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In vitro Assessment of Antischistosomal Effect of the Marine *Hyrtios* sp. Sponge Extract against *Schistosoma mansoni* Adult Worms

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ABSTRACT

Schistosomiasis is a prevalent epidemic disease in Egypt. As a result of the exclusive use of praziquantel (PZQ) in the treatment of schistosomiasis, the parasite may become resistant to the medication. Accordingly, there has been a growing interest in research with the aim of developing novel antischistosomal medicines from natural sources. The marine *Hyrtios* sponge exhibits bioactive compounds, qualifying it to explore novel drugs. Hence, the current study aimed to assess the *in vitro* activity of the *Hyrtios* sponge extract (HSE) against schistosomiasis through the detection of mortality and tegumental alterations in *S. mansoni* adult worms. The mortality rates of *S. mansoni* worms exceeded 50% after 2 and 3 hours of exposure to a concentration of 200µg/ ml of HSE. After 24 hours, mortality rates surpassed 50% at concentrations of both 100 and 200µg/ ml. Additionally, after 36 and 48 hours, mortality rates reached above 50% across concentrations ranging from 60 to 200µg/ ml. Furthermore, after 48h, the mortality rate of *S. mansoni* worms reached 100% at a 200µg/ ml concentration. After exposing the *S. mansoni* worms to HSE for 24h, the lethal concentration values (LC_{50} and LC_{90}) were 115.40 and 241.19µg/ ml, respectively. These lethal concentrations became 59.36 for LC_{50} and 144.11µg/ ml for LC_{90} after 48h of exposure. Scanning electron microscopy revealed morphological changes in both male and female worms at concentrations of 100 and 200µg/ ml following a 48-h *in vitro* treatment of *S. mansoni* worms with the HSE compared to the control group. Morphological abnormalities in male worms included swollen or flattened bodies, self-coiling contractions, tegument shrinkage, invaginations, erosion, deformed gynechophoric canal, distorted suckers, and smooth tubercles with blebs between them. The female body displayed morphological alterations, including swollen body, shrinkage of the ventral sucker, a pitted tegument with corrugation, folds, invaginations, wrinkles, erosions, and blebs in addition to the absence of body spines. The death of *S. mansoni* worms caused by HSE exposure could be associated with its effect on their tegumental surface and may be related to the existence of different antioxidant phytochemicals like alkaloids, saponins, phenolics, and flavonoids in this extract. In conclusion, HSE has an effective antischistosomal impact and can be considered an adjuvant medication for PZQ in *S. mansoni* infection.

INTRODUCTION

Schistosomiasis (bilharzia) is a highly common parasitic disease among humans worldwide caused by the trematode worm of the genus *Schistosoma* **(McManus *et al.*,**

2020; Llanwarne & Helmby, 2021). In Egypt, schistosomiasis is still one of the most prevalent epidemic diseases although various control programs exert many attempts to get rid of this infection **(Abdella *et al.*, 2024)**. The most widespread type of schistosomiasis among the Egyptian Nile Delta's rural inhabitants is *S. mansoni* **(Ahmed *et al.*, 2021)**. Schistosomiasis principally affects children, and its long-term infection causes significant health problems, including anemia, child growth impairment, fatigue, and impaired mental abilities **(King & Dangerfield-Cha, 2008; Ezeamama *et al.*, 2018)**.

Schistosomiasis control exclusively depends on chemotherapy through a single anthelmintic praziquantel (PZQ) medicine **(Kura *et al.*, 2022)**. Intensive and exclusive use of a single drug to treat schistosomiasis for decades may eventually give rise to legitimate worries that the disease may become resistant to PZQ **(Cioli *et al.*, 2014; Abou-El-Naga *et al.*, 2019)**. Therefore, significant attention has been paid to developing new, safe, and effective antischistosomal medications mostly derived from natural sources.

Marine invertebrates are rich sources of marine natural products with various medicinal benefits to improve human health **(De Zoysa, 2012)**. The isolated chemicals or prepared extracts from marine invertebrates have demonstrated a broad range of therapeutic properties, including anticoagulant **(Karthikeyan *et al.*, 2012)**, anthelmintic **(Mona *et al.*, 2012)**, anti-inflammatory **(Senthilkumar & Kim, 2013)**, antiprotozoa **(Lhullier *et al.*, 2020)**, antiviral, antimicrobial **(Mona *et al.*, 2021)**, anticancer **(Singh *et al.*, 2021)**, antioxidant **(Rasyid & Putra, 2023)**, and other medicinal effects. Therefore, marine invertebrates became a trend for discovering more novel treatments for diseases affecting humans.

Among marine invertebrates, sponges are the most promising source of biologically active compounds and are used for exploring novel medications **(Perdicaris *et al.*, 2013; Mehbub *et al.*, 2014)**. *Hyrtios* sponge **(**class, Demospongiae; order, Dictyoceratida; **Duchassaing & Michelotti, 1864)** is reported to be a plentiful source of various kinds of bioactive secondary metabolites of different classes **(Shady *et al.*, 2017a, b)**. The prominent bioactive compound classes from this genus include macrolides **(Kobayashi *et al.*, 1993)**, alkaloids **(Salmoun *et al.*, 2002; Utkina, 2009; Youssef *et al.*, 2013)**, sesterterpenes **(Youssef *et al.*, 2002; Qiu *et al.*, 2004; Mahidol *et al.*, 2009)**, sesquiterpenes **(Longeon *et al.*, 2011)**, flavonoids, and saponins **(Wardany & Nirmala, 2023)**. Numerous studies have indicated that bioactive compounds isolated from *Hyrtios* are shown to exhibit pharmacological activities, including antioxidant **(Utkina, 2009)**, anticancer **(Lim *et al.*, 2014)**, anti-inflammatory **(Muthiyan *et al.*, 2018)**, antitrypanosomal **(Shady *et al.*, 2018)**, anti-Alzheimer **(Nabil-Adam *et al.*, 2020)**, and antiplasmodial activities **(Mahfur *et al.*, 2022).** Despite the previous studies, there have yet to be any relevant reports on the anthelmintic effect of *Hyrtios* marine sponge. Therefore, the current study aimed to explore the *in vitro* activity of the methanolic extract prepared from the *Hyrtios* sponge against schistosomiasis by detecting mortality and morphological alterations of *S. mansoni* adult worms.

MATERIALS AND METHODS

1. *Hyrtios* sponge collection

Marine sponge *Hyrtios* sp. was collected from Hurghada, Egypt, in February 2022 (Fig. 1). Sampling was done using snorkeling and self-contained underwater breathing apparatus (SCUBA) diving technique at a depth between 13 and 20m. Freshly collected samples were washed with sterilized artificial seawater to remove sediments and impurities, immediately frozen, and kept till usage at –20°C.

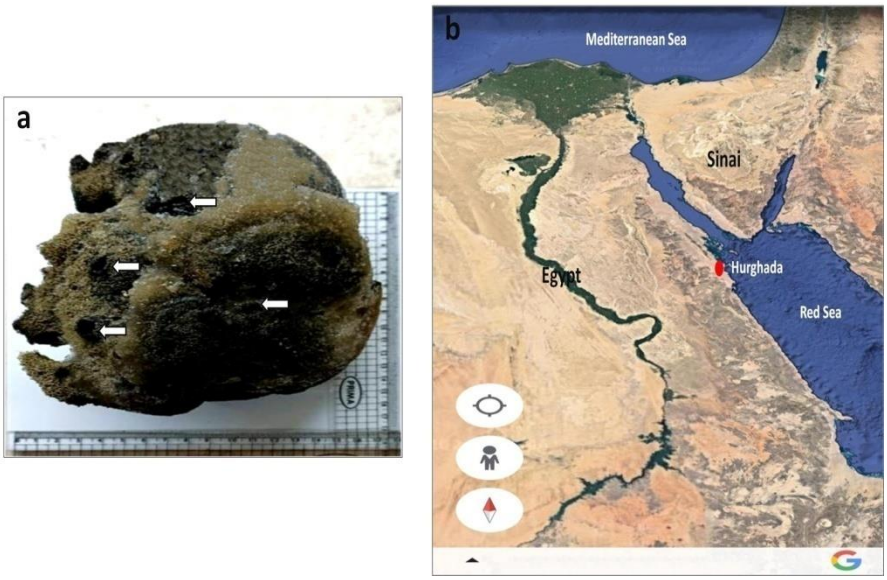

Fig. 1. a: A black round ball shaped of *Hyrtios* marine sponge with scattered oscula (white arrows) and spongin fibrous skeleton. **b:** Google map showing the sampling site for collection of *Hyrtios* sponge (Hurghada indicated by red dot) on the Red Sea coast, Egypt

2. Preparation of the methanolic crude *Hyrtios* sponge extract

The extraction process was conducted following the maceration method, as reported in studies by **Purushottama *et al.* (2009)** and **Hutagalung *et al.* (2014)**. In brief, sponge tissues were lyophilized for two days at room temperature (25– 27°C), cut into small pieces, and pulverized until they became powder. The grounded sample was then soaked in absolute methanol as a solvent (1: 20 *w/ v*) for 5h with genital stirring at room temperature. A vacuum rotary evaporator operating at 40°C was used to evaporate the solvent after the solution had undergone filtration through Whatman Filter Paper No. 1. Following the lyophilization of the residual solvent, the dried *Hyrtios* sponge extract (HSE) was preserved at –20°C for additional biochemical tests.

