Acta Biologica Hungarica 67(3), pp. 247–260 (2016)
DOI: 10.1556/018.67.2016.3.3

SCHISTOSOMA MANSONI: ANTIPARASITIC EFFECTS OF ORALLY ADMINISTERED *NIGELLA SATIVA* OIL AND/OR *CHROOCOCCUS TURGIDUS* EXTRACT

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(Received: January 12, 2016; accepted: March 11, 2016)

Schistosoma mansoni is one of the parasites causing schistosomiasis, a disease which threatens millions of people all over the world. Traditional chemical drugs are not fully effective against schistosomaisis due to the evolving drug resistant worm strains, so exploring new remedies derived from natural products is a good way to fight schistosomiasis. In the present investigation two natural products, *Nigella sativa* oil and *Chroococcus turgidus* extract were used separately or in a combination to explore their effect on *S. mansoni*. The infected mice treated with *Chroococcus turgidus* extract or/and sativa seed oil showed a significant decrease in the total worm burden. The total number of deposited eggs by females of *S. mansoni* was significantly decreased in the liver of mice treated with *Chroococcus turgidus* extract or/and sativa seed oil. However, in the intestine, the number of eggs was significantly reduced in mice treated with algal extract and those treated with both algal extract and oil. Fecundity of female *S. mansoni* showed a significant decrease from mice treated with algal extract or/and sativa seed oil. According to SEM investigations the tegmental surface, oral and ventral suckers of worms also showed considerable changes; as the tubercles lost their spines, some are swollen and torn out. The suckers become edematous and enlarged while the tegmental surface is damaged due to the treatment with *Chroococcus turgidus* extract or/and sativa seed oil. In conclusion, the *Nigella* sativa oil and *Chroococcus turgidus* extract are promising natural compounds that can be used in fighting schistosomiasis.

Keywords: Black seed – *Chroococcus turgidus* – Cyanophyte – Electron microscopy – *Schistosoma*

INTRODUCTION

Schistosomiasis is one of the most common human parasitic diseases in the world, and the second most important parasitic infection after malaria in terms of public health and economic impact [9] of which 207 million people in the developing countries and with 779 million, mostly children, are at risk of the infection in 76 tropical and subtropical countries, especially in Africa, Asia and Latin America. *Schistosoma mansoni* (causing one type of intestinal Shistosomes) represents the dominant species in America [18, 22, 56]. Schistosomiasis is still one of the most prevalent epidemic diseases in Egypt like in many other developing countries, regardless of the many

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efforts to control this parasitic infection [17]. Schistosomiasis mostly affects the liver and intestine with granuloma formation; necrosis and fibrosis are more common in hepatic tissues [16]. Praziquantel (PZQ), a drug used for treatment of schistosomiasis, was developed by Seubert et al. [52]. Praziquantel and Oxamniquine have been extensively used in the treatment of schistosomiasis with good efficiency and low toxicity [32]. But PZQ is not effective in the treatment of immature forms or preventing reinfection [33, 37, 62]. Meanwhile, the repeated use of oxamniquine and PZQ in endemic areas has resulted in the appearance of drug-resistant Schistosoma strains [29, 41, 64].

