

*Full Length Research Paper*

# ***In vitro* effect and scanning electron microscopic changes of *Nigella sativa* loaded chitosan nanoparticles on *Schistosoma mansoni* adult**

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**Nanoparticles can act as drug carriers that can modulate pharmacokinetics, increase bioavailability and target release with minimal toxic effects. The present work aimed to assess the therapeutic effect and electron microscopic changes of *Nigella sativa* loaded Chitosan Nanoparticles (NSLCN) on adult *Schistosoma mansoni* *in vitro*. Adult worms were removed from the portal and mesenteric veins of infected mice after 90 days, and then three to five mature worms including both sexes were cultured. *Schistosoma* adult was exposed to NSLCN at concentrations of (10, 20, 40, 60, 80, and 100 µg/ml) for 24, and 48 h. Examination for worm viability was done after 24, and 48 h using a stereomicroscope comparing with control negative and control positive groups. The mortality rate in worms reached 88.9% in the group treated with 100 µg and 80 and 84.6% in groups treated with 80 and 60 µg respectively (p-value <0.001). After 48 h of incubation with the same concentration, there were variable effects on motility and death of worms, the death rate reached 100% in all groups treated with nanoparticles. After 24 h incubation, the live worms have sluggish motility and reached dead score at 48 h of incubation. By (SEM) there were tegumental changes of both dead male and female in the form of loss of spines, swollen suckers and swollen inter tubercular ridges in male and loss of smooth architecture of female tegument with multiple pores. In conclusion, NSLCN appears as a new potential candidate drug against schistosomiasis. We successfully applied nanoemulsion preparation against the adult stage of *S. mansoni* *in vitro*.**

**Key words:** *Schistosoma mansoni*, *Nigella sativa*, chitosan nanoparticles, scanning electron microscope.

## **INTRODUCTION**

Schistosomiasis has been estimated to infect more than 207 million people with 779 million people at risk of infection (Steinmann et al., 2006). Schistosomiasis represents a major public health problem in about 78

tropical and subtropical countries with the majority (up to 90%) of the cases are located mainly in sub-Saharan Africa (WHO, 2013).

Praziquantel is effective against all species of

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*Schistosoma* infecting humans and has been used for the last decades. It is well tolerated, easily administered in tablet form, and cheap (Cioli and Pica-Mattoccia, 2003). However, the development of resistant strains has been reported, leading to schistosomiasis treatment failure that highlighted the importance of developing new and more effective drugs for this disease (De Moraes et al., 2013). As a consequence, in the last years, important efforts have been made in the search for new active compounds against *Schistosoma*, mainly those obtained from plants (Allegretti et al., 2012). Recently promising studies have been developed for the use of natural compounds derived from plant extracts as drugs against *Schistosoma* spp., being safe and with less medical side effects (Parreira et al., 2010).

Abaza (2013) reviewed all herpes that were used in the treatment of schistosomiasis including Chinese medicine, *Carvacrol* (essential oil of *Origanum vulgare* obtained from pepperwort), *Myrrh* (oleo-gum-resin from *Commiphora molmol*), *artemisinin* derivatives isolated from *Artemisia annua*, *curcumin* (*C. longa*), *quinine* lack seeds and *quinidine* (*Cinchona officinalis*), garlic extract (*Allium sativum*), black seed (*Nigella sativa*) and other several native plants from Brazil. The essential oil of *N. sativa* is one of the promising alternative drugs of plant origin that have antischistosomal effects (Mostafa and Soliman, 2002; Mohamed et al., 2005).

Nanoparticles can act as drug carriers that can modulate pharmacokinetics, increase bioavailability and target release with minimal toxic effects (Khalil et al., 2013). In this study, we used chitosan nanoparticles (CS NPs) as it is biodegradable and nontoxic (Yien et al., 2012). Several studies used scanning electron microscopy (SEM) to determine the alterations in the surface topography of *Schistosoma* for the evaluation of several drugs/compounds since the tegument of *Schistosoma* is an important target for such drugs (Jiraungkoorskul et al., 2005). Our study aimed to assess the therapeutic effect and electron microscopic changes of NSLCN on adult *S. mansoni* *in vitro*.

