300 units penicillin and 160µg gentamycin/100 ml medium.

***In vitro* evaluation of antischistosomal activities of medicinal plants:**

The method used in this study is similar to that by **Yousif et al. (2007)** and **Ramirez et al. (2007).** The present experiments were conducted in flat bottomed plates with 24 wells. Sterilized tissue forceps was used to place three pairs of worms in each well. All plates were then incubated at 37 C and humid atmosphere containing CO2. Adult Schistosomes were monitored every 12 hours. Also sdult Schistosomes incubated in Praziquantel (0.1 µg/ml) were used as positive control while the negative control was parasites incubated in DMSO. The stereomicroscope was used to test the viability of the worm after 24 h. Adult Schistosomes showing no sign of motility for one minute, together with worm deformity, such as blackening, turning, contraction etc., were considered dead. The efficacy of the extract was determined by the measurement of the number of dead worms compared to the total number of worms.

SEM: Dead Schistosome worms were washed with distilled water and were processed according to previous studies for visualization using scanning electron microscopy (Hayat, 1981). Specimens were processed, examined, and photographed at the SEM Unit, South Valley University, Qena, Egypt.

Ethical considerations: The present study was approved by the Ethics Committee and Review Board, Faculty of Medicine, South Valley University.

Results

***In vitro* effects of *Artemisia annua* extract on adults *Schistosoma mansoni* at different concentrations:** After incubation of the adult worms of *S. mansoni* with the different doses of the ethanolic extract of *Artemisia annua*, we recognized a significant reduction in the parasites movements and the survival of the adult worms with different extract concentrations. The duration of survival of *S. mansoni* adult worms was directly proportional with both incubation period and concentration of tested extract.100% of parasites were dead within 24 hours when incubated with extract of concentration of 100 & 50µg/ml. While *Artemisia annua* ethanolic extract with

concentration of 25µg/mL needed more incubation period (72 h) to kill 100% of incubated parasites. But, lower concentrations of the extraxct showed less effect on *chistosoma. mansoni* adults, 10 & 5µg/mL concentrations of *Artemisia Annua* ethanolic extract caused death of 45% and 20 % of parasites at 72h of incubation (**Table.1**). In the present work there was no difference was between male and female as per survival rates in response to different concentrations of the used extracts. The positive control treated by Praziquantel showed total death of the parasites (100%) after 24 h of incubation. All negative control groups were killed at 72 h of incubation the end point of experiment.

SEM: After 24h of in vitro incubation of *Schistosoma mansoni* adults with different concentrations of ethanolic extracts of *Artemisia annua* plant, different tegumental changes were observed using the scanning electron microscopy in comparison with negative control group. These changes were also dose dependent. Numerous ultramorphological changes were reported in the form of contractions, atrophic changes and peeling of tubercles and spine especially on its dorsal surface, suckers alteration or destruction (**Fig.1**). The positive control (Praziquantel treated group) showed similar tegumental alteration in 100% of Schistosome worms.

Table 1.The percentage of dead worms and worms with tegumental alteration in relation to incubation periods with PZQ and *Artemisia annua* in different concentrations.

Groups	Incubation period (h)	Dead worms (%)	Worms with tegumental alteration (%)
Control	24	0	0
	48	0	0
	72	0	0
PZQ	24	100	100
	48	100	100
	72	100	100
***Artemisia annua* 100 µg/mL**	24	100	100
	48	100	100
	72	100	100
50 µg/mL	24	100	100
	48	100	100
	72	100	100
25 µg/mL	24	0	0
	48	65	65
	72	100	100
10 µg/mL	24	0	0
	48	0	0
	72	45	45
5 µg/mL	24	0	0
	48	0	0
	72	20	20