In the last few years, there has been an increase in using anti-parasitic drugs of natural sources, especially from plants [13, 14, 36]. Accordingly, it is important to explore new effective natural compounds in a trail to treat and prevent schistosomiasis [27, 46]. Although different plant products throughout the world have been used in traditional medicine for the treatment of human helminthes [26, 27] and schistosomiasis [1, 28, 50, 51], only a few plant derivatives have been screened for activity against adult *Schistosoma mansoni* [28, 34]. *Nigella sativa* oil is one of the promising drugs of a plant origin that have an anti-Schistosoma effect [2, 44, 58]. Cyanophytes (blue–green algae or cyanobacteria) are among the oldest photoautotrophic prokaryotes. These microscopic, widespread algae inhabit all kinds of ecosystems [7]. They can evolve oxygenic photosynthesis, and they could change the Earth's atmosphere from anoxic to oxic [49]. Genetically, cyanophytes are a very diverse group of organisms with a wide range of physiological and biochemical characteristics. These organisms can live in complex habitats submitted to very extreme conditions. To enable rapid adaptation to new environmental conditions, cyanophytes produce a great variety of highly interesting bioactive secondary metabolites that cannot be found in other organisms [8]. These secondary metabolites include polysaccharides, polyphenols, tannins, enzymatic and non enzymatic antioxidants, pigments, vitamins, fatty acids, immuno-diagnostic agents, therapeutics, nitrogenous compounds and cyclic poly-ethers [3, 54]. Currently, cyanophytes extracts exhibit exciting biological activities ranging from immunosuppressant, antibiotics, anticancer, antiviral, anti-inflammatory agents and more. The variability of bioactivities pointed to the pharmaceutical potential of cyanobacteria [24, 38, 60]. It is well-known that, cyanophytes possess economical advantages over higher plants and seaweeds not only for their easier culture without organic substrates, but also because they don't compete as a human food [30]. Recent studies reported that, terrestrial (edaphic) cyanophytes represent renewable sources of different biologically active metabolites [38], studies on the anti-helminthic and anti-parasitic activities of cyanophytes (particularly soil chroococcales members) are missing. Moreover many studies were performed to test the anti-parasitic activity of some macro-algal extracts [20]. On the basis of anti-parasitic activity of medicinal plants and natural products, the aim of the present study was to test the anti-Schistosoma activity of *Nigella sativa* oil and/or the terrestrial *Chroococcus turgidus* (Kutz.) Nag. extracts as a novel oral treatments against schistosomiasis *mansoni* in mice.

MATERIALS AND METHODS

Blue-green alga treatment

Isolation, purification and identification of the cyanophyte taxon

In this work, *Chroococcus turgidus* was isolated from the reclaimed sandy soil of West of El-Bohyrat Almora (Egypt). The soil sample was collected in clean air-tight plastic bags from the surface strata down to a depth of about 17 cm [21]. This soil sample was brought immediately to laboratory. For culturing, isolation and purification of algae, sterilized liquid and solid blue green BG-11 media were used. Under aseptic conditions, about 0.1 g from the investigated sandy soil was inoculated in 50 ml of sterilized liquid medium. This was incubated at 25 °C under continuous shaking and continuous florescent, white daylight with intensity of 4000 lux. After 15 days, microscopic examination; identification and uni-algal isolation were done using different serial dilution on sterilized solid media (20 g agar-agar/one liter of liquid medium). Axenic culture of the investigated uni-algal isolate was obtained by repeating of uni-algal sub-culturing together with changing in pH (range from 4–8) of the previously used medium using NaOH and HCl buffer solution. pH of growth medium was measured by the laboratory pH meter (Knick digital pH meter, model: 643). When the algal growth of the tested taxon started to appear (after about one week), it was washed three times with sterilized distilled water. Finally microscopic examination was conducted by Bacterial Gram-Staining method and was applied three times for each inoculums to detect the presence or absence of bacteria (whatever dead or alive) in the nutritive culture. The studied cyano-algal taxon was photographically documented using an Olympus DP SOFT fitted with a Canon Powershot G12 digital camera.

Preparation of micro-algal extract

One hundred milligrams of the algal sample was extracted successively with 60 ml of methanol at room temperature. The extract was then clarified by centrifugation and the pellet was re-extracted twice with the same solvent. The supernatants were then pooled and filtered. The solvent was then removed from the filtrate by rotary evaporation and the dry crude extract was kept at 25 °C and protected under an atmosphere of nitrogen gas until use [23].

Experimental design

Forty male albino CD1 mice (20–30 gm) were supplied by the Schistosoma Biological Supply Program (SBSP) at Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Experiments were also performed at TBRI. All procedures contributing to this work

comply with legal ethical guidelines of the Medical Ethics Committee of the TBRI on the care and use of laboratory animals.

Mice were divided into four groups, 10 mice in each group. They were put in large plastic cages (50 × 30 × 23 cm); food and water were supplied daily. Mice were infected with *S. mansoni* cercariae. The first group was non-treated (control), the second group was treated with pure black seed *(Nigella sativa)* oil only, the third group was treated with *Chroococcus turgidus* extract only and the fourth group was treated with black seed oil and blue-green algal extract together. Mice were dissected by the end of the 7th week of the experiment (counted from the day of infection).