## MATERIALS AND METHODS

The present study was carried out at the *Schistosoma* Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Giza, Egypt and at electron microscope unit, Faculty of Science, Ain Shams University during the period from June 2018 to August 2018.

### Preparation of NSLCN

93% degree of deacetylation, sodium tripolyphosphate, Phosphate buffered saline (PBS) and an acetic acid were purchased from Sigma-Aldrich, USA. *N. sativa* (Baraka) was obtained from Pharco, Egypt. Chitosan nanoparticles (CS NPs) were synthesized via the ionotropic gelation of chitosan with Tripolyphosphate (TPP) anions. TPP has been used to prepare (CS NPs) as it is nontoxic, multivalent and its ability for gel formation via ionic interactions. The

charge density of TPP and chitosan can control this interaction, under the influence of the solution pH. Chitosan was dissolved at various concentrations of an acetic aqueous solution; 1, 2 and 3 mg/ml. The chitosan concentration was 1.5 times lower than that of the acetic acid in aqueous solution. The TPP solution (1 mg/ml) was prepared with double-distilled water. CS NPs were made-up with the drop wise adding about 5 ml of the chitosan solution to 2 ml of TPP solution under 1000 rpm magnetic stirring for 1 h at room temperature. The suspension was performed under the same above-mentioned conditions. Separations of the nanoparticles were done by centrifugation at 20000 g at 14°C for 30 min, and then they were freeze-dried and stored at 4°C. NSLCN was made by adding a chitosan solution to TPP solution (containing *N. sativa* a concentration of 500 mg/ 2 ml). NSLCN were separated from the suspension by centrifugation (20000 g at 14°C) for 30 min. Then, sediment was collected and weighed. The total protein content/mg of chitosan encapsulating powder was calculated by dividing the protein concentration of the loaded *N. sativa* by the nanoparticles weight (Danesh-Bahreini et al., 2011). The loading capacity efficiency of the nanoparticles was determined:

$$\%LC = [(A-B)/C] \times 100$$

A is the total amount of *N. sativa*, B is the free amount of *N. sativa* and C is the weight of nanoparticles.

### Characterization of NSLCN

Their weights were measured, and they were characterized using the transmission electron microscope (TEM) (JEOL 100 CX) at the electron microscope unit, Faculty of Science, Ain Shams University.

### Parasites and culture media

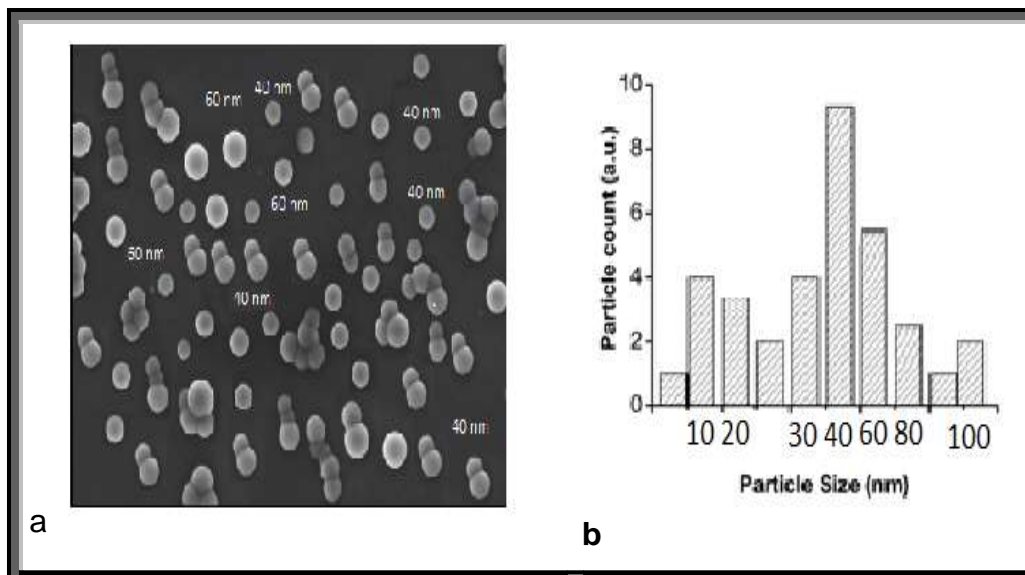
Adult of *S. mansoni* Swiss albino mice CD-1, weighing 18-22 g each, were obtained from SBSC, kept under environmentally-controlled conditions (temperature 25°C; humidity 70%; 12 h light and 12 h dark cycle) and acclimatized for one week before infection. The maintenance and care during the experimentation of animals were compliant with international guidelines for the human use of laboratory animals. Adult worms were removed from the portal and mesenteric veins of infected mice after 90 days (Duvall and Dewitt, 1967) sexed and counted (Xiao et al., 2009). Three to five mature worms including both sexes were cultured per well in 24- well plates containing RPMI medium at 37°C and 5% CO<sub>2</sub>, immediately after animal perfusion to ensure their vitality.