3. Determination of bioactive compounds in *Hyrtios* sponge extract

3.1. Total phenolics

The Folin-Ciocalteu procedure was utilized to evaluate the extract's total content of phenolics **(Taga *et al.*, 1984)**. Two milliliters of 2% Na2CO3 solution were combined with 100µl of either the sample or gallic acid (GA) standard. Subsequently, 100µl of 50% Folin-Ciocalteu reagent was added to the mixture. This mixture was preserved at room temperature for a period of 30min. A spectrophotometer was utilized to indicate the absorbance of the solution at 750nm wavelength. By comparing the results to the GA calibration curve, the total content of phenolics was calculated as milligrams of gallic acid equivalent (GAE) per gram of extract.

3.2. Total flavonoids

The extract's total content of flavonoids was estimated following the method of **Zhishen *et al.* (1999)**. Firstly, one ml of sample was combined with 4ml of distilled water (dis. H_2O) and 0.3ml of $NaNO_2$ (1: 20). Secondly, after 5min, 3ml of $AlCl_3$ (1: 10) was added and allowed to stand for a period of 6min. Finally, 2ml of 1.0M NaOH was added to the solution, and the volume was increased by adding dis. H_2O to 10ml. After thoroughly mixing the solution, a spectrophotometer was used to measure the solution's absorbance at 510nm against a blank. Quercetin was employed as a standard solution. By comparing the total flavonoid concentration to the reference calibration curve, it was calculated as milligrams of quercetin equivalent (QE) per gram of extract.

3.3. Total alkaloids

The estimation of the total content of alkaloids in the extract was based on the reaction between alkaloids in the extract and acetic acid forming a salt, and the liberation of alkaloids was stimulated with ammonia **(Harbone, 1973)**. About 0.2ml of 10% acetic acid in ethanol was mixed with 5mg of extract, and the mixture was permitted to stand overnight. The filtrate was concentrated in a water bath to be a quarter of its initial volume. The filtrate was gradually mixed with concentrated NH_4OH until precipitation occurred. The entire solution was left to settle overnight, and finally, the collected precipitate was washed with 0.1M NH_4OH and filtered. The total content of alkaloids was calculated as milligrams per gram of extract after the alkaloid residue was weighed and dried.

3.4. Total saponins

The quantification of the total content of saponins in the extract occurred following the method of **Espinoza *et al.* (2021)**. About 0.8ml of the extract was hydrolyzed in an aliquot using an equivalent amount of 6N HCl for a period of 2h at 110°C and then cooled for 5min in an ice-cold water bath. Then, after neutralizing it with an ammonia solution, it was centrifuged for 5min at 3000 ×g. After extracting the supernatant, 3ml of ethyl acetate was added for every 5ml sample. After that, the fractions were mixed and filtrated on a bed of anhydrous Na_2SO_4. About 250µl of this final extract was shaken vigorously with 1000µl of the reagent mixture (glacial CH_3COOH/H_2SO_4, 1:1 *v/ v*) for a period of 30sec and, and then heated at 60°C in a water bath for 30min to develop light

purple color. The absorbance of the cooled mixture was estimated at a specific wavelength of 527nm. Total saponin content was quantified as milligrams of sapogenin equivalent (SE) per gram of extract.

4. Determination of antioxidant capacity of *Hyrtios* sponge extract using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant capacity of *Hyrtios* extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, which was suggested by **Brand-Williams *et al.* (1995)** and **Molyneux (2004)**. Ascorbic acid concentrations ranging from 0.1 to 0.5mg/ ml were prepared to establish a standard curve. In brief, 1.0ml of 0.2Mm DPPH in methanol (0.0078 g/100 ml) was prepared and added to 1ml of the extract (5mg/ ml) and/or standard solution at varying concentrations. The mixture was incubated in the dark at an ambient temperature for 30min. Subsequently, the absorbance (Abs) of the resulting reduction in DPPH color intensity was determined at 517nm utilizing a spectrophotometer. The DPPH radical-scavenging activity was determined as the percentage of DPPH inhibition using the subsequent equation:

$$\text{DPPH inhibition \%} = (\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}})/\text{Abs}_{\text{Control}} \times 100$$

For DPPH control, all reagents were added except the tested sample. The calibration curve was employed to express the antioxidant capacity as milligrams of ascorbic acid equivalent (AAE) per gram of dried extract.

5. *In vitro* screening of antischistosomal activity of *Hyrtios* sponge extract

Adult *S. mansoni* worms of Egyptian strain were obtained from *Schistosoma* Biological Supply Center, Theodor Bilharz Research Institute, Embaba, Giza, Egypt. The used culture medium was Roswell Park Memorial Institute (RPMI-1640) medium (Sigma, USA) enhanced by sterilizing antibiotics (100µg/ ml of streptomycin and 100U/ ml of penicillin; Sigma, USA), 2g/ L glucose, 0.39g/ L glutamate, 20g/ L $NaHCO_3$, and 20% fetal calf serum (Gibco, USA) as a nutrient agent.

The antischistosomal activity of the methanolic crude HSE was evaluated *in vitro* on adult *S. mansoni* worms according to the method of **Melek *et al.* (2012)** and **Abou El-Nour and Fadladdin (2021)**. The known weight of the HSE was dissolved in dis. H_2O to obtain a stock solution from which the desired concentrations (20, 40, 60, 80, 100, and 200µg/ ml) were prepared. Adult worms incubated with medium and dis. H_2O served as a negative control, and the reference drug PZQ (0.2µg/ ml) was utilized as a positive control. Under sterile conditions, eight adult *S. mansoni* worms of both sexes were added to the culture media in each well of a flat-bottom 24-well tissue culture plate and preserved at 37°C in a moist condition containing 5% CO_2 for 2h for adaptation before the addition of treatments. After that, 1.0ml of PZQ and/or different tested extract concentrations were added to each well in triplicate and re-incubated for 48h. The adult worms were monitored at different intervals (1, 2, 3, 24, 36, and 48h). After each incubation period, the viability and mortality of worms were checked under a stereomicroscope and recorded. Worms that remained motionless for one minute were classified as dead. The antischistosomal activity of HSE was calculated as the number of dead worms relative to the total number of worms and compared with the negative and

positive controls. The extract lethal concentrations for 50 and 90% mortality of the exposed *S. mansoni* adult worms (LC_{50} and LC_{90}, respectively) were evaluated using linear regression equations at all-time intervals.

6. Scanning electron microscopic examination of *Schistosoma mansoni* adult worms

S. mansoni adult worms were rinsed multiple times in 9% saline. Then, they were primarily fixed in 4% paraformaldehyde (PFA) + 2.5% glutaraldehyde solution and were kept overnight at 4°C. After that, the worms were submerged in a phosphate buffer for 15min to remove any remaining fixative residue. Post-fixation of worms occurred in 1% osmium tetroxide phosphate buffer for one hour. Then, the worms were given two rounds of washing in the phosphate buffer before being dehydrated using ethyl alcohol (30, 40, and 50%) for 15min each. After being left in 70% ethanol for 30min, the worms were cleaned twice in 80, 90, and 100% ethanol, mounted on copper stubs holders, and covered with gold using a sputter coater. Finally, the worms were examined and microphotographed utilizing a scanning electron microscope (SEM) (Jeol–JSM 6510 LV) at Mansoura University **(Mona *et al.*, 2012)**.

7. Statistical analysis of data

All experiments were conducted in triplicate, and the resulting data were presented as means ± standard deviations (SD). This study utilized linear regression analysis to estimate LC_{50} and LC_{90} of HSE against *S. mansoni* adult worms. The one-way analysis of variance (ANOVA) test and Tukey's test for multiple comparisons were conducted to analyze the obtained results using the Statistical Package for the Social Sciences (IBM-SPSS Version 20.0) software. The differences were statistically significant at $P \leq 0.05$ **(Kirkpatrick & Feeney, 2012)**.