Mice infection and treatment

Mice and *S. mansoni* cercariae were supplied by SBSP. Mice were infected with 50–70 *S. mansoni* cercariae via subcutaneous route. Cercariae were suspended in 0.1 ml distilled water. Infected mice were then treated orally day after day from the fifth-week till the end of the 7th week post-infection with *Chroococcus turgidus* extract at a dose of 25 µl/mouse (25 µl extract + 75 µl distilled water) and/or *N. sativa* oil at a dose of 250 µl/kg body weight. The black seed oil was suspended in sun oil (75 µl) before oral administration to mice. The oral administration of *C. turgidus* extract and *N. sativa* oil was performed by a stomach tube. Black-seed oil is obtainable from drug stores in the form of gelatin capsules with the name of "Baraka" (Pharco Pharmaceuticals, Alexandria, Egypt). *Chroococcus turgidus* extract was supplied from Department of Botany, Faculty of Science, Ain Shams University, Cairo, Egypt.

Worm recovery

Worms were recovered from the hepatic portal system and mesenteric blood vessels of infected mice at 7-weeks post infection. Worm recovery was performed by perfusion technique according to the method of Clegg and Smithers [12]. A perfusion apparatus (Filamatic®; National Instrument Co., Baltimore, MD) was used to obtain adult worms. The worms were examined and counted (total number of male and female worms) under a binocular microscope. After counting, worms were fixed in a 3% glutaraldehyde on ice for scanning electron microscopy.

Egg count

Small parts of liver and intestine (ileum) of infected mice were incubated over night in 4% potassium hydroxide (KOH) as described by Cheever [11], and then centrifuged at 200 *g* for 10 min. Twenty five milliliters of saline was added to the pellet, then 25 µl from each sample was placed in a flat bottom plate (Coaster) for counting

the eggs in liver and intestine. Total egg count in liver and intestine of infected mice can be calculated by knowing the weights of the liver and the intestine.

Fecundity

By knowing the total egg count in liver and intestine in addition to the number of female worms from the perfusion process in each mouse, fecundity of the female worms can be easily calculated according to the following formula:

$$\text{Fecundity of a female} = TE/TFW,$$

where TE is the total number of eggs in liver and intestine; and TFW is the total number of female worms

Electron microscopy

Adult worms of *S. mansoni* (7 weeks old) were fixed in 3% glutaraldehyde in sodium cacodylate buffer for 2 h then post-fixed in 1% osmium tetroxide. Worms were then dehydrated with ethanol and at critical point dryer. Specimens were mounted on metal stubs, coated with carbon and gold then examined with a JEOL JEM-1200 EXII electron microscope at the Department of Zoology, Faculty of Science, Ain Shams University, Cairo, Egypt.

Statistical analysis

All values were tested for normality. Student's unpaired *t*-test and the Mann-Whitney test were used to analyse the statistical significance of differences between mean experimental and control values, and a P value of ≤ 0.05 was considered significant.

RESULTS

Algal description

Mass growth of the studied cyanophyte (blue-green algae) taxon appeared after two weeks from the incubation time. This was identified according to monograph of Desikachary [15] as: *Chroococcus turgidus* (Kutz.) Nag. It is belonging to Kingdom: Monera, Division: Cyanophyta, Class: Cyanophyceae, Order: Chroococcales and Family: Chroococcaceae. Its cells and colony are microscopic, coccoied spherical and single or in groups of mostly 2–4 cells. Colonies are 8–32 μm in diameter with colorless sheath (Figs 1A and B). They became pure and axenic at pH = 5.

