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Praziquantel-encapsulated niosomes against *Schistosoma mansoni* with reduced sensitivity to praziquantel

Niosomas encapsulados en praziquantel contra *Schistosoma mansoni* con sensibilidad reducida al praziquantel

Praziquantel niosomes against tolerant *S. mansoni*

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Introduction: Praziquantel (PZQ) is the only commercially available drug for schistosomiasis. The current shortage of alternative effective drugs and the lack of successful preventive measures enhance its value. The increase in the prevalence of PZQ resistance under sustained drug pressure is, therefore, an upcoming issue.

Objectives: To overcome the tolerance to PZQ using nanotechnology after laboratory induction of a *Schistosoma mansoni* (*S. mansoni*) isolate with reduced sensitivity to the drug during the intramolluscan phase.

Materials and methods: Shedding snails were treated with PZQ doses of 200 mg/kg twice/week, followed by an interval of one week, and then repeated twice in the same manner. The success of inducing reduced sensitivity was confirmed in vitro via the reduction of cercarial response to PZQ regarding their swimming activity and death percentage at different examination times.

Results: Oral treatment with a single PZQ dose of 500 mg/kg in mice infected with cercariae with reduced sensitivity to PZQ revealed a non-significant reduction (35.1%) of total worm burden compared to non-treated control mice. Orally inoculated PZQ-encapsulated niosomes against *S. mansoni* with reduced sensitivity to PZQ successfully regained the pathogen's sensitivity to PZQ, as evidenced by measuring different parameters in comparison to the non-treated infected animals with parasites with reduced sensitivity to PZQ. The mean total worm load was 1.33 ± 0.52 with a statistically significant reduction of 94.09% and complete eradication of male worms. A remarkable increase in the percentage reduction of tissue egg counts in the liver and intestine (97.68% and 98.56% respectively) was obtained associated with a massive increase in dead eggs and complete absence of immature stages.

Conclusion: PZQ-encapsulated niosomes restored the drug sensitivity against laboratory-induced *S. mansoni* adult worms with reduced sensitivity to PZQ.

Key words: *Schistosoma mansoni*; drug resistance; liposomes; praziquantel.

Introducción. El praziquantel (PZQ) es el único fármaco disponible comercialmente para la esquistosomiasis. La escasez actual de medicamentos alternativos eficaces y la falta de medidas preventivas eficaces aumentan su valor. El aumento de la prevalencia de la resistencia al PZQ bajo una presión prolongada del fármaco es, por tanto, un tema emergente.

Objetivos. Superar la tolerancia a PZQ mediante nanotecnología después de la inducción en laboratorio de un aislamiento de *Schistosoma mansoni* (*S. mansoni*) con sensibilidad reducida al fármaco durante la fase intramolusca.

Material y métodos. Los caracoles que liberaban cercarias se trataron con dosis de PZQ de 200 mg / kg dos veces por semana, seguido de un intervalo de una semana, y luego se repitieron dos veces de la misma manera. El éxito de inducir una sensibilidad reducida se confirmó in vitro mediante la reducción de la respuesta de las cercarias al PZQ con respecto a su actividad de natación y el porcentaje de muerte en diferentes momentos de examen.

Resultados. El tratamiento oral con una dosis única de PZQ de 500 mg / kg en ratones infectados con cercarias con sensibilidad reducida a PZQ reveló una reducción no significativa (35,1%) de la carga total de gusanos en comparación con los ratones de control no tratados. Los niosomas encapsulados en PZQ inoculados por vía oral contra *S. mansoni* con sensibilidad reducida a PZQ permitieron reestablecer con éxito la sensibilidad del patógeno a PZQ, como lo demuestra la medición de diferentes parámetros en comparación con los animales infectados no tratados con parásitos con sensibilidad reducida a PZQ. La carga media total de gusanos fue de 1,33 ± 0,52 con una reducción estadísticamente significativa del 94,09% y la erradicación completa de los gusanos machos adultos. Se obtuvo un aumento notable en el porcentaje de reducción del recuento de huevos en tejido en el hígado y el

intestino (97,68% y 98,56% respectivamente) asociado con un aumento masivo de huevos muertos y ausencia total de estadios inmaduros.

Conclusión. Los niosomas encapsulados en PZQ restauraron la sensibilidad al fármaco contra gusanos adultos de *S. mansoni* inducidos en laboratorio con sensibilidad reducida a PZQ.

Palabras clave: *Schistosoma mansoni;* resistência a medicamentos; lipossomas; praziquantel.

Schistosomiasis is a major health problem in tropical and sub-tropical areas. The global health burden of schistosomiasis is estimated at 3.3 million disability-adjusted life years (DALYs), a value similar to that of malaria and tuberculosis (1). Chemotherapy remains the primary intervention for such a disease (2), and since praziquantel (PZQ) is essentially the only drug currently available (3), such a reliance on a single drug for a disease of this magnitude represents a precarious situation, particularly in light of reports concerning schistosome isolates having reduced susceptibility to PZQ in the field (4,5).

Drug resistance depends on the selective pressure of drug exposure (6). Unfortunately, the mechanism of action of PZQ is not yet completely understood (7). Therefore, the mechanism of PZQ tolerance and the methods for overcoming this issue remain unclear. Ways to overcome such a knowledge shortage include generation of laboratory-induced schistosome isolates with reduced sensitivity to PZQ to permit comparison between them and susceptible parasites. Under laboratory conditions, induction of resistance has been achieved via two approaches in either the definitive or intermediate hosts. In the first approach, mice infected with *Schistosoma mansoni* (*S. mansoni*) were initially treated with sub-curative doses of PZQ followed by increasing the dose in animals for several passages in mice/snails to complete the parasite's life cycle (8,9). Alternatively, the second approach involves selecting PZQ-resistant parasites during the asexual stages of the life cycle of the snail intermediate host (10,11). An important mechanism regarding PZQ resistance involves an increase in PZQ efflux by the multidrug resistance (MDR) transporters, in which the glycoprotein transporters such as SmMDR2 and multidrug resistance-like proteins (SmMRP1) are implied to be important (12).

Many strategies, including nanotechnology, have been adopted to increase the effectiveness of the drugs (13). Lipid-based nanoparticles, including liposomes, solid lipid nanoparticles and nanostructured lipid carriers (the second generation of solid lipid nanoparticles), present promising oral drug-delivery candidates for PZQ (14-16). Furthermore, to circumvent MDR, nanotechnology provides an innovative and promising alternative to conventional chemotherapeutics. Successful examples of nanotechnology use in reversing drug resistance and regaining drug activity include studies conducted utilizing antibiotics (17,18), anticancer drugs (19) and antimalarial drugs (20). Recently, the co-delivery of chemotherapeutics and inhibitors of multidrug resistance transporters using lipid-based nanocarriers provided a promising approach for overcoming drug resistance and reducing the potential toxicity of chemotherapeutic drugs in different diseases (21). Niosomes are among the best lipid carriers. They are widely used as alternatives to liposomes and are even preferred over liposomes due to their high chemical stability and low cost. Moreover, niosomes possess other characteristics that make them promising candidates for clinical use (22). They improve the oral bioavailability of drugs, enhance their dissolution rate and protect them from being prematurely degraded/inactivated in addition to their known low toxicity and non-immunogenicity (23). Niosomes are essentially composed of cholesterol and non-ionic surfactant vesicles, in which they trap and retain the aqueous solution of the solute particles. In addition, various ionic amphiphiles are incorporated into their structure to achieve stability by inducing negative or positive charges (24). This unique structure of niosomes allows for the incorporation of hydrophilic drugs into its aqueous core and lipophilic drugs into its membrane bilayer (25). Thus, they were selected in the current work as a carrier system for overcoming reduced sensitivity to PZQ, primarily due to their unique character of surfactant inclusion which sensitizes resistant cells and

inhibits glycoprotein efflux transporters (26). This may aid in expanding the spectrum of niosome-loaded drugs in treating resistant organisms in the future. Therefore, in the present work, the laboratory induction of reduced sensitivity to PZQ in the *S. mansoni* asexual life cycle stages inside a *Biomphalaria alexandrina* snail host was performed; in view of the niosomes' potential as drug susceptibility enhancers, the effect of PZQ-loaded niosomes in mice infected with *S. mansoni* isolate with reduced sensitivity to PZQ was evaluated.