RESULTS

1. Characterization of the methanolic crude *Hyrtios* sponge extract

Phytochemical analysis of HSE indicated the presence of a high level of total alkaloid content at a concentration of 370± 10mg/ g of extract, followed by total contents of saponins, phenolics, and flavonoids at concentrations of 311.68± 5.23mg SE/ g of extract, 5.19± 0.32mg GAE/ g of extract, and 2.36± 0.16mg QE/ g of extract, respectively. Moreover, the antioxidant capacity of this extract was 101.70± 6.72mg AAE/ g of extract (Table 1).

Table 1. Phytochemicals and antioxidant capacity of the methanolic crude *Hyrtios* sponge extract (HSE)

Parameter	Concentration
Total phenolics (mg GAE/g of extract)	5.19 ± 0.32
Total flavanoids (mg QE/g of extract)	2.36 ± 0.16
Total alkaloids (mg/g of extract)	370 ± 10
Total saponins (mg SE/g of extract)	311.68 ± 5.23
Antioxidant capacity (mg AAE/g of extract)	101.70 ± 6.72

Each value is the mean of 3 replicates ± SD.

2. Screening of antischistosomal activity of the methanolic crude *Hyrtios* sponge extract

As shown in Table (2), there is a complete absence of mortality in the *S. mansoni* adult worms of the control group and 20µg/ ml HSE group. PZQ at a 0.2µg/ ml concentration eliminated all worms at 3, 24, 36, and 48h ($P< 0.001$). The rise in the mortality rate of *S. mansoni* adult worms depended on the increasing HSE concentration and longer exposure time. The HSE, ranging from a concentration of 60µg/ ml after 2 hours to 200µg/ ml after 48 hours, showed significant mortality percentages ($P\leq 0.001$) of adult *S. mansoni* worms compared to the control group. The *S. mansoni* adult worm's mortality of more than 50% occurred after 2 and 3h of exposure to HSE at 200µg/ ml, after 24h of exposure to HSE at 100 and 200µg/ ml, and after 36 and 48h of exposing to HSE at concentrations ranged from 60 to 200µg/ ml. The HSE achieved potent *in vitro* schistosomicidal activity on the adult worms of *S. mansoni* ($P< 0.001$) with 100% mortality at 200µg/ ml after 48h in comparison to the control group.

On 24 and 48h post-treatment, the mortality percentage of *S. mansoni* adult worms fluctuated between its maximum values (68.44 and 100%, respectively) at the highest applied concentration (200µg/ ml), and the minimum values (33.33 and 37.50%, respectively) at 40µg/ ml. The values of LC_{50} and LC_{90} for the HSE on the adult *S. mansoni* worms were estimated by the linear regression equation, as presented in Table (2). The HSE exhibited LC_{50} values of 115.40 and 59.36µg/ ml after 24 and 48h of exposure time, respectively. The LC_{90} values of HSE against the *S. mansoni* adult worms decreased dramatically with the increasing exposure time; they were 241.19 and 144.11µg/ ml after 24 and 48h of exposure time, respectively.

3. *In vitro* morphological changes of male and female *Schistosoma mansoni* adult worms

SEM examination of the adult worms of *S. mansoni* obtained from the control group illustrated that the adult male of *S. mansoni* was thicker, cylindrical, and shorter than the adult female. The male had a ventral gynaecophoric canal where the female was located (Fig. 2a). Body of the male had three distinguished regions; the anterior region, which comprises of oral sucker, ventral sucker, and a tegumental area connecting them, the middle region, and the posterior region. The male *S. mansoni* adult worm had an oval-shaped oral sucker at the top of the body anterior region with three notable regions; a thin rim, a large inner zone, and the mouth. The ventral sucker was round and consisted of a rim, inner zone, and central zone. The tegument of the body anterior region had different shapes of papillae, including hemispherical papillae (Fig. 2b). The outer margin of the gynaecophoral canal had tiny spines (Fig. 2c), and its inner surface appeared as folds with numerous spines (Fig. 2d). Moreover, the middle part of male body exhibited sponge-like tegument covered with many large spiny tubercles and macule papillae on the wrinkled tegument around them (Fig. 2e). Tegument of the body posterior region was consisted of spiny tubercles and macule papillae between them (Fig. 2f).

Table 2. The percentage of dead adult worms of *Schistosoma mansoni* after *in vitro* exposure to 0.2µg/ ml of praziquantel (PZQ) and different concentrations of *Hyrtios* sponge extract (HSE) during different incubation periods in comparison with the control group

Group	Concentration (µg/ml)	Percentage of dead adult worms of *S. mansoni* after different incubation periods (h)					
		1 h	2 h	3 h	24 h	36 h	48 h
Control	Distilled water	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
PZQ	0.2	0.00 ± 0.00^{a}	$33.33 \pm 11.54^{cd***}$	$100 \pm 0.00^{f***}$	$100 \pm 0.00^{e***}$	$100 \pm 0.00^{e***}$	$100 \pm 0.00^{e***}$
HSE	20	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	40	0.00 ± 0.00^{a}	12.50 ± 0.00^{ab}	$25 \pm 0.00^{b***}$	$33.33 \pm 7.21^{b***}$	$37.50 \pm 0.00^{b***}$	$37.50 \pm 0.00^{b***}$
	60	$17.50 \pm 4.33^{b***}$	$28.33 \pm 2.88^{bc***}$	$35 \pm 4.33^{c***}$	$35 \pm 4.33^{b***}$	$58.33 \pm 7.21^{c***}$	$70 \pm 8.66^{c***}$
	80	$27.38 \pm 2.06^{c***}$	$34.52 \pm 5.15^{cd***}$	$41.06 \pm 3.08^{cd***}$	$47.61 \pm 4.12^{c***}$	$60.71 \pm 3.09^{c***}$	$73.80 \pm 2.06^{c***}$
	100	$34.52 \pm 5.15^{cd***}$	$34.52 \pm 5.15^{cd***}$	$47.61 \pm 4.12^{de***}$	$54.76 \pm 4.12^{c***}$	$73.80 \pm 2.06^{d***}$	$86.90 \pm 1.03^{d***}$
	200	$41.06 \pm 3.08^{d***}$	$50.59 \pm 11.33^{d***}$	$54.76 \pm 4.12^{e***}$	$68.44 \pm 5.14^{d***}$	$82.14 \pm 6.18^{d***}$	$100 \pm 0.00^{e***}$
	F-ratio	122.95	24.57	401.18	206.37	310.07	507.98
	p-value	0.00	0.00	0.00	0.00	0.00	0.00
	Linear regression equation	$y = 0.239x + 0.132$ $R^2 = 0.76$	$y = 0.253x + 5.597$ $R^2 = 0.81$	$y = 0.253x + 12.81$ $R^2 = 0.68$	$y = 0.318x + 13.30$ $R^2 = 0.74$	$y = 0.375x + 20.83$ $R^2 = 0.64$	$y = 0.472x + 21.98$ $R^2 = 0.67$
	LC_{50}	208.65	175.50	146.99	115.40	77.78	59.36
	LC_{90}	376.01	333.60	305.09	241.19	184.45	144.11

PZQ: praziquantel, HSE: *Hyrtios* sponge extract, LC_{50}: Lethal concentration for 50% mortality of the exposed *S. mansoni* adult worms, LC_{90}: Lethal concentration for 90% mortality of the exposed *S. mansoni* adult worms, y: % Mortality value, and x: Concentration of the HSE. Each value is the mean of 3 replicates ± SD. Means in the same column with different superscript letters $^{(a, b, c, d, e, and f)}$ are significantly different. * Significant at *P*-value≤ 0.05, ** Significant at *P*-value≤ 0.01, and *** Significant at *P*-value≤ 0.001 (one-way ANOVA).

Regarding female adult *S. mansoni* obtained from the control group, SEM examination indicated that its body was cylindrical and elongated. The adult female's body was partitioned into three regions, as in males. The anterior part consisted of the oral sucker, ventral sucker, and the connecting tegumental area (Fig. 3a). Oral sucker was triangular with three regions; a thin rim, a large inner zone, and mouth. It had several spines oriented toward the mouth cavity. Hemispherical papillae were found around the oral sucker (Fig. 3b). Ventral sucker was circular, consisted of a rim, inner zone, and

central zone, and had several spines with consistent shape and size (Fig. 3c). Hemispherical and macule papillae spread over the dorsal surface of the tegument (Fig. 3d). The posterior part of the body had several spines and hemispherical papilla (Fig. 3e).

After 48h of *in vitro* exposure of adult worms to the HSE at 100 and 200µg/ ml concentrations, distinct morphological changes appeared in both sexes of *S. mansoni* adult worms compared to the control group. The morphological alterations were clear in the male adult *S. mansoni* worms after exposure to 100µg/ ml of HSE since they appeared to have abnormalities, including intensive contraction of the swelling body and the gynechophoric canal (Fig. 4a). Severe shrinkage and invaginations in the worm tegument were quite evident, particularly in the first part of the dorsal side of the body (Fig. 4b). A self-coiling phenomenon and deformation of the gynechophoric canal could also be observed after exposure to HSE at 100µg/ ml (Fig. 4c). Swelling of suckers was observed to the extent that makes the mouth opening scarcely recognizable (Fig. 4d). The tegumental tubercles became swollen with complete loss of spines and presence of blebs between them (Fig. 4e). Moreover, collapsed tubercles appeared on male tegument, and the sub- tegumental tissues were exposed as a result of extensive erosion in the integument (Fig. 4f).