### Evaluation of drug effect on *S. mansoni* adult worms

After *Schistosoma* adult was exposed to NSLCN at concentrations of (10, 20, 40, 60, 80, and 100 µg/ml) for 24, and 48 h. Examination for worm viability was done after 24, and 48 h using a stereomicroscope comparing with control negative (adults incubated with 0.5% DMSO plus culture media) and control positive (worms incubated with 1 µg/ml PZQ plus the culture media) groups. Worms showing no signs of motility for one minute, associated with worm deformity such as blackening, twisting, and contracting were considered dead. The activity of the drug was measured by calculating the number of dead worms relative to the total number of worms. In the case of any doubt about the viability of worms, they were allowed to recover in clean medium and re-examined.

### SEM study

To observe the morphological changes in the suckers and



**Figure 1a, b.** TEM of NSLCN showing regular, rounded shape with a smooth surface. Their mean size was 40 nm.

tegument of the adult parasites, *Schistosoma* was monitored using SEM following the standard procedure as described by Hassan et al. (2003). Adult male worms of *S. mansoni* were collected in glutaraldehyde- buffer solution (25%) as a fixative overnight at 4°C, then washed out of any of the fixative by keeping them overnight at 4°C in phosphate- buffered saline, then passed into rising concentrations of alcohol (30, 40 and 50%) each for 15 min and kept in 70% alcohol until the time of examination. Before the examination, they have washed twice for 30 min in 80 and 90% alcohol respectively. The last wash was for one hour in 100% alcohol. Worms were then mounted on stainless steel holders and put in a drier for about 30 min and then subjected to sputter coat of gold, the different parts of worms were examined using Joel JEM-1200 scanning electron microscope, provided with a camera fitted to it. Areas in the worms that showed specific changes were examined and photographed mainly, suckers and the tubercles on the tegument.

#### Statistical analysis

The collected data were analyzed using SPSS version 16 software, data were presented as number and percentage. Fissure extract test was used to detect the P-value.  $P < 0.05$  was considered significant

#### RESULTS

Nanoparticles characterization by the Transmission electron microscope (TEM), NSLCN were regular, rounded and have a smooth surface. Their mean size was 40 nm (Figure 1a, b). After 24 h incubation, the live worms have sluggish motility and reached dead score at 48 h of incubation (Table 1). There was no statistically significant difference ( $P > 0.05$ ) in its effect on males and females (Table 2). The death rate in worms reached

88.9% in the group treated with 100  $\mu\text{g}$  and 80 and 76.6 in groups treated with 80 and 60  $\mu\text{g}$  respectively ( $P$ -value  $< 0.001$ ) after 24 h of incubation (Table 1) and there were variable effects on motility. After 48 h of incubation with the same concentration and there was the death of all worms to reach 100% in all groups treated with NSLCN. Morphological alterations on the surface of male *Schistosoma* were in the form of worm deformity and swollen suckers. The tegument was swollen in some parts and flattened in other parts with shrinking and furrowing with edematous interpapillary ridges. *Schistosom* from negative control groups showed an intact tegument. The female tegument treated with NSLCN showed marked deformity in the form of wrinkles and furrowing and shrinking as shown in (Figure 2).