Material and methods

A brief summary of the methodology carried on in this work was presented in figure 1.

Animals and parasites

An Egyptian strain of *S. mansoni* was used in all experiments. Ten Swiss strain albino mice, aged 4-6 weeks and weighing 20-30 g, previously infected with *S. mansoni* were purchased from the Schistosome Biologic Supply Programme (SBSP), Theodor Bilharz Institute (TBI), Giza, Egypt.

In this study, work involving laboratory animals was conducted in accordance with the Egyptian National Animal Welfare Standards and was approved by the Ethics Committee of the Faculty of Medicine, Alexandria University, Egypt (protocol approval number: 020732).

Maintenance of the *S. mansoni* life cycle was conducted among laboratory snails and inbred Swiss albino mice raised and maintained at the Animal Unit, Department of Medical Parasitology, Faculty of Medicine, Alexandria University, Egypt. At seven weeks post infection (p.i.), the mice were administered intraperitoneal cal-heparin (5000 I.U/ ml; 0.1 ml each) and were euthanized by ether; their livers were used as a source of parasite eggs for snail infection (27).

Snail source, maintenance and infection

Four hundred laboratory-bred susceptible *Biomphalaria alexandrina* snails were supplied by SBSP/TBRI, Giza, Egypt, and were used for inducing PZQ reduced sensitivity during the intra-molluscan phase to harvest cercariae for the *in vitro* study and animal infection. Snails were maintained in the Department of Medical Parasitology laboratory, Faculty of Medicine, Alexandria University, Egypt, in transparent plastic aquaria; each contained 50 snails and was maintained at 26-28°C inside an incubator. Each container contained five liters of well-aerated aged dechlorinated tap water (DTW) that was replaced twice a week. Freshly washed lettuce leaves were supplied as snail food every couple of days, and soft chalk was added to all aquaria. Dead snails were regularly removed. Pieces of foam were placed inside the containers for egg deposition (28).

At seven weeks post infection, the purchased mice were sacrificed, and their livers became the source of parasite eggs for snail infection. The collected eggs were exposed to light to stimulate miracidial release. The snails were individually exposed to 8-10 vigorously swimming, freshly hatched miracidia in direct sunlight for 3-4 hours. Afterwards, the snails were kept in the dark and maintained under the conditions previously described (29).

Induction of reduced sensitivity to PZQ in the intra-molluscan phase

Preparation and administration of the drug

To induce reduction in sensitivity to PZQ, 100 g of 99.5% pure PZQ white powder, C19H24N2O2, of molecular weight 312.40606 g/mol was kindly provided by Alexandria Company for Pharmaceuticals & Chemical Industries, Egypt. The drug was incorporated into mouse chow (purchased from the local market) (11). The chow was ground up with calcium carbonate in a 9:1 ratio. The ratio was reconstituted with water until it became pasty. The snails were individually weighed to calculate the drug

dosage for each snail. PZQ was administered in doses of 200 mg/kg twice/week, followed by an interval of one week, and then repeated twice in the same manner (30). The PZQ dose to be administered per kg was incorporated into 100 mg of food (the amount of food administered for each snail). The amount of food provided each day was allowed to be totally ingested by each snail, suggesting that the snails received the entirety of the drugs offered in the ration (10).

The snails infected with *S. mansoni* were checked for cercarial shedding four weeks after infection. Two hundred shedding snails were divided equally into two groups. Group A contained the infected PZQ-unexposed snails used as a source of PZQ-susceptible cercariae.Group B contained the infected PZQ-exposed snails. Both snail groups were maintained in glass containers and DTW was changed every 24 hours.

Cercarial harvest

Biomphalaria alexandrina snails, infected with the Egyptian strain of *S. mansoni* in each group, were used for cercarial harvest to check the cercarial response to PZQ *in vitro* and for evaluating the efficacy of PZQ against adult *S. mansoni* with reduced sensitivity to PZQ in animal models using nanotechnology. Each snail was placed in a 200 ml beaker containing dechlorinated tap water (DTW) at 30°C and was kept under intense illumination at a distance of 50 cm from the light source. After two hours, cercarial suspension was collected. Only 50 µl of the cercarial suspension were stained by Lugol's iodine to facilitate cercarial count under a stereo microscope while the remaining cercarial suspension was used to complete the study (31).

Confirmatory test for cercarial response to PZQ

A stock solution of 0.1% dimethyl sulfoxide (DMSO) in distilled water was used as a drug solvent and a control. Solutions of $5x10^{-6}$ M PZQ in 0.1 % DMSO were prepared and stored for a maximum of two weeks in the refrigerator. The assay was performed

using ordinary glass slides without using a cover slip. Three sets of slides were used for each group. For each set, the number of cercariae was 5–7 in 20 µl of DTW per drop, two drops per slide (36 *S. mansoni* cercariae). The first set of cercariae received no drug, the second received 20 µl of 0.1% DMSO per drop and the third received 20 µl of the PZQ solution per drop. Cercariae were examined under an ordinary microscope every ten minutes for one hour. They were carefully observed and classified as unaffected, affected or dead.

To avoid the slides drying up and the resulting increase in the PZQ concentration, and to stabilize the temperature during the 60-minute examination period, the slides were kept in a plastic box on a thin (3 mm), wet sponge. The temperature of the sponge was adjusted to 28^{o}C by adding warm water (32). This study was performed in triplicate.

Efficacy of PZQ on adult S. mansoni with reduced sensitivity to PZQ using nanotechnology

Drug preparation

PZQ suspension

500 mg of PZQ powder was dissolved in one ml 60% ethanol and then suspended in a phosphate buffer saline (7 ml) to create a total volume of 8 ml. Each mouse was administered a single dose of PZQ (500 mg/kg) as 0.2 ml PZQ suspension (33).

Preparation and characterization of PZQ-encapsulated niosomes:

Preparation of niosomes

Niosomes were prepared via the thin film hydration method; Span 60 and cholesterol in a 7:6 molar ratio were dissolved in 10 ml of chloroform and ethanol mixture (7/3, v/v), as an organic solvent, in a round-bottom flask of a rotator evaporator. Extracting the solvent from the nanodroplets was achieved via evaporation at 55°C in the rotator evaporator under reduced pressure (200 mmHg) for 15 minutes until a thin film was created in the

inner wall of the flask, leading to the formation of nanoparticles by precipitating macromolecules. The dry lipid film was hydrated with 10 ml PBS (pH 7.4), shaken for 15 minutes at low speed at 55 °C and hand shaken for 15 minutes at room temperature to obtain a lipid suspension**.** The particles were then downsized by sonication via a bath-type sonicator operated at a frequency of 55 KHz (kilo Hertz) for 5–10 minutes at 42°C (the transition temperature of the lipid) (34).