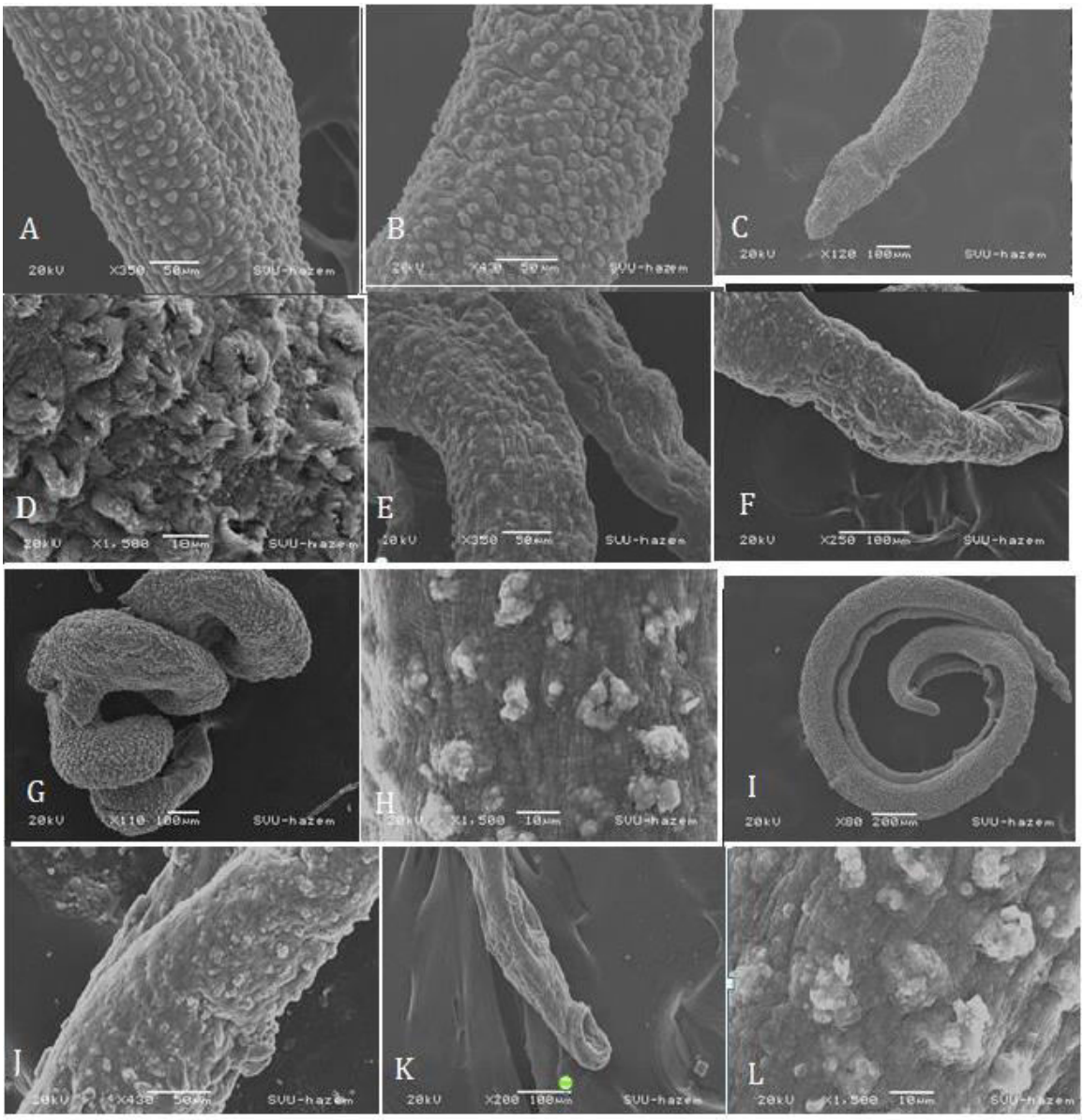

Fig.1. (A, B, C, D): SEM of the normal adult *Schistosoma mansoni* kept in RPMI-1640 alone; showed normal morphology with preservation of tubercles and spines. Fig.2: (E, F, G, H): SEM of adult *Schistosoma mansoni* following the exposure to ethanolic extracts of *Artemisia Annua*. (e) loss of male and female copula; (f) Male anterior end showing damaged oral sucker and loss of tubercles (g) Destruction of tubercles and contractility of the adult worm (h) higher magnification showing male dorsal surface with lost tubercles and spines. Fig.3: (I, J, K, L): SEM of adult *Schistosoma mansoni* worms kept in Praziquantel (0.1µg/ml) (i) Loss of male and female copula (j) Male dorsal surface showing complete destruction of tubercles and spines. (k) Male ventral surface with damage of ventral sucker (l) higher magnification showing male dorsal surface with lost tubercles and spines.

Discussion

Praziquantelis the drug of choicefor treatment of all species of *Schistosoma*. This drug has been widely used for more than three decades with good efficacy and low toxicity. But unfortunately the emergence of praziquantel resistant Schistosomes was recorded (Wang,

2012),so production of new treatments for schistosomiasis is needed. plants are a rich source in bioactive compounds were tested for the production of new drugs (**Tonuci et al, 2012**).In vitro screening tests are efficient and inexpensive methods to detect potential antiparasitic agents for in vivo experiments (**Keiser, 2010**).