#### DISCUSSION

The in vitro test with *Schistosoma* is one of the useful tools to explore the antischistosomal properties of a known effective drug and also helps to analyze the mode of action against *Schistosoma* (Doenhoff et al., 2009). The seeds of *N. sativa* were subjected to a range of pharmacological investigations in recent years. These studies have shown a wide spectrum of activities such as antibacterial (Sasikumar et al., 2011), antitumor (David et al., 1998), anti-inflammatory (Mutabagani and El-Mahdy, 1997), CNS depressant and analgesic (Ramadhan et al., 2011), hypoglycemic (Boseila and Messalam, 2011), smooth muscles relaxant (Aqel and Shaheen, 1996), cytotoxic and immunostimulant (Swamy and Tan, 2000).

Besides, the essential oil was shown to have

**Table 1.** Effect of *N. sativa* loaded chitosan nanoparticles on motor activity of adult *S. mansoni* *in vitro*.

Groups	Time of incubation (h)	Total exam	Normal Score=3	Slow Score=2	Sluggish Score=1	Dead Score=0	FET	P-value
Control -ve		10	0	0	0	0		
Control +ve(treated with pzq)		12	0	0	0	12		
<b>Nano-treated</b>								
100 µg	24	9	0	0	1	8(88.9)	11.92	<0.001**
	48		0	0	0	9	15.2	<0.001**
80 µg	24	10	0	0	2	8(80)	10.21	0.001**
	48		0	0	0	10	16.2	<0.001**
60 µg	24	13	0	0	3	10(76.6)	13.0	<0.001**
	48		0	0	0	13	19.11	<0.001**
40 µg	24	11	0	0	7	4(36.4)	2.44	0.09
	48		0	0	0	11	17.18	<0.001**
20 µg	24	12	0	0	9	3(25)	1.16	0.22
	48		0	0	0	12	18.15	<0.001**
10 µg	24	12	0	0	9	3(25)	1.16	0.22
	48		0	0	0	12	18.15	<0.001**

P-value between total control –ve and treated with nano at different concentrations and different incubation times. FET used to estimate P-value.

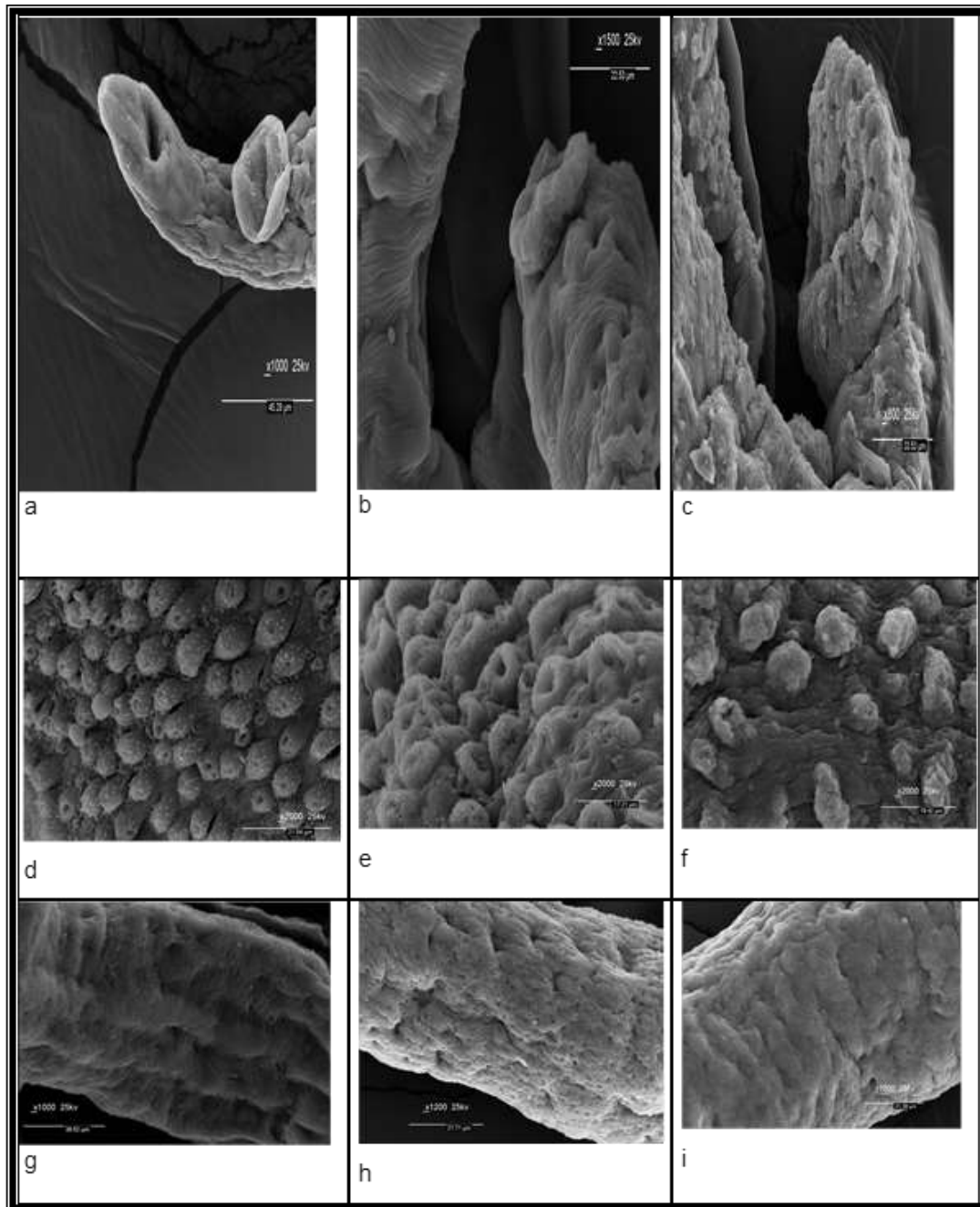
**Table 2.** Effect of *N. sativa* loaded chitosan nanoparticles on male and female.