Preparation of PZQ-encapsulated niosomes

The same method of niosomes preparation was followed in preparing PZQ-encapsulated niosomes with the addition of 500 mg of PZQ to Span 60 and cholesterol in the first step.

Lyophilization of PZQ-encapsulated niosomes

PZQ-niosomes solutions were placed in 50 ml tubes, frozen in liquid nitrogen and freeze-dried using a vacuum freeze-drying machine at a pressure of 26.5 pascal. The lyophilized particles were then characterized (35).

Characterization of PZQ-encapsulated niosomes

Particle size analyzer

Distributing the size of PZQ-encapsulated niosome particles was determined through laser light scattering on a Beckman coulter particle size analyzer. PZQ-encapsulated niosomes were added to the sample dispersion unit containing the stirrer and were stirred to reduce the aggregation between the PZQ-encapsulated niosomes while the laser obscuration range was maintained at 15–20%. The mean particle size was measured after performing the experiment in triplicate (36).

Transmission electron microscopy (TEM)

The physical size and shape of the prepared PZQ-encapsulated niosomes were determined using a transmission electron microscope. For this purpose, the particle

suspension was diluted 10 times with distilled water and deposited dropwise onto a 400-mesh copper grid coated with carbon film and were allowed to dry in the air before being examined under the microscope (37).

Zeta potential

The zeta potential (a key indicator of colloidal dispersion stability) of PZQ-encapsulated niosomes dispersed in phosphate buffer solution (pH 6.5) was determined via laser Doppler anemometry. The nanovesicle suspension was diluted to 4 ml with a phosphate buffer (pH 6.5). An electric field of 150 millivolts (mV) was applied to observe the electrophoretic velocity of the vesicles. All measurements were made at 25 ºC in triplicate at the same ionic concentration (35).

Determination of encapsulation efficiency of niosomes

The encapsulation efficiency (EE) of PZQ was expressed as a ratio between the PZQ concentration in the niosomes and the concentration of PZQ added to the system. The amount of PZQ in the niosomes was determined by recording the absorbance of the loading solution at λ_{max}= 490 nm (after removing niosomes by centrifugation at 10,000 rpm for 30 minutes). The unloaded PZQ in the supernatant was determined using a spectrophotometer at λ_{max} 490 nm. The EE of niosomes was calculated according to the following equation: $EE = [(D_t-D_u)/D_t] \times 100$, where D_t represents the total amount of drug and D_u represents the amount of free drug (38).

Experimental design

One hundred and sixty laboratory-bred Swiss strain albino mice aged 4-6 weeks and weighing 20-30 g were individually infected with 100 cercariae using the paddling tail-immersion technique based on the method described by Smithers and Terry (39). The mice were divided equally into two main groups: **Group I:** Mice were infected with *S. mansoni* susceptible cercariae from PZQ-unexposed snails (group A) and **Group II:**

mice were infected with *S. mansoni* cercariae with reduced sensitivity to PZQ from PZQ-exposed snails (group B). Each group was further subdivided equally into four main subgroups: **Subgroup a:** infected non-treated mice**; Subgroup b:** infected PZQ-treated mice, in which each animal was inoculated with a single oral dose of 500 mg/kg (in two divided doses on the same day)**; Subgroup c:** infected niosomes-treated mice; and **Subgroup d:** infected PZQ-niosomes-nanoparticles-treated mice. Mice treated with niosomes or PZQ-encapsulated niosomes were inoculated orally with 400 µl single therapy of the prepared suspension divided into two doses on the same day.

Drugs were administrated, against the adult *S. mansoni* stage, 42 days post infection. All mice were perfused from the hepatic and mesenteric vessels 49 days after cercarial challenge (39). The experiment was repeated thrice. Having the average replicate, the data from one of the independent experiments is presented.

The following parameters were done for assessing drugs against PZQ-susceptible and *S. mansoni* adult isolates with reduced sensitivity to PZQ:

Adults worm burden

Recovered worms were counted and sexed under a stereo microscope.

Tissue egg count

Egg counts in both the liver and intestine were performed according to Cheever's technique (40). The mouse liver and intestine were digested by overnight incubation in 4% (w/v) potassium hydroxide at 37°C. The digested tissue suspensions were thoroughly stirred, and eggs were counted in 2x50 µl samples on microscope slides under 10x magnification. The mean of the duplicate egg count was determined for each mouse. The treatment-induced percentage reductions in worm and egg burdens were calculated as P = (C-V/C) x100.

(Where P is the percentage of worm/egg counts reduction; C is the mean number of worms/eggs recovered from control mice; and V is the mean number of worms/eggs recovered from treated mice).

Egg developmental stages (oogram pattern)

Assessment of therapeutic efficacy is based on quantitative and qualitative oogram techniques following the criteria described by Pellegrino & Faria (41). After perfusion of the portal system, 5–10 cm of the middle portion of the small intestine was opened longitudinally with a pair of scissors and rinsed in a petri dish of saline solution, and three 10 mm fragments were cut off and processed for oogram. One hundred eggs were counted in each fragment and classified, according to the different stages of development, as immature, mature or dead.

Statistical analysis

The statistical package for social sciences (SPSS) software package version 20.0 statistical programme was utilized for both data presentation and statistical analysis of the results. The descriptive measures used for the results were arithmetic mean, standard deviation (SD), minimum (Min.), maximum (Max.) and median. The level of significance selected for this study was *p* equal to or less than 0.05 ($p \leq 0.05$). The Kolmogorov-Smirnov test was used to verify the normality of distribution. Kruskal Wallis test was used for abnormally distributed quantitative variables to compare between more than two studied groups, and Mann Whitney test for pairwise comparisons (42).

Results

***In vitro* cercarial response to PZQ**

The onset of the first cercarial re-shedding from PZQ-exposed snails occurred eight weeks after the cessation of drug exposure. The effect of PZQ on the harvested cercariae from the unexposed and exposed groups (group A and group B) was

observed and classified into three stages. Stage 1: the unaffected live cercariae demonstrating normal swimming activity (rapid linear progressive swimming). The cercariae exhibited body contractions during movement with or without coiling of rami of bifurcation (figures 2A and 2B). Stage 2: affected live cercariae demonstrating a: intermittent spins (cercariae remained momentarily motionless before the body and tail suddenly rotated around their central axis with no linear progressive movement); b: a tilted head (a cercaria weakly moving its head or tail without any progressive movement) (figure 2C); c: sluggish head movement with complete stoppage of body movement. A loss of body contractions occurred with or without coiling of rami of bifurcation (figures 2D and 2E). Stage 3: dead cercariae with complete stoppage of head and tail movement. Some cercariae exhibited separation of the tail (figure 2F). At the beginning of the experiment, the re-shed cercariae from PZQ-exposed snails in group B revealed no detectable differences regarding the cercarial activity compared with cercariae harvested from snails in group A (stage 1). Immediately after adding PZQ, an initial increase in the cercarial activity of snails in group B (prompt linear progressive and zigzag swimming motions) for approximately 20 seconds was observed. At the various examination points, statistically significant differences were recorded between cercariae harvested from both snail groups (group A and group B). After the first 10 minutes, 91.67% of cercariae from the group B snails were completely unaffected (stage 1) with no recorded cercarial deaths, while the remaining cercariae exhibited intermittent spins (stage 2). In contrast, after the same duration, 50.92% of cercariae from PZQ-unexposed snails (group A) died (stage 3). The cercarial death percentage in the same group increased up to 87.3% after 20 minutes of drug exposure, with total cercarial deaths occurring at the 30-minute examination point. On the other hand, by increasing the duration of drug exposure, the influence of PZQ on

the harvested cercariae from the PZQ-exposed group (group B) displayed slow and gradual enhancing effects.