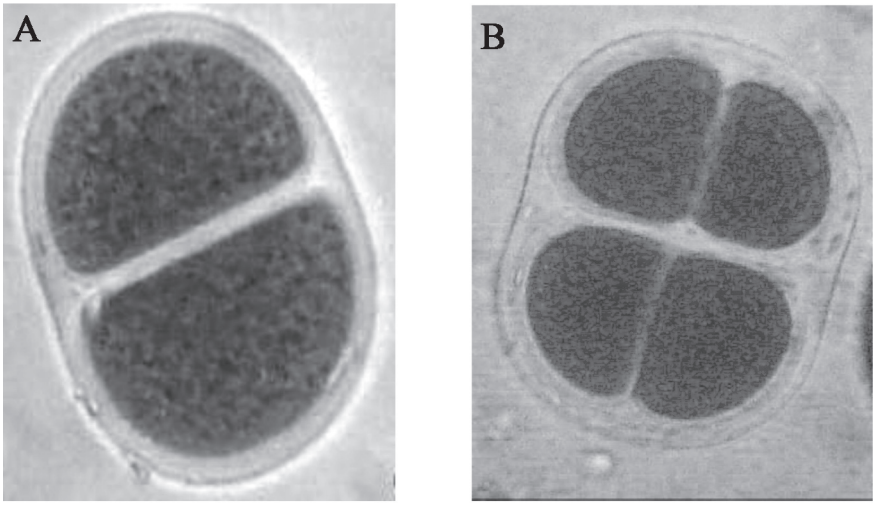

Fig. 1. Chroococcus turgidus (Kutz.): (**A**) two-cells stage, (**B**) four-cells stage (×450)

Worm reduction

Schistosoma mansoni infected mice treated with *Chroococcus turgidus* or sativa seed oil showed a significant decrease in the total worm burden ($P<0.05$), with 45% and 57% total worm reduction in *Chroococcus turgidus* or sativa seed oil treated groups, respectively. Forty seven percent was the total worm reduction in the case of combined treatment (*Chroococcus turgidus* and sativa seed oil together) (Table 1). On the other hand, statistically non-significant ($P>0.05$) reductions ranging between 21%, and 41% were observed in female worm burdens in different treated groups compared to the control group (Table 1), while sativa oil treated groups showed a significant reduction in male worm burden ($P<0.05$) by 64%.

Table 1
Worm reduction in infected CD1 mice treated with multiple doses of algae or/and sativa seed oil

Mice groups	Total worms	Red. (%)	*P*	Female worms	Red. (%)	*P*	Male worms	Red. (%)	*P*
Infected control	24.67±3.79	–	–	7.67±1.16	–	–	16.33±4.16	–	–
Infected treated with algae	13.50±0.71	45	0.029*	5.50±0.71	28	0.104	8.00±1.41	52	0.079
Infected treated with oil	10.50±3.54	57	0.024*	4.50±2.12	41	0.111	6.00±1.41	64	0.048*
Infected treated with algae + oil	13.00±1.41	47	0.028*	6.00±1.41	21	0.240	8.50±3.54	49	0.119

Red. (%): percent of reduction and *P:* P-value.

Tissue egg load

Significant reductions *(P* < 0.05) of 56% followed by 65% and 74% in number of eggs in liver tissue per gram were observed from mice treated with algal extract, or those treated with oil, or those treated with both algal extract and oil. In addition, significant reductions ($P < 0.05$) of 47% and 62% in number of eggs per gram tissue of small intestine were observed in groups from mice treated with algal extract and those treated with both algal extract and oil. On the other hand, a non-significant reduction ($P > 0.05$) of 44% in small intestine egg load per gram was observed in mice treated with oil only. Worm fecundity showed significant reductions ($P < 0.05$) by 32% and 22% in mice treated with algal extract, and mice treated with oil respectively, while mice treated with both algal extract and oil showed a highly significant reduction ($P < 0.01$) in worm fecundity (60%) compared to the infected control group (Table 2).

Table 2
Tissue egg load and fecundity reductions

Mice groups	Liver	Red. (%)	*P*	intestine	Red. (%)	*P*	Fec.	Red. (%)	*P*
Infected control	340±53	–	–	236±22.2	–	–	73.3±5.3	–	–
Infected treated with algae	149±16.4	56	0.02*	126±33.9	47	0.021*	50.1±3.8	32	0.014*
Infected treated with oil	119±28.9	65	0.017*	131±72.1	44	0.086	56.6±4.2	22	0.035*
Infected treated with algae + oil	90±14.1	74	0.011*	90±42.4	62	0.014*	29.7±2.4	60	0.002**

Red. (%): percent of reduction and Fec.: fecundity.