As a result of exposure of the male *S. mansoni* adult worms to HSE at a concentration of 200µg/ ml, severe body alterations occurred. The body became flattened and smooth with a loss of tubercles and spines (Fig. 5a). The marked distortion of the suckers, like the flattening of the ventral sucker, was also recorded (Fig. 5b). The gynechophoric canal was distorted, seemed open, and devoided of its ridges (Fig. 5c). Tegumental sloughing appeared on lateral side of the gynechophoric canal (Fig. 5d). Most parts of the tegument were collapsed and/or ruptured tubercles were arranged in irregular rows with no spines (Fig. 5e, f).

In vitro treatment with the HSE at 100µg/ ml resulted in observable morphological changes on the female *S. mansoni* adult worms, like severe shrinkage of ventral sucker, tegumental pits with corrugation, tegumental folds including invaginations, and tegumental sloughing (Fig. 6). At the same time, HSE at 200µg/ ml caused destructive impacts on the female *S. mansoni* adult worms, including swelling of the whole body, abnormal oral sucker surrounding with blebs, marked shrinkage of ventral sucker with pulling it inside. Furthermore, severe alterations in the tegumental architecture, like wrinkling, invaginations, folding, erosions, and tegumental blebs with the absence of spines, were recorded (Fig. 7).

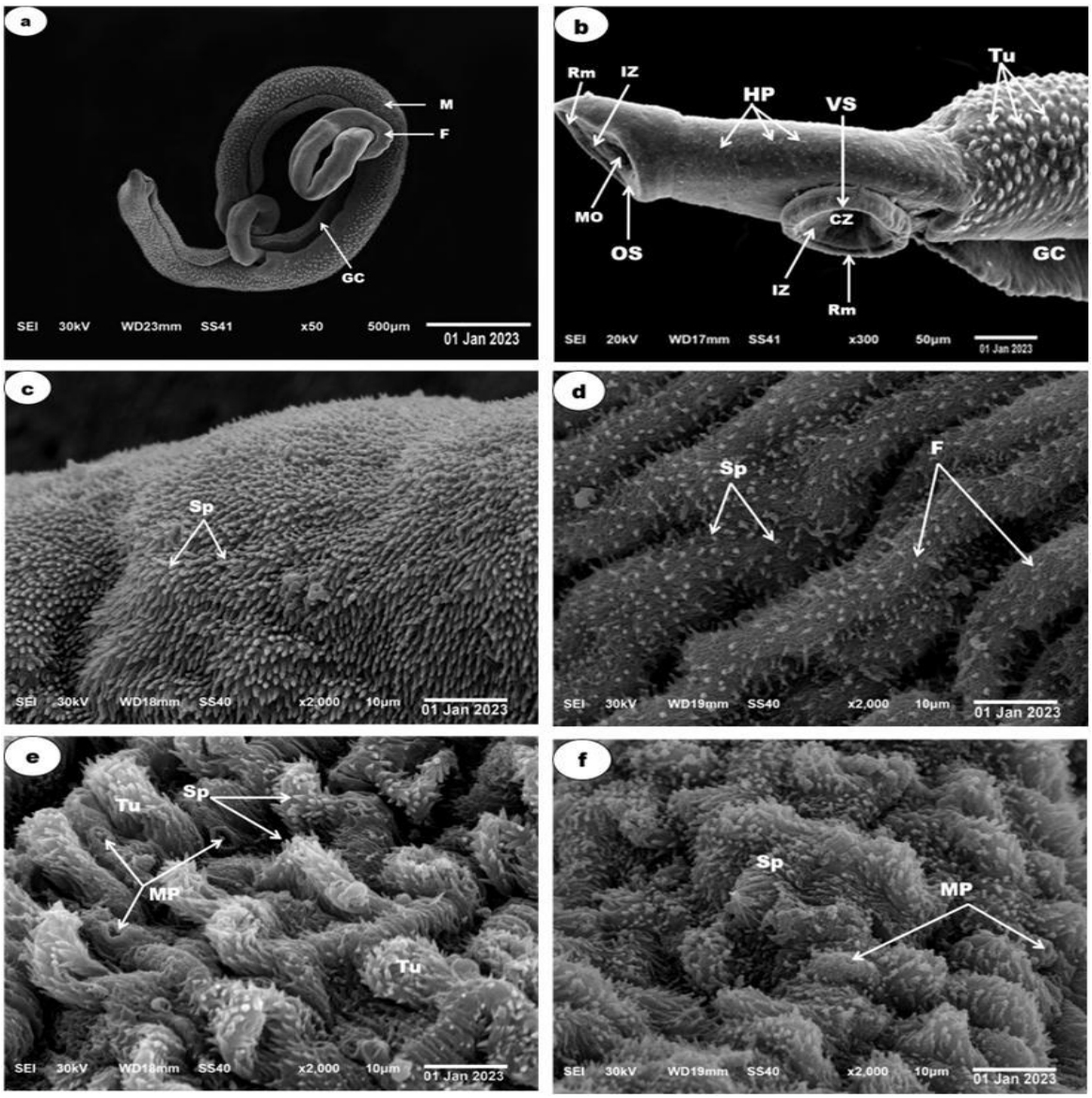

Fig. 2. Scanning electron micrographs (SEM) of the male adult worms of *Schistosoma mansoni* from the control group showing: **(a)** Copulation with female in the gynechophoric canal of male, **(b)** The anterior region of the body with an oval-shaped oral sucker having three notable regions (rim, inner zone, and the mouth), a round-shaped ventral sucker distinguished into three regions (rim, inner zone, and central zone), and hemispherical papillae on the tegument between the two suckers, as well as the beginning of the middle region of the body with gynechophoric canal and tubercles, **(c)** The outer margin of the gynechophoric canal with tiny spines, **(d)** Inner surface of the gynechophoric canal with folds and several spines, **(e)** Sponge-like tegument in the middle part of the body of adult male covered with tubercles armed with apically directed spines and appearance of macule papillae on the wrinkled tegument around them, and **(f)** Tegumental surface of the body posterior region with spiny tubercles and macule papillae between them. (M) Male, (F) Female, (GC) Gynechophoric canal, (OS) Oral sucker, (VS) Ventral sucker, (Mo) Mouth, (Rm) Rim, (IZ) Inner zone, (CZ) Central zone, (HP) Hemispherical papillae, (Tu) Tubercles, (Sp) Spines, (F) Folds, and (MP) Macule papillae