After 30 minutes, up to 65.75% of cercariae harvested from group B (PZQ-exposed snails) remained unaffected. The sum of the affected and dead cercariae markedly increased at the 50-minute and 60-minute examination points, reaching approximately 80.55% at both points. At these examination points, complete death was achieved by 33.33% and 54.64% of the cercariae, respectively (figure 3).

Characterization of PZQ-encapsulated niosomes

The TEM of the prepared niosomes and PZQ-encapsulated niosomes revealed spherical, smooth surface nanoparticles with average sizes of 69.9-120 nm (figure 4A) and 43.2-131 nm (figure 4B), respectively**.** The zeta potential of the niosomes was -16.7 mV, while that of the PZQ-encapsulated niosomes was -20.2 mV. In addition, the particle size distribution performed by the particle size analyzer revealed the niosomes without PZQ to have a mean particle size of 83.55 nm and showed a polydispersity index of 0.715 (i.e. less than 1), indicating the homogeneous nature of the formulation. On the other hand, the particle size distribution of the PZQ-encapsulated niosomes revealed a mean particle size of 97.14 nm, and the polydispersity index was 0.534 (i.e. less than 1).

The encapsulation efficiency of PZQ was expressed as the ratio of the PZQ concentration in the niosomes and the PZQ concentration added to the system. This formula produced a percentage of encapsulation efficiency of 66.8%.

In vivo efficacy of PZQ and PZQ-encapsulated nanoparticles on adult *S. mansoni*

Adults worm burden

Recovered worms collected 49 days p.i. from all subgroups were counted and sexed under a dissecting microscope. A marked reduction of the drug potency was detected

against adults collected from PZQ-treated mice infected with cercariae with reduced sensitivity to PZQ (subgroup IIb) compared with its control (non-treated mice infected with cercariae with reduced sensitivity to PZQ (subgroup IIa), with a total worm reduction of 35.1%. This result was statistically non-significant ($p > 0.05$), whereas, under the same circumstances, the reduction of the female adult worm burden was statistically significant (47.37%; $p \leq 0.05$) (table 1).

No activity of niosome nanoparticles against the adult stage was detected in niosomes-treated mice infected with cercariae either PZQ-susceptible or with reduced sensitivity to PZQ (subgroups Ic and IIc) compared with their controls (subgroups Ia and IIa). The percentage reductions were 2.69% and 5.78%, respectively that were statistically non-significant ($P > 0.05$). Meanwhile, oral treatments with PZQ-encapsulated niosomes (subgroups Id and IId) revealed the highest drug efficacy among all the studied subgroups. Highly significant reductions were obtained by comparing subgroups Id and IId with their controls (subgroups Ia and IIa). The mean total worm load was 1.33 ± 0.52 with a statistically significant reduction of 94.09%, and the complete elimination of adult male worms was detected in *S. mansoni* with reduced sensitivity to PZQ treated with PZQ-encapsulated niosomes (subgroup IId) ($P \leq 0.05$). However, in subgroup Id (PZQ-susceptible *S. mansoni* treated with PZQ-encapsulated niosomes), a slight enhancement of the drug efficacy was reported with a statistically significant reduction of 95% ($p \leq 0.05$).

Tissue egg count and oogram patterns

Niosomes-treated mice infected with PZQ-susceptible cercariae or cercariae with reduced sensitivity to PZQ, (subgroups Ic and IIc, respectively), did not reveal statistically significant differences in each total egg count in liver and intestinal tissues and their oogram patterns ($p > 0.05$). However, oral treatment with PZQ or PZQ-

encapsulated niosomes in either the mice infected with PZQ-susceptible cercariae (subgroups Ib and Id) or the mice infected with cercariae with reduced sensitivity to PZQ (subgroups IIb and IId) demonstrated statistically significant reductions in the egg counts in both hepatic and intestinal tissues compared with those of their control subgroups ($p \leq 0.05$) (table 2).

For the PZQ-susceptible isolate, PZQ and PZQ-encapsulated niosomes-treated mice (subgroups Ib and Id) revealed a statistically significant increase in the dead eggs, with mean percentages of 47.67% and 69.90%, respectively, and a statistically significant reduction in the immature eggs, with mean percentages of 11.83% and 8.95%, respectively ($P \leq 0.05$) (table 3).

On the other hand, mice infected with less sensitive cercariae to PZQ, orally treated with PZQ (subgroup IIb), exhibited a much lower reduction in the number of immature eggs or an increase in the number of dead eggs. No significant changes in the percentage of the mature eggs were detected in subgroup IIb compared with subgroup IIa ($p > 0.05$). However, a remarkable increase in the percentage of dead eggs was detected in subgroup IId, in which the animals received PZQ-encapsulated niosomes, with a mean percentage of 87.67% ± 16.93.

Discussion

In the present work, schistosomes with reduced sensitivity to PZQ were obtained by drug selection during the asexual stages of the parasite in the snails. Shedding snails were treated with PZQ doses of 200 mg/kg twice/week, followed by an interval of one week, and then repeated twice in the same manner and reared in glass containers with changing the DTW every 24 hours. This approach is far less expensive, time consuming and labour intensive than other strategies applying drug pressure through multiple intra-mammalian stage passages (10). This regimen and rearing conditions

decreased the stress to which the snails were exposed, by dividing the dose of the drug and decreased time for the protein content of the paste in water to be fermented especially in a warm climate. Additionally, glass containers might offer a suitable temperature for snail growth and survival. Glass, for instance, is a very good insulator at room temperature, but becomes a conductor only when heated to a very high temperature. It is worthy to mention that, the approximate temperature for snail breeding and reproduction is 15 to 25 °C; snails cannot survive at > 29 °C, and may die within several hours at > 40 °C (43).

PZQ-exposed snails demonstrated cessation of cercarial shedding for eight weeks after the termination of drug exposure. This finding can be partially explained according to Mattos *et al.* (44), who reported that a sublethal dose of PZQ induces morphological and metabolic alteration on the sporocysts, thereby interrupting the shedding. As the effect of PZQ on the sporocysts is temporary and reversible (33), re-shedding of the cercariae occurred after recovery.

The success of inducing reduced sensitivity to PZQ in the present study was confirmed *in vitro* by reducing the PZQ susceptibility of cercariae harvested from the PZQ-exposed snails compared with those from the PZQ-unexposed snails. Similarly, cercariae from snails infected by resistant isolates induced either experimentally or from the field exhibited reduced susceptibility to PZQ (32,45). Furthermore, the development of reduced sensitivity to PZQ was confirmed experimentally. The therapeutic efficacy of 500 mg/kg PZQ was greatly diminished such that the 35.1% reduction in the total worm load in treated mice infected with cercariae with reduced sensitivity to PZQ (subgroup IIb) was not significant compared with its control. According to Coles and Kinoti (46), this value for worm recovery is sufficient to provide an isolate resistant to PZQ.