Ultrastructural examination

Scanning electron microscopy revealed that the dorsal surface of *S. mansoni* male worms recovered from infected control group had intact tegument bearing large numerous tubercles with evenly distributed spines. Intertubercular surface was composed of circular folding with minute sensory papillae (Fig. 2A). Oral and ventral suckers were obvious; they were rounded to oval in shape (Fig. 2B).

In the majority of male worms obtained from mice treated with *Chroococcus turgidus* extract, the most frequent feature was a slight change in the aspect and form of the tubercles which appeared reduced in some instances, accompanied with the loss of some of their spines, and, in addition, there was shrinkage and wrinkling in the areas between the tubercles (Fig. 2C).

In addition to distortion of oral and ventral suckers of some worms (Fig. 2D), the tubercles of the male worms obtained from sativa seed oil-treated mice were heavily wrinkled and collapsed, being short and blunt. Also, swellings and pronounced

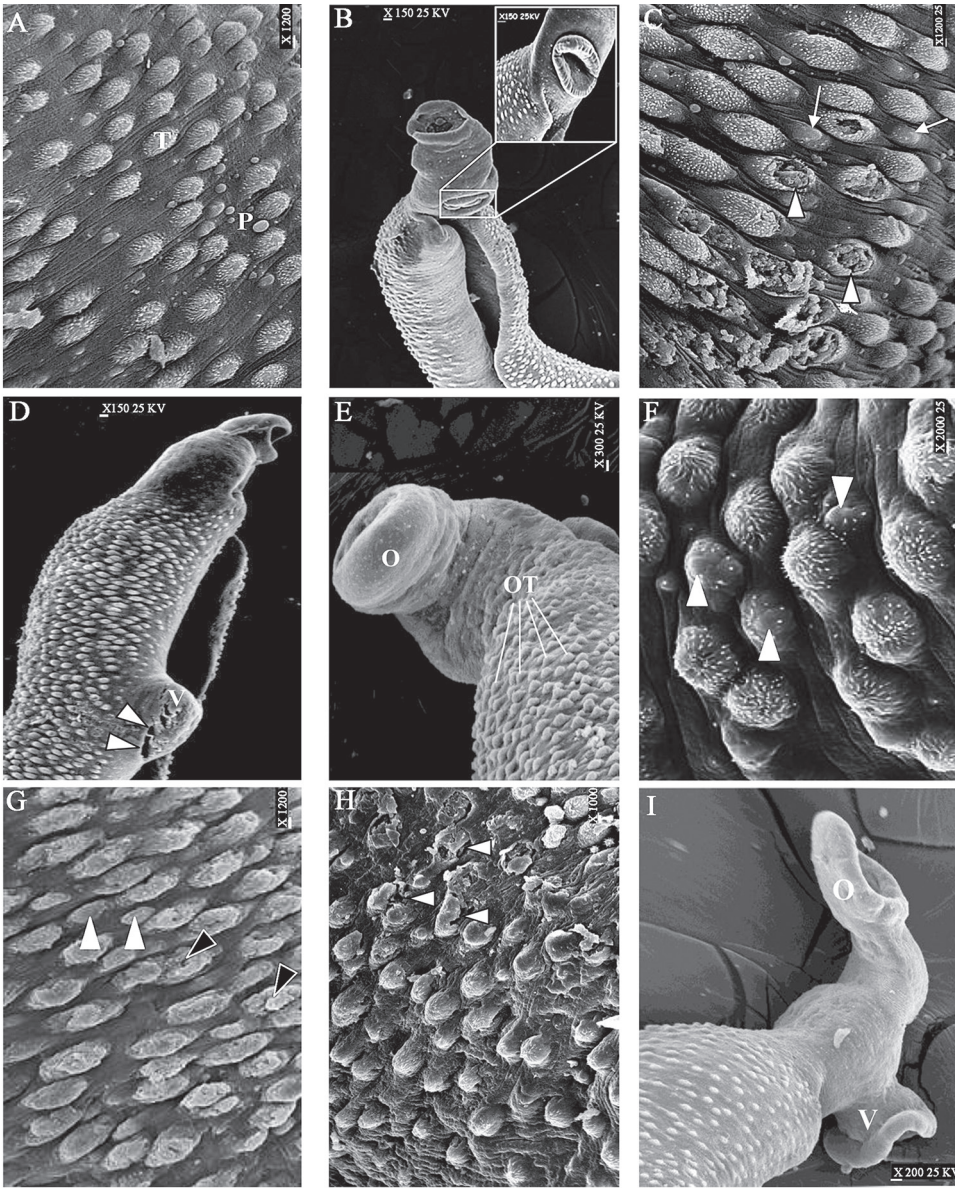

Fig. 2. Electro-micrographs showing the tegument of *Schistosoma mansoni* (untreated or treated with seed oil or/and algal extract); **(A)** Tegument of untreated worms has numerous tubercles (T) with evenly dispersed spines. Inter-tegmental areas have circular folding with minute sensory papillae (P). (×1200); **(B)** Oral (O) and ventral suckers of untreated worms with normal architecture; they were rounded to oval in shape. Inset showing an enlarged ventral sucker. (×150); **(C)** Tegmental surface of worms treated with algal extract, some tubercles appeared small (arrows), some were torn out, however lost some of their spines (arrow heads). Shrinkage and wrinkling in the areas between the tubercles was observed. (×1200);

(D) Distortion of oral (O) and ventral (V) suckers with some torn (arrow heads) in worms treated with seed oil. (×150); **(E)** Inter-tegmental areas of worms treated with seed oil have swellings and pronounced oedema. The oedema also involved the tubercles (OT), which appeared thickened with raised knobs. Sever dilation and oedema in the oral sucker (O) was also observed. (×300); **(F, G)** tegmental surface of worms treated with both seed oil and algal extract; oedematous tegument with tubercles