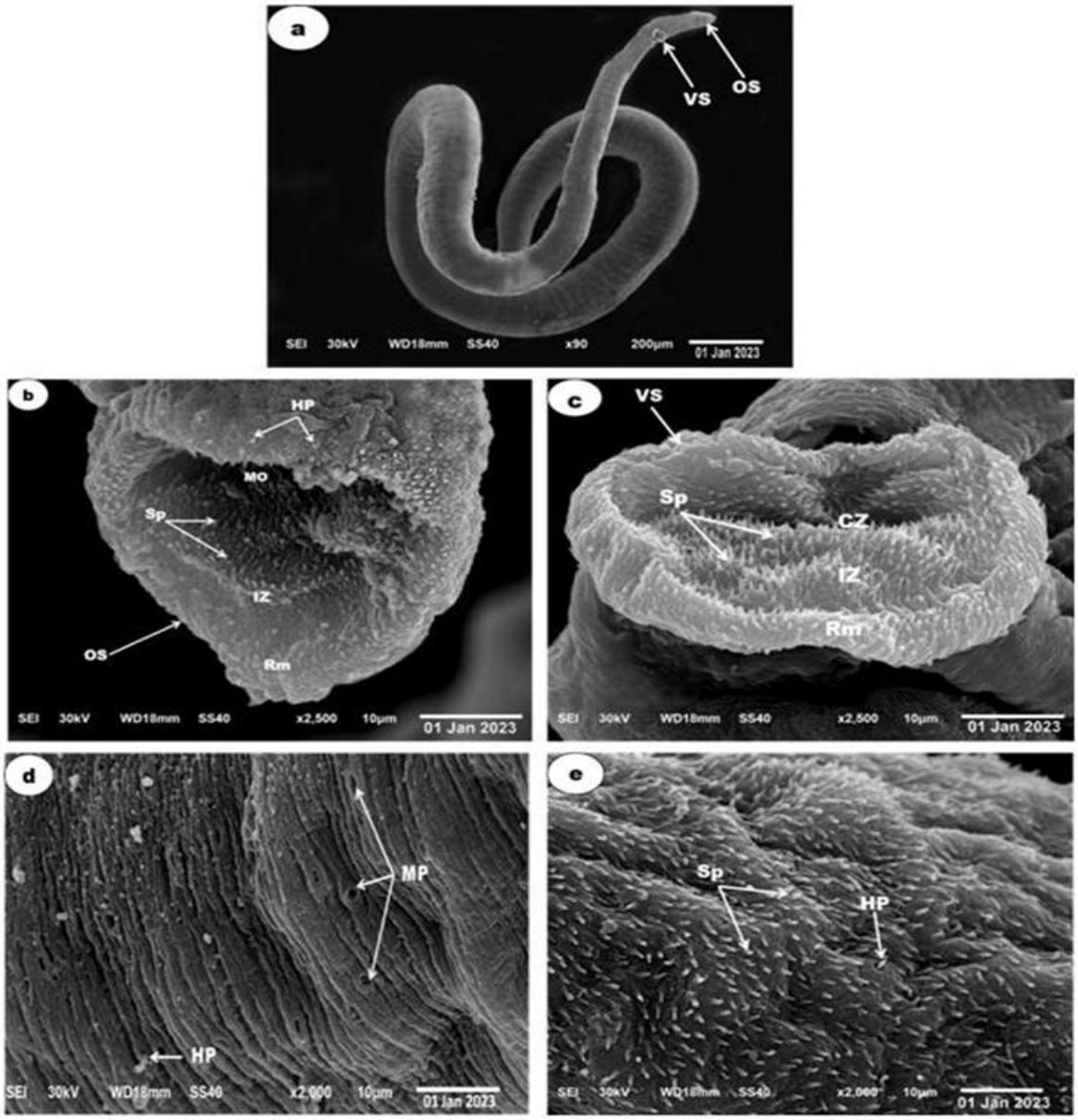

Fig. 3. Scanning electron micrographs (SEM) of the female adult worms of *Schistosoma mansoni* from the control group showing: **(a)** Cylindrical and elongated body of female worm with oral and ventral suckers, **(b)** Magnification of triangular-shaped oral sucker with three regions: thin rim, inner zone, and mouth and presence of numerous spines oriented towards the mouth cavity and hemispherical papillae surrounding the oral sucker, **(c)** Magnification of circular-shaped ventral sucker with three regions: rim, inner zone, and central zone and presence of several spines with consistent shape and size, **(d)** An intact tegument of the dorsal surface with hemispherical and macule papillae, and **(e)** Tegumental surface of the posterior part of the body with numerous spines and hemispherical papilla. (OS) Oral sucker, (VS) Ventral sucker, (Sp) Spines, (Mo) Mouth, (Rm) Rim, (IZ) Inner zone, (HP) Hemispherical papillae, (CZ) Central zone, and (MP) Macule papillae

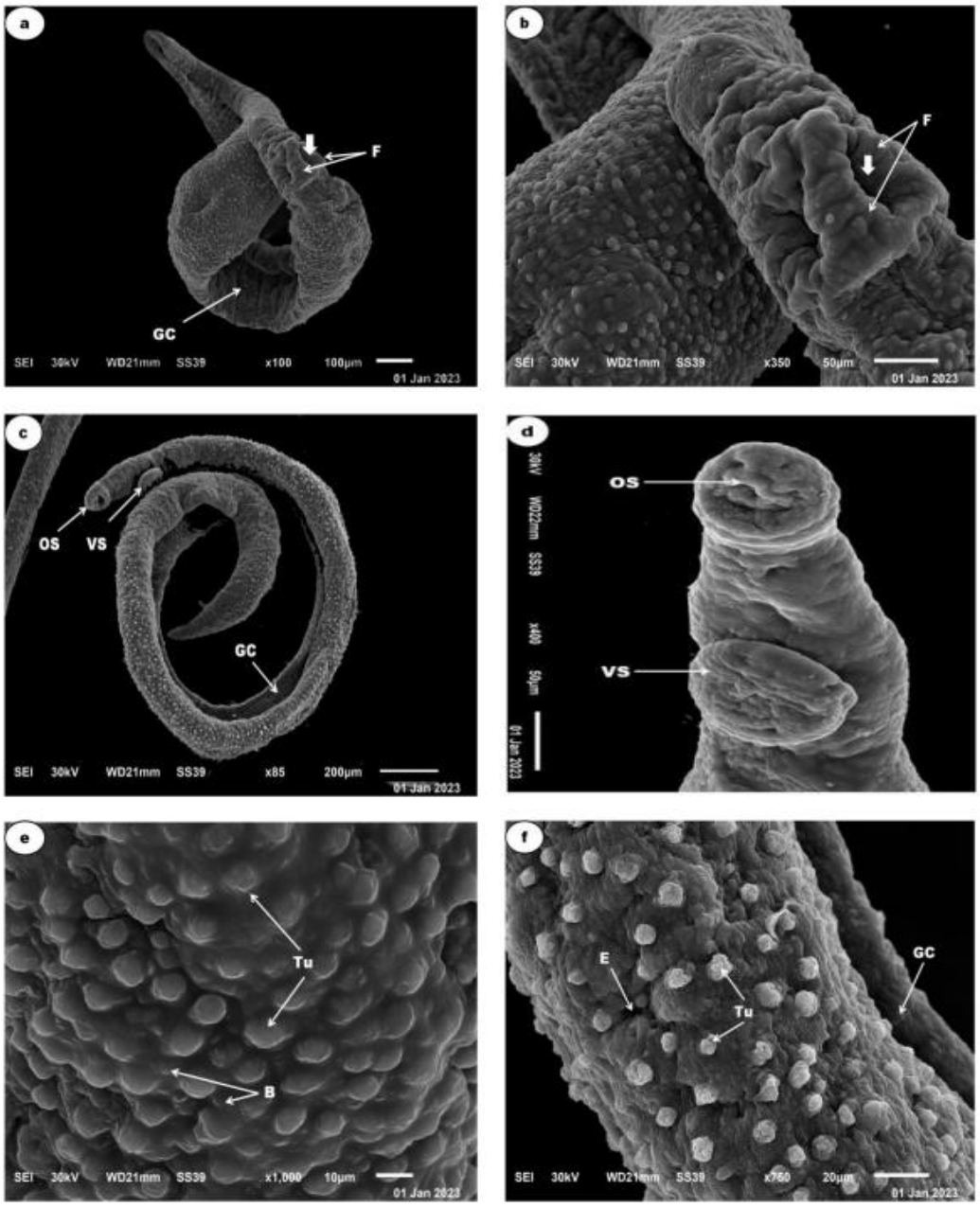

Fig. 4. Scanning electron micrographs (SEM) of the male adult worms of *Schistosoma mansoni* after 48h of *in vitro* treatment with the methanolic crude *Hyrtios* sponge extract (HSE) at 100µg/ ml showing: **(a- b)** Intensive contraction of the swelling body, including the gynechophoric canal and severe tegumental shrinkage with an obvious two folds including an invagination on the anterior part of the dorsal side, **(c)** Self-coiling phenomenon and deformation of gynechophoric canal, **(d)** Severe swelling of oral and ventral suckers, **(e)** Completely naked swollen tubercles and presence of blebs between them, and **(f)** Collapsed tubercles and tegumental erosion. (White wide arrow) Invagination, (F) Folds, (GC) Gynechophoric canal, (OS) Oral sucker, (VS) Ventral sucker, (Tu) Tubercles, (B) Blebs, and (E) Erosion