Parasite and/or host factors could serve as underlying reasons for such unresponsiveness. In schistosomes, PZQ tolerance has been linked to over-expression of sarco/endoplasmic reticulum Ca $^{2+}$ ATPases, heat shock protein 70 in addition to glycoproteins or other multidrug transporters which lead to increase drug efflux (13,47,48). In an investigation on the possible host factors involved in the PZQ unresponsiveness, Hanallah *et al.* (49), reported that, PZQ insusceptible *S. mansoni* isolates possesses a different immunogenic makeup, both qualitatively and/or quantitatively when compared to isolates susceptible to PZQ. Furthermore, according to Botros *et al*. (50), the decreased sensitivity to PZQ could be due to a lower inhibition of hepatic drug-metabolizing enzymes, mainly cytochrome P 450 (CYP 450) in host infected with resistant schistosoma isolates with a consequently higher metabolic transformation of PZQ and lower level of serum drug concentration. Inhibition of activities of hepatic drug metabolizing enzymes in PZQ susceptible *S. mansoni* infected mice was previously reported (51). Such inhibition was attributed to possible denaturation of CYP 450 to its inactive form (CYP 422) as a result of the inflammatory reaction following egg deposition (52). Therefore, lower inhibition of such enzyme could occur in association with the demonstrated significant reduction of the hepatic egg counts in *S. mansoni* resistant isolate.

When mice infected with *S. mansoni* isolate with reduced sensitivity to PZQ were treated with PZQ, a significant reduction in the female worm load in subgroup IIb compared with its control was demonstrated. The sex-specific sensitivity between male and female schistosomes could be explained by the fact that female schistosomes are more metabolically active than males. Interestingly, Kasinathan *et al*. (12) revealed an expression of higher levels of *S. mansoni* multidrug resistance-like protein transporters in males than in female worms that is responsible for resistance. This can

consequently explain the significant egg count reduction in the livers and intestines in mice infected with *S. mansoni* isolate with reduced sensitivity to PZQ and treated with PZQ (subgroup IIb) despite the non-significant reduction in the total worm load compared with their control (subgroup IIa).

We reported the lack of any significant therapeutic effect of niosomes nanoparticles (NPs) orally administrated alone against adult *S. mansoni* with reduced sensitivity to PZQ. In contrast to metals, metal oxides and polymer-based NPs (which are well known for their highly potent antimicrobial effect), biological nanoparticles such as lipids are used only for drug delivery (13,18,53).

Regarding niosomes´ safety, it is generally believed that lipids are biocompatible. A niosome is a non-ionic surfactant-based liposome. They are formed mainly by cholesterol incorporation as an excipient. In this study, Span 60 was the non-ionic surfactant used for preparing the niosomes particles. Span 60 is one of the alkyl esters, which are considered non-toxic and non-irritant materials (54). The Food and Drug Administration revised them in 2015 to be one of the food additives (55). These biophysical properties of niosomes support their tissue non-toxicity.

Oral administration of niosomes in conjugation with PZQ against susceptible adult *S. mansoni* (subgroup Id) enhanced the therapeutic efficacy of the drug regarding the studied parasitological parameters. Due to the presence of hydrophilic, amphiphilic and lipophilic moieties in their structure, drug molecules with a wide range of solubility can be accommodated. These moieties may act as a depot, releasing the drug in a controlled manner. Similarly, solid lipid nanoparticles loaded with PZQ was effective in reducing the worm load and the tissue egg count (56). In this work, niosomes were able to promote a high concentration of the drugs in the target cells, and when

combined with sodium stibogluconate, they were found to be more effective than liposomes against experimental murine visceral leishmaniasis (57).

The low aqueous solubility of PZQ is considered a limiting factor regarding its bioavailability (58). On the other hand, niosomal drug delivery enhances drug bioavailability by crossing the anatomical barrier of the gastrointestinal tract via cell transcytosis of Peyer's patches in the intestinal lymphatic tissues (23).

In the present work, the regimen using single therapy of PZQ-encapsulated niosome in mice infected with cercariae with reduced sensitivity to PZQ was shown to be capable of successfully overcoming the tolerance of *S. mansoni* to PZQ, as revealed by our results displaying statistically significant reductions in all the evaluated indicators of drug susceptibility compared with all the relevant studied subgroups. Kulsirirat *et al.* (26) demonstrated that non-ionic surfactant had an inhibitory effect on P-gp ATPase activity transporters, which have been linked to PZQ resistance in schistosomes (12). This finding identified a possible mechanism by which niosomes could overcome the reduced sensitivity to PZQ. In agreement with the reported data, the development of new therapeutics or compounds that target these transporters proved useful in enhancing the efficacy of the drugs (6,59). Verapamil and Tariquidar are considered P-gp inhibitors and can enhance the cytotoxic effects of chemotherapeutic drugs against resistant parasites (6,60). Moreover, a combination of the P-gp transporter inhibitors and PZQ has been successfully used against resistant *S. mansoni* (9,59).

In the current study, we report that PZQ-encapsulated niosomes enhanced and restored drug sensitivity against susceptible and laboratory-induced *S. mansoni* adult worms with reduced sensitivity to PZQ, respectively. Interestingly, the primary mechanism of overcoming the drug resistance could be related to the surfactant inhibitory effect on P-gp

efflux transporters. Further investigations are necessary to verify the exact mechanisms by which niosomal nanoparticles exert their effect against tolerant parasites.

Conflicts of interest

The author declares absence of any conflict of interest.

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Figures:

Fig. 1 Flowchart showing a brief summary of the methodology carried on in this work.