Groups	No. &% of dead males in relation to total males no.			No. &% of dead females in relation to total females no.			FET	P-value
	Total	Death	Death%	total	death	Death%		
Control -ve	6	0	0	4	0	0		
Control +ve(treated with pzq)	6	6	100	6	6	100		
<b>Nano treated</b>								
100 µg	6	6	100	3	2	66.7	0.14	0.33
80 µg	6	6	100	4	2	50	1.28	0.13
60 µg	7	7	100	6	3	50	1.28	0.13
40 µg	6	2	33.3	5	2	40	0.0	1.0
20 µg	6	1	16.7	6	2	33.3	0.0	1.0
10 µg	6	1	16.7	6	2	33.3	0.0	1.0

antihelminthic activity (Agarwal et al., 1979) and the seeds were effective against cestodes and nematodes (Akhtar and Rifaat, 1991). In the last decades, plant extracts were widely used for the treatment of *Schistosoma* infection (Sparg et al., 2000). However, *N. sativa* seeds essential oil was recently found to have antihelminthic activity against *S. mansoni* infection

(Mahmoud et al., 2002).

Many studies used nanoparticles as vehicles to deliver drugs for the improvement of their therapeutic efficacy (El-Temsahy et al., 2016). In this study, we used chitosan as a drug carrier for *N. sativa* to improve its efficacy. Chitosan is a natural polymer used in nanomedicines, for its attractive characteristics for drug delivery and its



**Figure 2.** Tegumental changes of adult (male and female) *S. mansoni* after NSLCN. SEM showing normal suckers of normal control group (a) tegument of normal control male showing normal tubercles with intact spines (d) smooth tegument of normal control female (g). The group treated with nanoparticles showing partial loss of spines and swelling of the inter-tubercular ridges (e) and swelling of the ventral sucker (b) and marked changes of female tegument (pores, furrows and shrunken tegument (h)). The group treated with PZQ showing complete loss of spines and marked deformity of tubercles in male (f) and edema of female tegument (i) and suckers deformity (c).

formulated nanoparticulate form proved to be effective. Its cationic character and its solubility in aqueous medium have been reported as important properties for the success of this polysaccharide (Grenha et al., 2010). This

study aimed to assess the therapeutic effect and electron microscopic changes of NSLCN on adult *S. mansoni in vitro*.