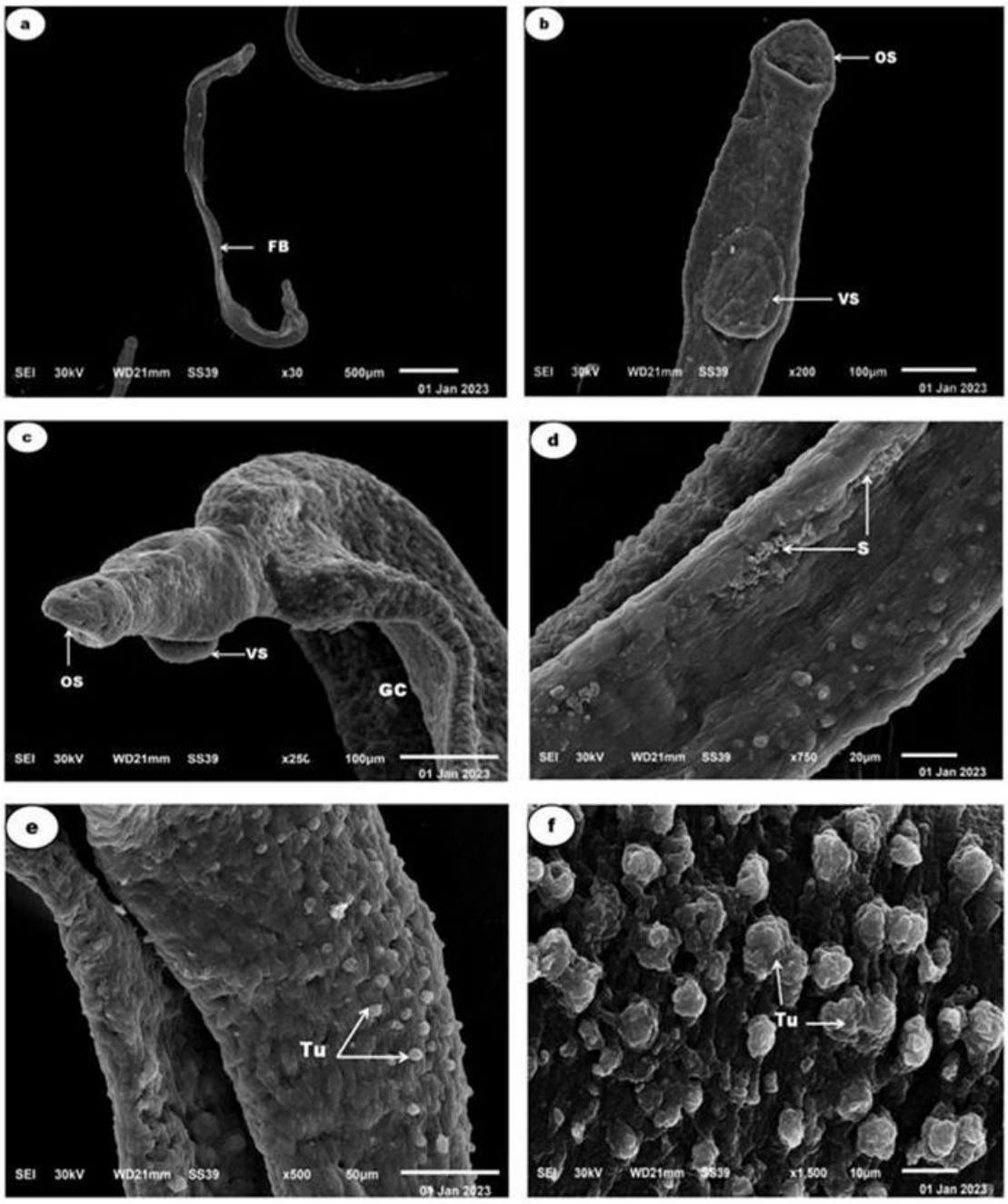

Fig. 5. Scanning electron micrographs (SEM) of the male adult worms of *Schistosoma mansoni* after 48h of *in vitro* treatment with the methanolic crude *Hyrtios* sponge extract (HSE) at 200 µg/ml showing: **(a)** Flattened body with loss of tubercles and spines, **(b)** Marked distortion of the suckers; oral and ventral suckers, **(c)** Male worm body with oral and ventral suckers and deformation of gynechophoric canal, **(d)** Lateral side of the gynechophoric canal with tegumental sloughing, and **(e- f)** Presence of collapsed and ruptured tubercles arranged in irregular rows and have no spines. (FB) Flat body, (OS) Oral sucker, (VS) Ventral sucker, (GC) Gynechophoric canal, (S) Sloughing, and (Tu) Tubercles.

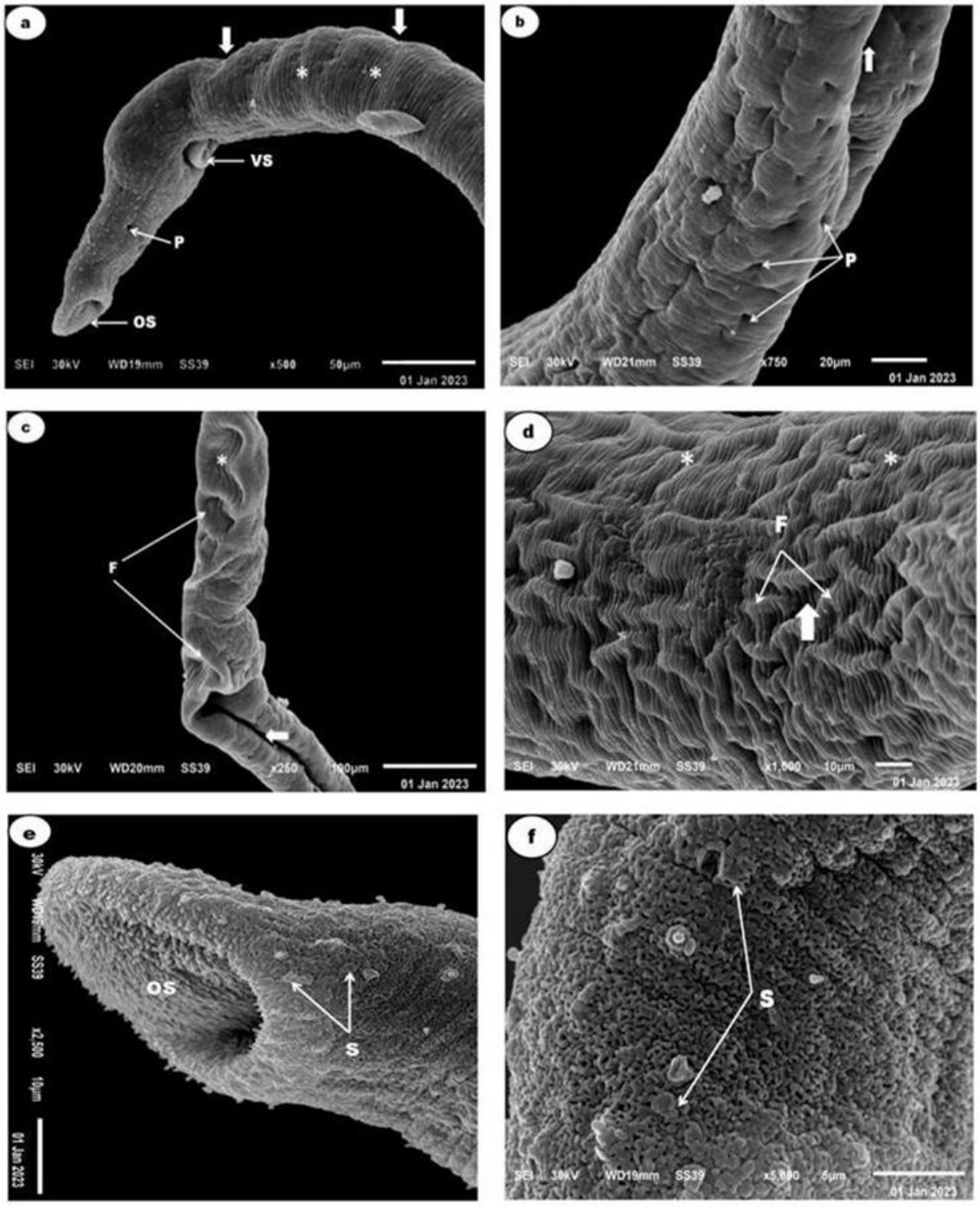

Fig. 6. Scanning electron micrographs (SEM) of the female adult worms of *Schistosoma mansoni* after 48h of *in vitro* treatment with the methanolic crude *Hyrtios* sponge extract (HSE) at 100 µg/ml showing: **(a- b)** Female body with oral sucker, shrinkage of ventral sucker, and tegumental pits with corrugation and invaginations, **(c- d)** Corrugation of the tegument with tegumental folds and invaginations, **(e)** Oral sucker surrounding by tegumental sloughing, and **(f)** Body with tegumental sloughing. (OS) Oral sucker, (VS) Ventral sucker, (Asterisk) Corrugation of the tegument, (White wide arrow) Invagination, (P) Pits, (F) Folds, and (S) Sloughing