In the current study, we evaluated the lethal effect of various doses of the ethanolic extract of *A. annua* leaves against *Schistosoma mansoni* adult worms in vitro. This lethal effect was evaluated by recording the survival time and tegumental alterations with different concentrations of the extract(100, 50, 30, 20, 10 & 5µg/ml). Also the observed lethal effects were dose-dependent. Highest concentration of *Artemisia annua* ethanolic extract (100 & 50µg/ml) caused death of all parasites within 24hr of incubation with marked tegumental alteration and limitation of motor activity in all examined parasites. While the 30 & 20µg/ml caused death of 100% of parasites within 72 h of incubation with also tegumental alteration and limitation of motor activity in all examined parasites. But, the 10 & 5µg/ml concentrations of *Artemisia annua* ethanolic extract caused death of 45% and 20 % of parasites at 72h of incubation. This result agreed with de **Almeida et al. (2016)** who found that 100% mortality in adults, as well as tegumental alterations and significant decrease in motor activity. Also, it agreed with **Ekanem and Brisibe (2010)** who reported that *A. annua* ethanolic (70%) extract killed and dislodged the monogeneantrematodes *Dactylogyrus* and *Gyrodactylus* that infect catfish. Results of the present agreed with **Utzinger et al. (2001)** and **Keiser et al. (2006; 2010)** who found that leaves of *A. annua* and its semisynthetic derivatives have antischistosomal properties by oral doses of 6 mg/kg in randomized controlled clinical trials.

Artemisinin is mainly an antimalarial drug, its mode of actions involves a reaction of the Artemisin in peroxide group with heme iron generated by the parasite degradation of hemoglobin, resulting in the release of free radicals inside the cells of the parasite (**O'Neill et al, 2010**). Also, *A. annua* contains over 40 flavonoids, some of which might potentiate artemisinin by inhibiting CYP450 enzymes that degrade Artemisinin (**Ferreira et al, 2010**).

As regards its antischistosomal effects, Artemisinin was first discovered to possess anti-schistosomal properties (Chen *et al,* 1980), followed by artemether, artesunate and DHA being reported to be effective against *S. japonicum, S. haematobium* and *S. mansoni* in schistosomiasis models in mice, rabbits, dogs and hamsters, resulting in significant worm burden reduction of at least 86% (**Xiao et al., 1995; Shuhuaet al., 2000; Xiao, 2005; Utzingeret al., 2007**).

In the present study, leaves of *A. annua* ethanolic extract caused marked damage of the suckers and tubercles as recognized by SEM. This agreed with **Shuhua et al. (2000)** who found that Artemether caused damage to tegument and musculature of schistosomulae, and exert its helminthotoxic effect through synergy with heme containing compounds.

In vivo studies on the effectiveness of Artemisinins stated that there is a prominent effectiveness against juvenile infections and only moderately effective against adult infections (Keiser and Utzinger, 2007). So, these drugs particularly show clinical benefit when used as prophylactic treatments.

In meta-analysis study done by Liu and colleagues, stated that administration of multiple low doses of artemether or artesunate over a 1-2-week period achieved a protection rate of 65-97% against schistosomiasis japonicum (**Pérez et al., 2012**). A decreased risk was observed

using artemether for the prevention of *S. mansoni* and *S. haematobium* infections (**Utzinger et al, 2000a**). **Keiser and Utzinger (2007)** reported high efficacy, low to moderate cure rates were observed.

In comparison of the lethal effect of leaves of *A. annua* ethanolic extract in the present study with some other herbal extracts as Pomegranate extracts results agreed with **Yones et al., (2016)** who stated that the documented antischistosomal activity of Pomegranate extracts on *Schistosoma mansoni* adult worms concerning the following (mating, motility, survival time, and tegumental alterations) but with the uses of higher concentrations (100, 300, & 500µg/ml) in comparison to our study. Also, **Hussein and Al-Almubark (2019)** found that *Punicagranatum* methanolic extracts showed significant effect on adult worms of *Schistosoma mansoni* after 24h in vitro cultivation: 100% of worms were dead with both leaves and peels extracts in different concentrations. **El-Shenawy et al. (2008)**, using natural compounds as chemotherapeutic agents, reported remarkable reduction in worms, tissue egg load and alteration in oogram pattern in animal groups treated with either garlic extract or *Nigella sativa* oil.

In the present work, *Artemisia annua* ethanolic extracts at 10 and 5µg/mL caused death of 45% and 20 % of parasites after 72h of incubation which induced little morphological destructions. Also from our study there was damage in the oral sucker.

Lastly from our study is no difference in the lethal effects was observed between male and female adult worms in response to different concentrations of the used extracts about the survival time and tegumental alterations.

Conclusion

Leaves of *Artemisia annua* ethanolic extract provided lethal effects on the adult *Schistosoma mansoni* in vitro with faster and complete effect in the higher concentration, so more studies especially in vivo on the efficacy of this extract on different *Schistosoma* stages.

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