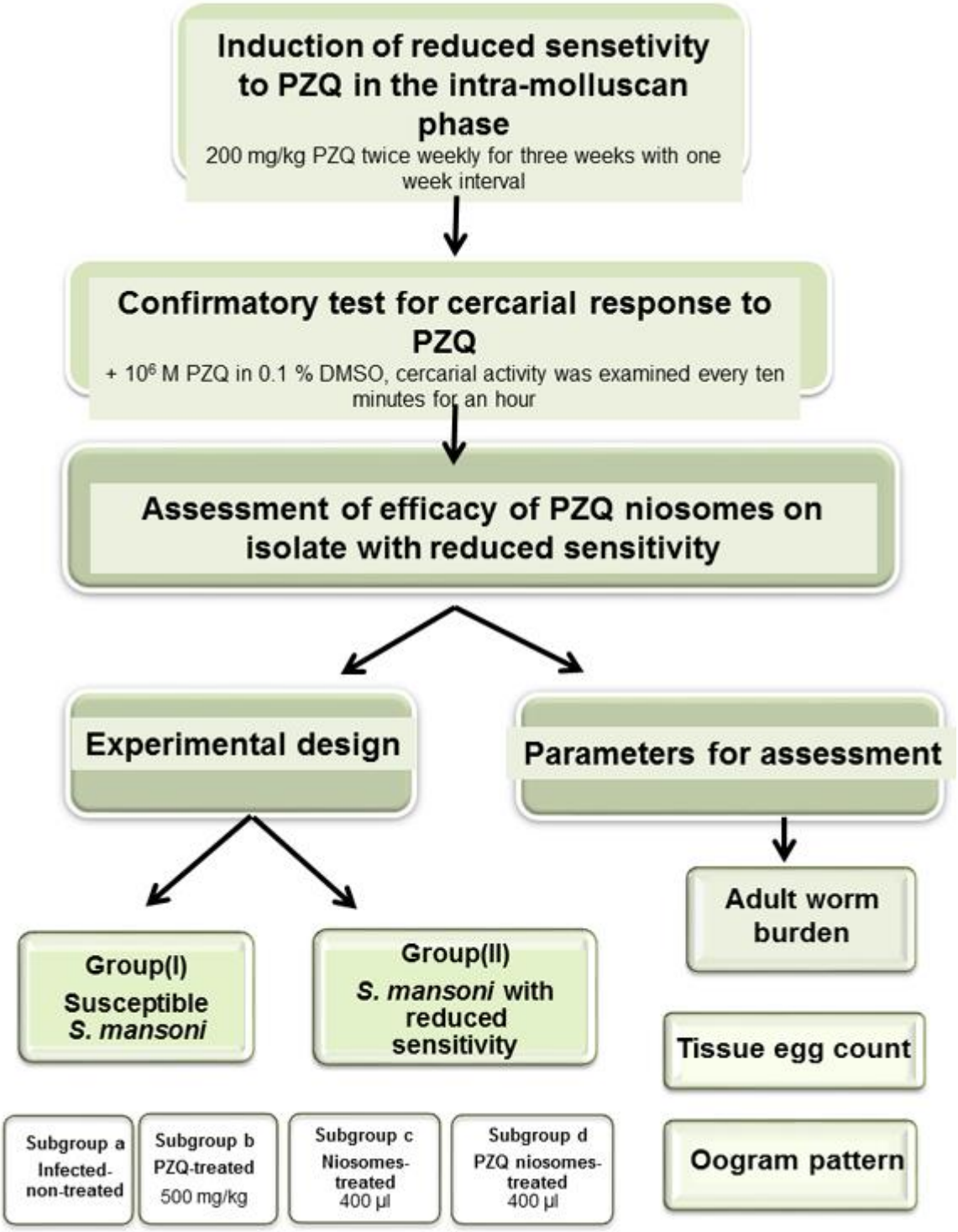

Fig. 2 *In vitro* cercarial response to PZQ (x100) (scale bar 100µ). Cercariae harvested from group B snails showing; (A): tail constrictions (arrow) with two coiled rami of the bifurcation; (B): cercariae showing tail constrictions (arrows) with no coiling of the ramus of the bifurcation; (C): cercariae showed tilting of the head (stage 3); (D): cercariae showing disappearance of tail constrictions (stage 4) with two rami of the bifurcation coiled; (E): cercariae showing disappearance of tail constrictions (stage 4) with no coiling of ramus of the bifurcation; (F): dead cercariae showing separation of the tails (stage 5).

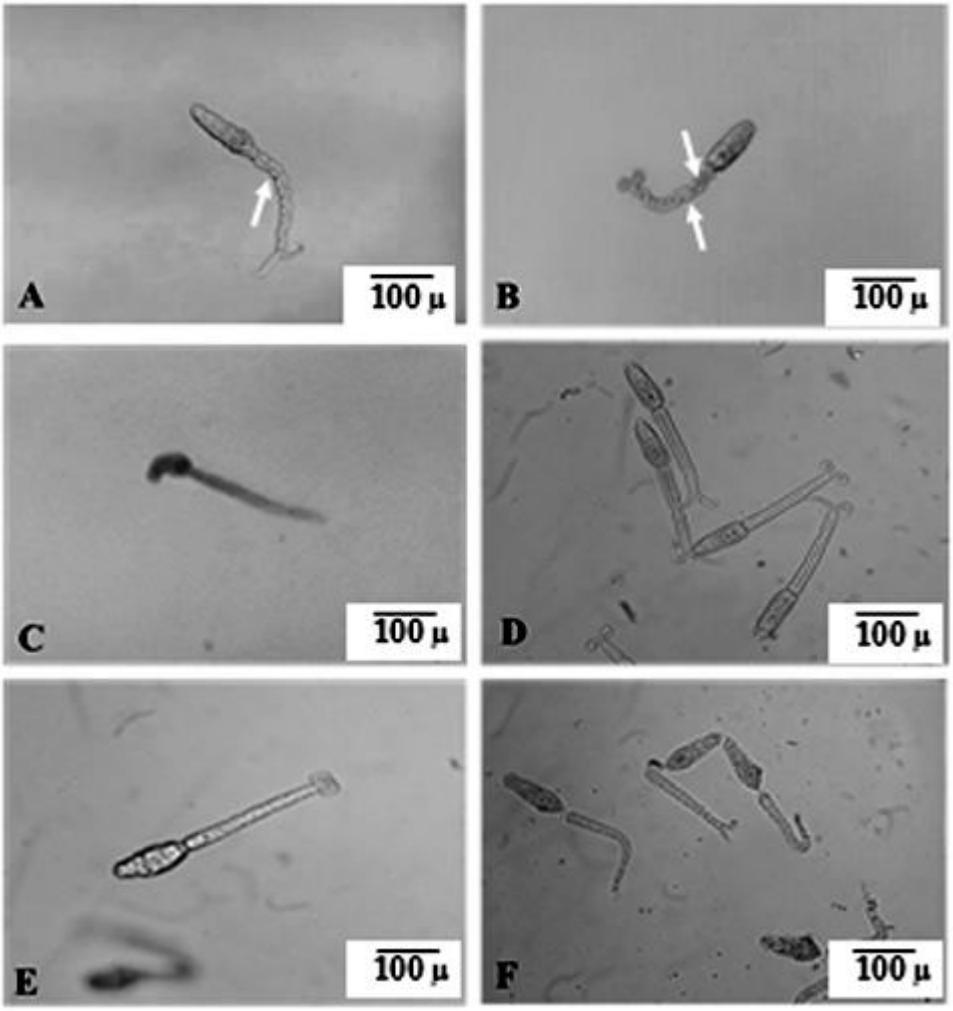

Fig. 3 *In vitro* effect of $5x10^{-6}$ M PZQ on the cercariae (n= 36) harvested from **(A):** PZQ unexposed control group (group A) and **(B)**: PZQ exposed (group B) snails at a dose of 200 mg/kg PZQ twice/week followed by an interval of one week, and then repeated twice in the same manner. Lines represent mean count of un-affected, affected and dead cercariae after drug exposure every ten minutes for one hour. Error bars represent standard deviations of three cercarial sets per group.