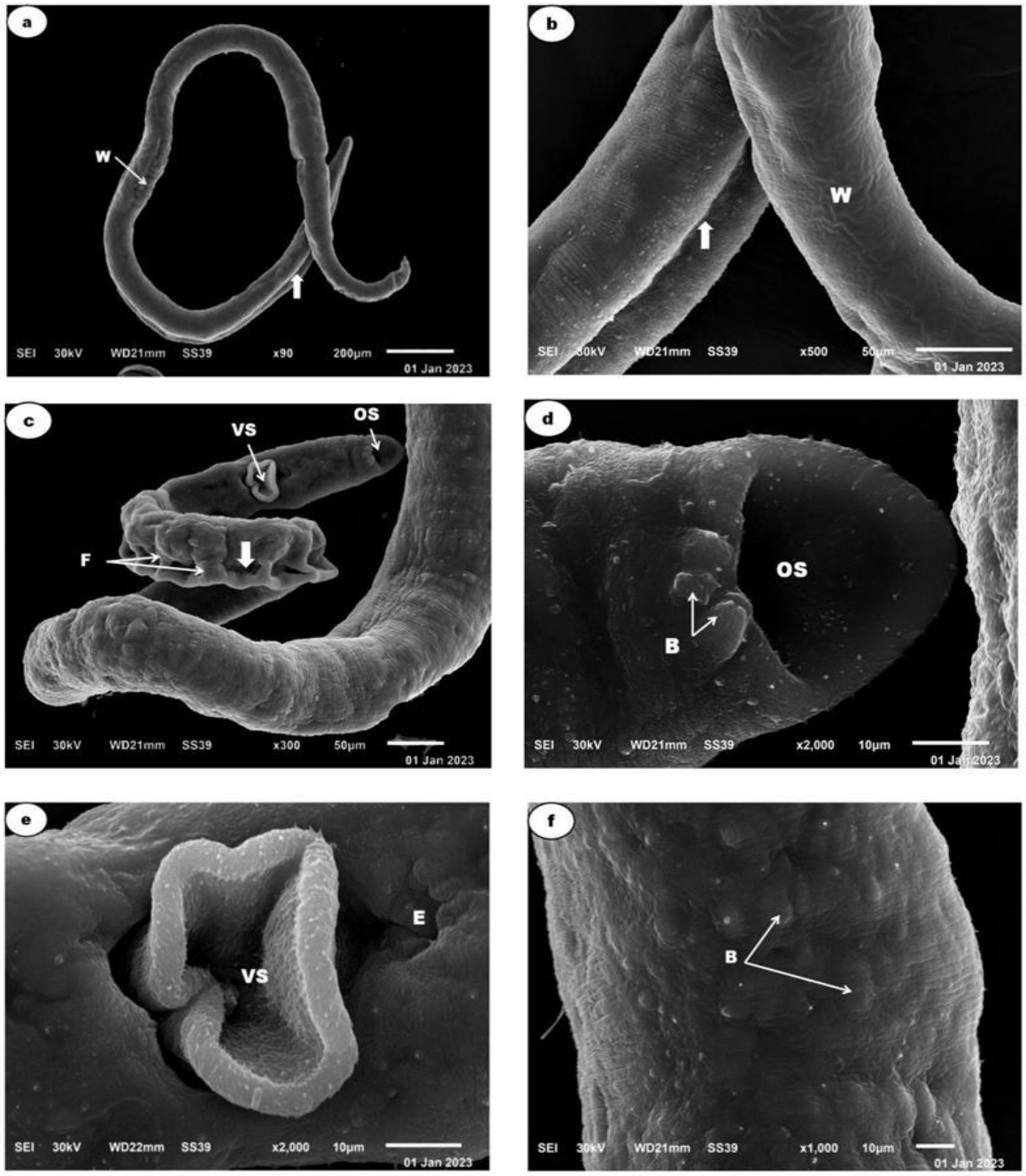

Fig. 7. Scanning electron micrographs (SEM) of the female adult worms of *Schistosoma mansoni* after 48h of *in vitro* treatment with the methanolic crude *Hyrtios* sponge extract (HSE) at 200µg/ ml showing: **(a- b)** Swelling of the whole body with wrinkles and longitudinal invagination, **(c)** Swelling of the whole body along with abnormal oral sucker, shrinkage of ventral sucker, tegumental folds, and surface invaginations, **(d)** Blebs around the oral sucker, **(e)** Marked shrinkage in ventral sucker with pulling it inside and tegumental erosion, and **(f)** Smooth tegument without spines and presence of blebs. (White wide arrow) Invagination, (W) Wrinkles, (OS) Oral sucker, (VS) Ventral sucker, (F) Folds, (B) Blebs, and (E) Erosion

DISCUSSION

Schistosomiasis is one of the common diseases related to using water sources contaminated by sewage and unsanitary practices. PZQ is the only drug that has been used against schistosomiasis for decades, but regrettably, an emergence of PZQ-resistant Schistosomes was recorded **(Melman *et al.*, 2009; Wang, 2012; Shaaban *et al.*, 2019).** Additionally, PZQ exhibits genotoxicity, mutagenic effects, and inadequate efficacy against juvenile forms **(Vale *et al.*, 2017)**. Therefore, efforts are focused on developing different approaches to treat schistosomiasis alongside PZQ **(Lago *et al.*, 2019)**. Natural marine products have a great interest as possible sources of new drugs for schistosomiasis **(Mona *et al.*, 2012; Stein *et al.*, 2015; Abd-Allah *et al.*, 2022)**. Among marine animals, sponges recorded a high rank in the priority of natural products research due to identifying a variety of bioactive chemical components and secondary metabolites with possible medicinal uses **(Perdicaris *et al.*, 2013)**. *Hyrtios* marine sponge has different chemicals with a broad spectrum of antiparasitic activities, such as antitrypanosomal **(Shady *et al.*, 2018)** and antiplasmodial activities **(Mahfur *et al.*, 2022)**. However, this study is considered the first one to assess the *in vitro* activity of the methanolic extract prepared from *Hyrtios* sponge (HSE) of adult worms of *S. mansoni* by estimating the mortality and observing the tegumental alterations.

The present study recorded different phytochemicals in HSE with a high level of alkaloids content, followed by saponins, phenolics, and flavonoids, as reported in the previous studies **(Salmoun *et al.*, 2002; Utkina, 2009; Youssef *et al.*, 2013; Wardany & Nirmala, 2023)**. This finding is confirmed by the estimation of the antioxidant capacity of this extract, which was 101.70mg AAE/ g of extract, as these phytochemicals are well-known antioxidant secondary metabolites possessing free radical scavenging activity through their redox potentials that enable them to function as reducing agents, through donation of hydrogen and singlet oxygen quenching **(Francis *et al.*, 2002; Ozsoy *et al.*, 2009; Formagio *et al.*, 2014; Zou *et al.*, 2016)**.

The *in vitro* test is a helpful tool to investigate the antischistosomal effect of novel drugs. It is considered an initial step to understand its mechanism of action against *Schistosoma* **(Doenhoff *et al.*, 2009)**. The current findings show that the mortality rates of adult worms of *S. mansoni* increased with the duration of exposure to HSE and occurred in a dose-dependent manner. The positive control group exposed to 0.2µg/ ml of PZQ demonstrated a significant increase in worm mortality, scoring 100% death of adult worms after 3h of incubation. The antischistosomal mechanism of PZQ is based on embedding itself into the outer membrane of the worm tegument and reacting with it, thus resulting in a lipid phase transition followed by a destabilizing effect on the membrane and cutting it dramatically **(Abou El-Nour & Fadladdin, 2021)**.

At the same time, the mortality rates of adult worms of *S. mansoni* were more than 50% after 2 and 3h of exposure to HSE at 200µg/ ml, after 24h of exposure to HSE at 100 and 200µg/ ml, and after 36 and 48h of exposing to HSE at concentrations ranging from 60 to 200µg/ ml. Moreover, it achieved 100% mortality at 200µg/ ml after 48h. The values of LC_{50} and LC_{90} of HSE against the *S. mansoni* adult worms were 115.40 and 241.19µg/ ml, respectively, after 24h of exposure time, and they were 59.36 and 144.11µg/ ml after 48h of exposure time, respectively. The antischistosomal efficiency of HSE is considered better when compared with the findings observed in previous studies

on different types of extracts prepared from natural sources. **Reda *et al.* (2016)** recorded that values of LC_{50} and LC_{90} of *Olea europaea* leaves extract were 121.4 and 436.17µg/ ml, respectively, after 24h of incubation of *S. mansoni* adult worms with this extract. **Tekwu *et al.* (2017)** reported that all adult worms exposed to 250µg/ ml of *Rauwolfia vomitoria* root and stem bark died within 120h of incubation. **Nkondo *et al.* (2022)** found that the value of LC_{50} of the hydroethanolic extract of *Pedilanthus tithymaloides* stem bark was 731.17µg/ ml after 48h of *S. mansoni* adult worms exposure to this extract.

The development of new antischistosomal drugs requires sufficient knowledge of *Schistosoma* tegumental constituents since it functions as an intermediary between the parasite body and the host's intravascular environment **(El-Shabasy *et al.*, 2015; Kamel & El-Shinnawy, 2015)**. The tegument also has a role in regulating the osmotic balance, metabolite secretion, and nutrition intake **(Reda *et al.*, 2016)**. Changes in the worm tegument lead to impairment in the immunological efficiency of the worms, and thus, it becomes easy for the host's immune system to attack **(Xiao *et al.*, 2000, 2002)**. Furthermore, the tegumental alterations cause the worms to be unable to adhere to host blood vessel walls, move by the bloodstream from mesenteric veins till they reach intravenous hepatic capillaries, and become trapped in the liver, causing disintegration and death of the worms **(Gnanasekar *et al.*, 2009; Riad *et al.*, 2009)**. Therefore, the schistosomicidal activity of HSE in the present study was supported by the SEM study to assess its effect on the *S. mansoni* tegument.