lost their spines (white arrow heads) and appeared torn at their tips (black arrow heads) (Fig. F ×2000, Fig. G ×1200); **(H)** Intertubercular areas showed extensive surface swellings with damage of tubercles (arrow heads) and with disruption of tegmental surface architecture (×1000); **(I)** A distinct deformation and dilation of the oral (O) and ventral (V) suckers; loss of spines was also recorded. (×200)

oedema were noted in the inter tegmental areas. The oedema also involved the tubercles, which appeared thickened with raised knobs (Fig. 2E). Severe dilation and oedema in the oral sucker with loss of its spines was also observed (Fig. 2E).

While combined treatment with both sativa seed oil algal extract led to a pronounced change in the tegument, that appeared oedematous with tubercles lost their spines and appeared torn at their tips (Fig. 2F, G). Tubercles and inter tubercular areas showed extensive surface swellings and lesions with disruption of the sensory pores (Fig. 2H). A pronounced deformation and dilation of the oral and ventral suckers with loss of spines was also recorded (Fig. 2I).

DISCUSSION

In the present work, the total worm burden was reduced from 45 to 57%, which is more or less similar to that found by Mostafa et al. [47]. However, the reduction of the total worm burden is 31–39% after treatment with arachidonic acid [18] which is lower than that of the present study. While the percentage of egg reduction was 56–74% in the liver which is much more if compared to egg reduction in that treated with ginger [47]. A higher number of males were recovered from mice if compared to female worms obtained from the same mice (Table 1). Several authors tried to explain the higher male proportion in *Schistosoma*-experimentally infected animals [6], but Boissier and Moné [4] found that male–female interactions could play an important role in the establishment of higher male proportion. Boissier and Moné [5] supposed that in a very permissive host, a lot of genotypes of the parasites could develop, inducing a strong competition between females and thus an increase in the male proportion. Another explanation is that males are larger in size with larger suckers, so males could be easily fit inside the blood capillaries; so the number of males is more than that of females.