Our results showed that both male and female

parasites are susceptible to NSLCN. We observed that the *Schistosoma* exposed to NSLCN showed motility changes in the form of sluggish contractions after 24 h of incubation. Furthermore, it caused 100% mortality of parasites at all concentration after 48 h of incubation, and affect male and female but the difference was a statically insignificant difference. These results are in harmony with Mahmoud et al. (2002) who stated that, administration of the black seed essential oil to *S. mansoni* infected mice showed high activity against adult worms.

On the other hand, De Araújo et al. (2007) using nanoemulsion of a new schistosomicidal drug (BphEA) showed that male worms moved slowly at the end of 48 h whereas all the female worms died. The recorded decreased worm motility produced by NSLCN has been described to be as a result of smooth muscle relaxation effect of *N. sativa* (Khazdair, 2015). This agrees with Jahromy et al. (2014) who observed that, *N. sativa* (100 mg/kg) significantly improved the muscle rigidity score starting at the 40th minute, while animals treated with extract (50 mg/kg) had no significant difference with the control group (received water). Moreover, *N. sativa* (200 mg/kg) significantly improved the muscle rigidity score starting at all times measured in comparison with the control group. This is attributed to the improvement of penetration of NSLCN through parasite tegument, a result of increase passage of hydrophilic pits in *Schistosoma* tegument and enhanced diffusion of nanoparticles. This occurs as a result of the increased solubility of the tested product in biological media of the parasite.

Alterations in the surface ultrastructure of *Schistosoma* worms were used by several investigators for the evaluation of antischistosomal drugs (Mostafa, 2005). Drug-induced tegumental changes have been described in *S. mansoni* worms after treatment with a variety of schistosomicidal drugs (Mohamed et al., 2005). It seems likely that the tegumental changes in the worms may be an important aspect of drug activity leading to the death and elimination of worms with the stopping of their egg production (Nosseir et al., 2000).

In this study, there were tegumental changes of both dead male and female in the form of loss of spines, swollen suckers and swollen inter tubercular ridges in male and loss of smooth architecture of female tegument with multiple pores. These results are in agreeing with Ali et al. (2016) who reported that there was edema of tubercles and sever dilatation and swelling of suckers of adult male *Schistosoma* treated with *N. sativa*.

The obtained results are in harmony with that observed by Mostafa and Soliman (2002) in their study of the surface topography of adult worms of *S. mansoni* harbored in albino mice treated with black-seed essential oil; they reported that the tubercles on the dorsal surface of the mature males developed in mice treated with black-seed essential oil from 0 days of infection showed extensive loss of spines. Spines may be partially or completely disappeared in some worms.

Moreover, the size of the tubercles was greatly reduced. The inter-tubercle tegumental regions showed extensive swelling (edema) while the erosion of the surface was observed. Also, Mostafa (2005) found that the surface topography of male worms obtained from mice treated with Sidr honey alone showed extensive loss of spines. The tegument of worms that developed in mice treated with black-seed essential oil showed moderate structural changes, since the tubercles on the dorsal surface of the male showed partial loss of spines. However, the worms developed in mice treated with Sidr honey and black-seed oil together showed the greatest changes they lost their normal surface architecture and erosion of the tegument and spines loss was noted.

Previous studies have identified that when *Schistosoma* exposed to an immune system containing anti-schistosomal antibody, neutrophils, complement and praziquantel (1 µg/ml), the damage to the worm tegument induced by the drug and attachment of neutrophils on the worm surface aggravated the tegument injury which resulted in worm death within 24 h (Xiao et al., 2009). This agrees with Gonçalves et al. (2013) by using SEM, demonstrated the peeling and erosion of the tegumental surface and swelling of spines, both in the collar of spines region and the eventual erosion of the oral sucker after PZQ treatment. These morphological alterations on the surface of the worm are similar to those found in other trematodes, however, surface blebs were not seen. To summarize, we successfully applied NSLCN preparation against the adult stage of *S. mansoni in vitro*. Further study is needed to highlighting its effect *in vivo*. The application of nanotechnology may offer a safe, effective, and cheap treatment.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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