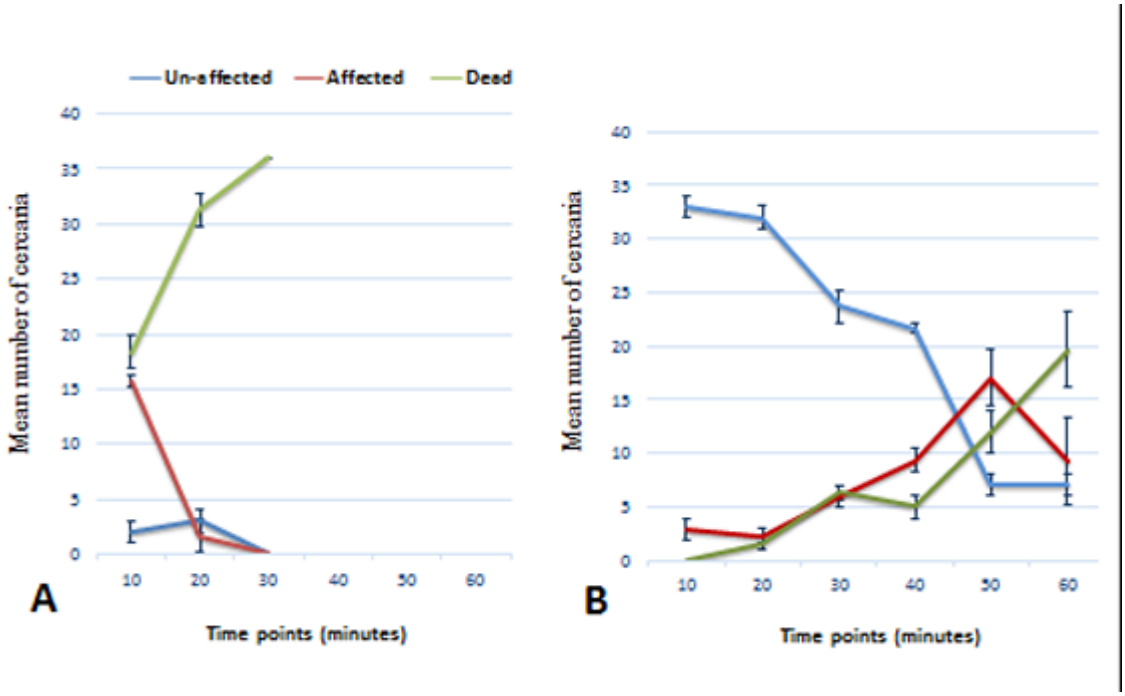

Fig. 4 Transmission electron microscope of the nanoparticles: **(A):** rounded smooth surface of free niosomes with average size of 69.9- 120 nm (5000X); **(B):** PZQ encapsulated niosomes with average size of 43.2 -131 nm (5000X).

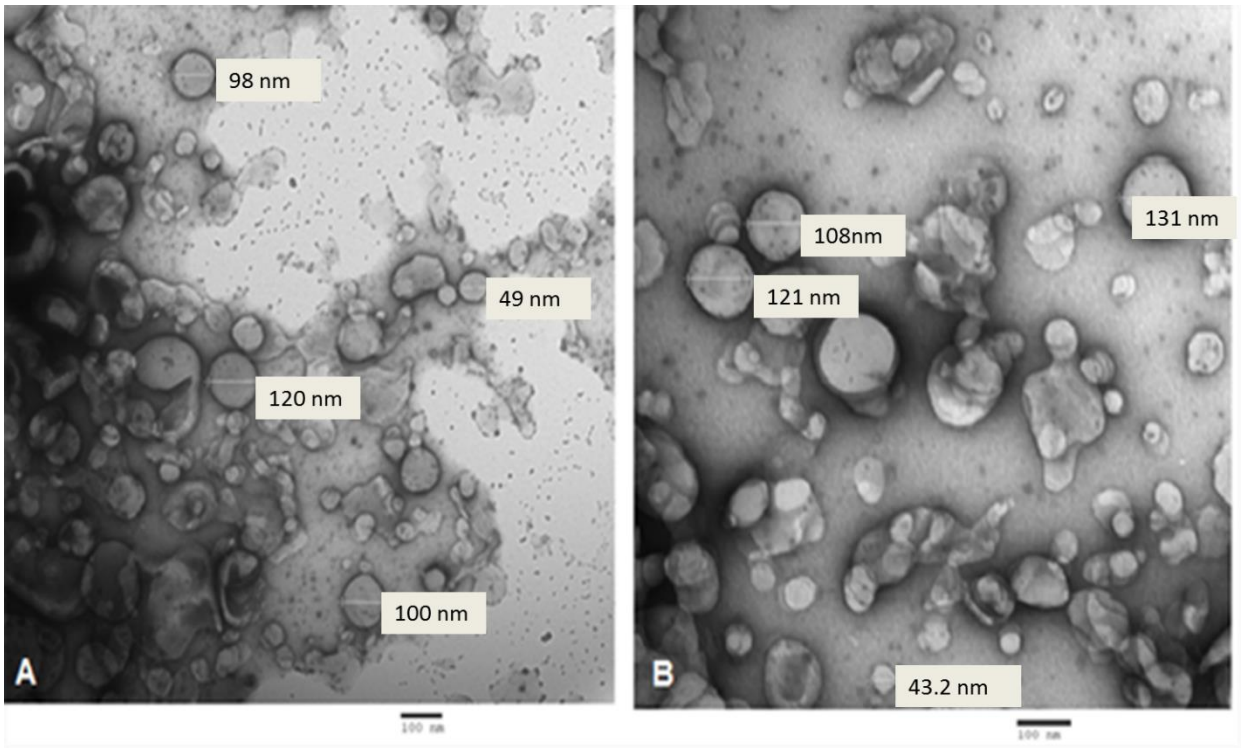

Table 1 Total and female adult *S. mansoni* worm loads among the different studied subgroups**.**

Subgroups / Worm Load	Group I (susceptible group)				Group II (group with reduced sensitivity to PZQ)			
	Ia (Control)	**Ib**	**Ic**	**Id**	**IIa (Control)**	**IIb**	**IIc**	**IId**
Total worm load								
Mean ± SD.	30.83 ± 9.43	3.0 ± 1.55	30.0 ± 5.10	1.55± 0.94	22.5 ± 6.06	14.60 ± 3.30	21.2 ± 2.32	1.33 ± 0.52
$P_{control}$		0.004*	0.747	<0.001*		0.009*	0.259	0.003*
Sig. bet. Groups		p_1=0.004*, p_2=0.020*, p_3<0.001*				p_4=0.001*, p_5<0.001*, p_6=0.003**		
Sig. bet. Groups	p_7= 0.092, p_8<0.001*, p_9= 0.004*, p_{10}=0.645							
% Reduction		↓90.27	↓2.69	↓95.0		↓35.1	↓5.78	↓94.09
Female worm load								
Mean ± SD.	15.7 ± 4.4	1.5 ± 0.84	14.0 ± 2.68	1.10 ± 0.79	13.3 ± 2.2	7.0 ± 5.6	12.2 ± 3.5	1.33 ± 0.52
$P_{control}$		0.003*	0.744	<0.001*		0.024*	0.220	0.003*
Sig. bet. Groups		p_1= 0.003*, p_2=0.254, p_3<0.001*				p_4= 0.053, p_5= 0.182, p_6= 0.003*		
Sig. bet. Groups	p_7= 0.255, p_8= 0.190, p_9= 0.170, p_{10}=0.553							
% Reduction		↓90.45	↓10.83	↓93.0		↓47.37	↓8.27	↓90.0

Group I (susceptible group): mice infected with PZQ susceptible cercariaee**; Ia:** non treated; **Ib:** PZQ treated; **Ic:** niosomes nanoparticles treated; **Id:** PZQ niosomes nanoparticles treated.