Mahmoud et al. [35] observed that the black seed oil was efficient in reducing *Schistosoma mansoni* worms' number in the liver, it also decreased the number of ova deposited in both liver and intestine. It increased the number of dead ova in the wall of the intestine, as well. Administration of black seed oil concomitantly with an antihelminthic drug lowered further the number of dead ova, indicating that the oil potentiates the action of the drug. Moreover, in this investigation, the effect of combination

of both black seed oil and *Chroococcus turgidus* extract lead to a higher reduction of eggs in both the liver and intestine, so it is concluded that, the addition of another compound to the sativa oil may enhance its effect. Mohamed et al. [43] used the natural product of blue green algae, alone or combined with praziquantel PZQ as a therapeutic agent on *Schistosoma mansoni* infected mice. Mansour et al. [38], Viviana and Vitor [60] and Mukund et al. [48] reported that, *Chroococcus turgidus* contained a good amount of active metabolites with higher antiviral (against rabies virus), antimicrobial and antioxidant potential. However, gas chromatography-mass spectroscopy (GC-MS) analysis of *Chroococcus turgidus* methanol extract [3] revealed a list of volatile organic compounds such as 1,4-Benzenedicarboxylic acid, bis (2-methylpropyl) ester, 1,2,3-Benzenetricarboxylic acid, 1,2-Benzenedicarboxylic acid, butyl cyclohexyl ester, Ethyliso-allocholate 16-Octadecenoic acid, methyl ester which all are commonly used as strong antioxidants and antimicrobial biologically active molecules. These antioxidant compounds can be used as promising effective protecting agents against various diseases [43].

In the present study, the most prominent effects of the black seed oil and the *Chroococcus turgidus* extract were the reduction of worm burden and reduction of deposited eggs as well as the reduction of fecundity in females. These observations were also reported by Utzinger et al. [61], Suleiman et al. [57] and Mati et al. [39]. They found, the reduction in the worm recovery and egg density in treated mice was considered as a strong indication of the effectiveness of anti-Schistosoma drugs. Moreover Mohamed et al. [43] suggested that blue green algae can be considered as promising for development of a complementary and/or alternative medicine against schistosomiasis.

The current study also revealed that the tegument of male *Schistosoma* worms, treated with black seed oil or *Chroococcus turgidus* extract, led to deformities in the tegmental appearance, as tubercles were wrinkled and collapsed, some loss in spines, dilation and oedema in the oral sucker. In addition, some worms had a distorted ventral sucker. However, the combined treatment led to more obvious effects on the tegmental appearance as more deformation and spine loss were observed; the inter tubercular areas had extensive surface swellings and lesions with disruption of the sensory pores and much dilation of the oral and ventral suckers was observed. Mehlhorn et al. [41] reported that male worms use their tubercles and spines in holding to the wall of the blood vessels. In view of the fact that the treatment with black seed oil and/or algal extract induced damage to these structures, worms can be easily drifted in the blood stream. The damage happened to the suckers must result in a loss of ability of worms to adhere to blood vessels rendering ingestion of nutrients from the blood more difficult. The damage to the tegument along the worm's body would have impaired the functioning of the tegument and also destroyed the defense system of the worm, so that it could easily be attacked by the host's immune system [63]. Kusel et al. [31] reported some of the functions of glycoproteins in the parasite surface acting as a physical or immunochemical barrier against the host's immune system. Therefore, the tegmental alteration in *S. mansoni* worms after treatment with black seed oil and/or algal extract could have exerted an intense effect on the meta-

bolic activities of the parasite and in turn expose the tegument to the host's immune attack. Changes in the surface topography of *Schistosoma* worms were used by a number of investigators for the assessment of anti-Schistosoma drug actions, as the tegument is an important target for anti-Schistosoma drugs [18, 25, 46, 47]. Moreover, the change caused by anti-Schistosoma drugs was more distinct in the male tegument than in that of the female, where most of the female's body is enclosed in the gynaecophoric duct of the male and it is not in direct contact with the host's microenvironment [53, 55].

In conclusion, the present investigation opens the way to use different natural products in different combinations and at different concentrations to test their effects against *Schistosoma* worms. Soil algae derivatives may be considered as new interesting natural sources of functional ingredients that exhibit exciting biological activities. Further studies are needed to reach the good combination of selection and concentration of natural product(s) to effectively fight *Schistosoma*.

ACKNOWLEDGEMENTS

We gratefully thank Dr. Mohamed Salah, Dr. Sohair, Prof. Magdy Khalil and Prof. Shadia Hassan for their help in mice infection, perfusion and in electron microscope work.

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