The present study showed that the structural characteristics of male and female *S. mansoni* in the control group remained unchanged and are consistent with those found in previous studies **(Aboueldahab & Elhussieny, 2016; Ellakany *et al.*, 2019; Reda *et al.*, 2019; El-Morsy *et al.*, 2021)**. Spines are widely dispersed on both oral and ventral suckers, gynaecophoric canal, as well as along the tegumental surface of *S. mansoni* male worms and some parts of the body of female worms.

Concerning the ultrastructural findings of the *S. mansoni* tegument in response to HSE, the results showed that the male and female worms exhibited obvious modifications in the structure of the suckers and tegument after 48h of incubation with a methanolic extract of *Hyrtios* sponge in comparison to the negative control group. Among all tested HSE concentrations, 100 and 200µg/ ml concentrations were selected for the SEM study since they recorded the highest mortality rates of adult worms. The ultramorphological alterations in mature worms were visible in these two concentrations. The morphological changes in the male tegument included intensive contraction of the body with a self-coiling phenomenon, swelling or flattening of the body, severe shrinkage and invaginations in the worm body, deformation of the gynechophoric canal, marked distortion of suckers, extensive tegumental erosion and sloughing, and clear appearance of damaged smooth tubercles with blebs between them. At the same time, the treated females of *S. mansoni* with HSE suffered from the swelling of the whole body, severe shrinkage of the ventral sucker, tegumental pits, and other tegumental changes in the form of folds with invaginations, corrugation, wrinkling, erosions, and sloughing. Furthermore, the tegumental blebs with the absence of spines were recorded.

These alterations impede the worm's attachment to the blood vessel tissues and thus affect feeding. The denaturation of the ventral and oral suckers and tubercle's spines could result in an inability to adhere to blood vessel tissues. Thus, they would struggle to acquire nutrients from the blood efficiently. Additionally, the worm's ability to select an

appropriate attachment site during feeding is impacted by the elimination of the sensory papillae on the oral sucker **(Reda *et al.*, 2019)**. Additionally, tegumental damage may affect the tegumental functions, causing damage to the defensive mechanism in the worm, and therefore, it could be easily targeted by the host's immune system **(Xiao *et al.*, 2000; Kamel & El-Shinnawy, 2015; Shaaban *et al.*, 2019)**.

These findings are consistent with the comparable tegumental damages of *in vitro* and *in vivo* previous studies on adult worms of *S. mansoni* under exposure to natural antischistosomal extracts. **Abdella *et al.* (2024)** demonstrated that by incubating adult *S. mansoni* worms with ethanolic extracts of *Artemisia annua* at different concentrations for 24h, the worms showed contractions, atrophic changes, peeling of tubercles, i.e., tubercles became swollen with complete loss of spines, and sucker's alteration or destruction. Moreover, **Aboueldahab and Elhussieny (2016)**, **Hassan *et al.* (2016)**, **Shaaban *et al.* (2019)**, **Abou El-Nour and Fadladdin (2021)** and **El-Morsy *et al.* (2021)** coincided with these changes.

It is worth mentioning that the antischistosomal activity of HSE and its potency to cause morphological alterations in the body of adult *S. mansoni* worms may be attributed to the different phytochemical compounds in it, including alkaloids, saponins, phenolics, and flavonoids. *In vitro* exposure to countless alkaloids displays antischistosomal activity either by decreasing motor activity, separating all paired worms, or tegumental sloughing, causing death to worms **(Neves *et al.*, 2015)**. Saponins, triterpene glycosides, are potential natural compounds having hemolytic and anthelmintic activities based on either adhesive defense or toxic mechanisms **(Melek *et al.*, 2012; Shalaby *et al.*, 2016; Bahrami *et al.*, 2018)**. Flavonoid and phenolic compounds have been reported to exhibit pronounced anthelmintic activity against nematode worms **(Garcia-Bustos *et al.*, 2019)** and the *Opisthorchis felineus* liver flukes **(Mordvinov *et al.*, 2021)**, respectively. Moreover, flavonoids can cause a notable decrease in motor activity or pairing of the adult *S. mansoni* worms **(Neves *et al.*, 2015)**.

CONCLUSION

Given the obtained results, the current work concluded that the HSE has an effective *in vitro* antischistosomal impact against adult *S. mansoni* worms causing death and tegumental alterations. Thus, it can be considered a promising adjuvant medication for PZQ in *S. mansoni* infection. However, further *in vivo* studies are needed to examine its effect on the liver tissues of the infected mice.

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ARABIC SUMMARY

التقييم المختبري للتأثير المضاد للبلهارسيا لمستخلص إسفنج *Hyrtios* sp. البحرى ضد ديدان *Schistosoma mansoni* البالغة

سمير أحمد ابراهيم العباسى, دنيا نعيم على الهوارى, جيهان محمود الخضرى, أمل زكى غنيم, سلوى عبد الفتاح الصعيدى *

قسم علم الحيوان- كلية العلوم- جامعة دمنهور- مصر.

يعد داء البلهارسيا من الأمراض الوبائية المنتشرة في مصر. نتيجة للاستخدام الحصري والمتكرر للبرازيكوانتل في علاج داء البلهارسيا، قد يصبح الطفيلي مقاومًا للدواء. وبناءً على ذلك، اهتمت الأبحاث بتطوير أدوية جديدة مضادة للبلهارسيا من مصادر طبيعية. يحتوي إسفنج *هيرتيوس* البحري على مركبات نشطة بيولوجيًا من فئات مختلفة تؤهله لاستكشاف أدوية جديدة. ومن هنا هدفت الدراسة الحالية لتقييم النشاط المختبري لمستخلص إسفنج *هيرتيوس* (HSE) ضد داء البلهارسيا من خلال الكشف عن الوفيات والتغيرات فى جليد الديدان البالغة لل*شيستوسوما مانسونى*. كانت معدلات وفيات ديدان *شيستوسوما مانسونى* أكثر من 50% بعد ساعتين و3 ساعات عند تركيز 200 ميكروجرام/مل من HSE، و بعد 24 ساعة عند 100 و200 ميكروجرام/مل، و بعد 36 و48 ساعة عند تركيزات تتراوح بين 60 إلى 200 ميكروجرام/مل. علاوة على ذلك، بعد 48 ساعة، وصل معدل الوفيات إلى 100% عند جرعة 200 ميكروجرام/مل. بعد تعريض ديدان *شيستوسوما مانسونى* إلى HSE لمدة 24 ساعة، كانت قيم التركيزات المميتة (LC_{50} و LC_{90}) 115.40 و 241.19 ميكروجرام/مل، على التوالي. وكانت 59.36 و144.11 ميكروجرام/مل بعد 48 ساعة من وقت التعرض، على التوالي. كشف الفحص المجهري الإلكتروني عن تغيرات شكلية في كلا من الديدان الذكرية والأنثوية بعد المعالجة المختبرية لديدان *شيستوسوما مانسونى* باستخدام HSE عند تركيزات 100 و200 ميكروجرام/مل لمدة 48 ساعة، مقارنةً بالمجموعة الضابطة. تضمنت التشوهات المورفولوجية في ذكور الديدان أجسامًا منتفخة أو مسطحة، و متقلصة حول نفسها، مع وجود انكماش شديد وانغمادات فى جليد الدودة، وتشوه قناة الإحتضان، و وجود ممصات مشوه، وتآكل و انسلاخ الجليد، ووجود درنات ناعمة مع ظهور انتفاخات فيما بينها. أظهر جسم الديدان الأنثوية تغيرات شكلية، بما في ذلك تورم الجسم بالكامل، وانكماش شديد للممص البطنى، و ظهور جليد منقور مع تمويجات، وطيات، وانغمادات، وتجاعيد، وتآكل، و انسلاخ، و فقاعات، و عدم وجود أشواك. يمكن أن يرتبط موت ديدان *شيستوسوما مانسونى* الناجم عن تعرضها ل HSE بتأثيره على سطح الجليد المغلف لها و الذى قد يكون مرتبطًا بوجود مواد كيميائية نباتية مختلفة مضادة للأكسدة مثل القلويدات والصابونين والفينولات والفلافونويدات في هذا المستخلص. في الختام، HSE له تأثير فعال ضد البلهارسيا ويمكن اعتباره دواء مساعد للبرازيكوانتل في عدوى *شيستوسوما مانسونى*.