Group II (group with reduced sensitivity to PZQ): mice infected with cercariae with reduced sensitivity to PZQ; **IIa:** non treated; **IIb:** PZQ treated; **IIc:** niosomes nanoparticles treated; **IId:** PZQ niosomes nanoparticles treated.

% Reduction: Percentage reduction between each subgroup and the control subgroup
P: Kruskal Wallis test, Significance between groups was done using Mann Whitney test.
$P_{control}$: p value for comparing between Control and each subgroup
p_1: p value for comparing between Ib and Ic
p_2: p value for comparing between Ib and Id
p_3: p value for comparing between Ic and Id
p_4: p value for comparing between IIb and IIc
p_5: p value for comparing between IIb and IId
p_6: p value for comparing between IIc and IId
p_7: p value for comparing between Ia and IIa
p_8: p value for comparing between Ib and IIb
p_9: p value for comparing between Ic and IIc
p_{10}: p value for comparing between Id and IId

Table 2 Hepatic and intestinal tissue egg counts ($\times 10^2$) per gram of tissue among the different studied subgroups.

Subgroups / Egg counts	Group I (Susceptible group)				Group II (Group with reduced sensitivity to PZQ)			
	Ia (Control)	**Ib**	**Ic**	**Id**	**IIa (Control)**	**IIb**	**IIc**	**IId**
Liver egg count								
Mean ± SD.	347.2± 157.98	24.67 ± 32.05	244.2 ± 55.4	18.79 ± 1.81	193.8 ± 60.8	67.8 ± 33.4	139.2 ± 117.7	4.50 ± 3.6
$P_{control}$		0.004*	0.150	<0.001*		0.008*	0.109	0.004*
Sig. bet. Groups		p_1=0.004*, p_2=0.223, p_3<0.001*				p_4=0.078, p_5=0.004*, p_6=0.004*		
Sig. bet. Groups	p_7= 0.016*, p_8= 0.025*, p_9= 0.078, p_{10}<0.001*							
% reduction		↓89.54	↓29.67	↓94.59		↓65.02	↓28.17	↓97.68
Intestinal egg count								
Mean ± SD.	384.7 ± 136.7	57.3 ± 32.9	242.3 ± 102.7	42.30 ± 5.47	231.67 ± 74.4	137.7 ± 38.02	222.7 ± 73.1	3.33 ± 1.8
$P_{control}$		0.004*	0.055	<0.001*		0.037*	0.687	0.004*
Sig. bet. Groups		p_1=0.004*, p_2=0.031*, p_3<0.001*				p_4=0.055, p_5=0.004*, p_6=0.004*		
Sig. bet. Groups	p_7= 0.037*, p_8= 0.010*, p_9= 1.000, p_{10}<0.001*							
% reduction		↓85.12	↓37.02	↓89.0		↓40.56	↓3.87	↓98.56

Group I (susceptible group): mice infected with PZQ susceptible cercariaee**; Ia:** non treated; **Ib:** PZQ treated; **Ic:** niosomes nanoparticles treated; **Id:** PZQ niosomes nanoparticles treated.

Group with reduced sensitivity to PZQ (group II): mice infected with cercariae with reduced sensitivity to PZQ; **IIa:** non treated; **IIb:** PZQ treated; **IIc:** niosomes nanoparticles treat ed; **IId:** PZQ niosomes nanoparticles treated.

% Reduction: Percentage reduction between each subgroup and the control subgroup
P: Kruskal Wallis test, Significance between groups was done using Mann Whitney test.
$P_{control}$: p value for comparing between Control and each subgroup
p_1: p value for comparing between Ib and Ic
p_2: p value for comparing between Ib and Id

p_3: p value for comparing between Ic and Id
p_4: p value for comparing between IIb and IIc
p_5: p value for comparing between IIb and IId
p_6: p value for comparing between IIc and IId
p_7: p value for comparing between Ia and IIa
p_8: p value for comparing between Ib and IIb
p_9: p value for comparing between Ic and IIc
p_{10}: p value for comparing between Id and IId
*: Statistically significant at $P \leq 0.05$

Table 3 Oogram pattern (Egg developmental stages) of the different studied subgroups

Subgroups / Egg Developmental Stages	Group I (Susceptible group)				Group II (Group with reduced sensitivity to PZQ)			
	Ia (Control)	**Ib**	**Ic**	**Id**	**IIa (Control)**	**IIb**	**IIc**	**IId**
Mature eggs Mean ± SD.	33.92 ± 4.45	40.0 ± 20.12	27.50 ± 9.29	21.15 ± 5.93	35.33 ± 29.24	32.33 ± 16.26	45.67 ± 21.51	12.33 ± 11.71
$P_{control}$		0.054	0.261	<0.001*		0.748	0.337	0.024*
Sig. bet. Groups		p_1=0.054, p_2=0.014*, p_3<0.001*				p_4=0.748, p_5=0.337, p_6=0.423		
Sig. bet. Groups	p_7=0.337, p_8=0.520, p_9=0.128, p_{10}=0.027*							
Immature eggs Mean ± SD.	63.77 ± 4.94	11.83 ± 9.62	66.17 ± 6.11	8.95 ± 3.33	59.17 ± 26.57	29.50 ± 26.24	51.0 ± 22.12	0.0 ± 0.0
$P_{control}$		0.004*	0.469	<0.001*		0.045*	0.520	0.002*
Sig. bet. Groups		p_1=0.004*, p_2=0.575, p_3<0.001*				p_4=0.045*, p_5=0.520, p_6=0.199		
Sig. bet. Groups	p_7=0.520, p_8=0.200, p_9=0.335, p_{10}<0.001*							
Dead eggs Mean ± SD.	2.32 ± 1.80	47.67 ± 28.52	6.67 ± 5.47	69.90 ± 5.19	5.67 ± 3.56	32.17 ± 22.52	3.33 ± 1.51	87.67 ± 16.93
$P_{control}$		0.004*	0.090	<0.001*		0.043*	0.168	0.004*
Sig. bet. Groups		p_1=0.006*, p_2=0.014*, p_3<0.001*				p_4=0.036*, p_5=0.168, p_6=0.054		
Sig. bet. Groups	p_7=0.049*, p_8=0.423, p_9=0.257, p_{10}=0.123							

Group I (susceptible group): mice infected with PZQ susceptible cercariae**; Ia:** non treated; **Ib:** PZQ treated; **Ic:** niosomes nanoparticles treated; **Id:** PZQ niosomes nanoparticles treated.

Group II (Group with reduced sensitivity to PZQ): mice infected with cercariae with reduced sensitivity to PZQ; **IIa:** non treated; **IIb:** PZQ treated; **IIc:** niosomes nanoparticles treated; **IId:** PZQ niosomes nanoparticles treated.

% Reduction: Percentage reduction between each subgroup and the control subgroup

P: Kruskal Wallis test, Significance between groups was done using Mann Whitney test.
$P_{control}$: p value for comparing between Control and each subgroup
p_1: p value for comparing between Ib and Ic
p_2: p value for comparing between Ib and Id
p_3: p value for comparing between Ic and Id
p_4: p value for comparing between IIb and IIc
p_5: p value for comparing between IIb and IId
p_6: p value for comparing between IIc and IId
p_7: p value for comparing between Ia and IIa
p_8: p value for comparing between Ib and IIb
p_9: p value for comparing between Ic and IIc
p_{10}: p value for comparing between Id and IId
*: Statistically significant at $P \leq 0.05$