



## Pentoxifylline and/or praziquantel reduce murine schistosomiasis *mansoni* histopathology via amelioration of liver functions

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### ABSTRACT

Murine schistosomiasis represented a good lab model of granulomatous hypersensitivity reaction which could be elicited by *S. mansoni* eggs trapped in the host tissues which might lead to liver fibrosis. Pentoxifylline (PTX) is an immunomodulatory and antifibrotic substance. The present investigation aimed to study the action of PTX (4 weeks post infection) either alone or combined with PZQ (7 wk PI) on fibrosis developed from murine hepatic schistosomal granulomatous reaction. The experiments were carried out on naïve C75BL/6 mice that were divided into three groups. Two control groups were used one without infection (normal) and the other was infected without treatment. Mice were sacrificed 12 weeks post infection.

PTX treatment alone caused a partial toxic effect on worm burden; egg count; and ameliorated the liver functions. But, it increased immature and dead eggs, while it decreased the mature ones. PZQ administration alone or in combination with PTX showed a highly significant reduction in worm burden, egg count, disappearance of immature egg stage and a high increase in dead eggs. Also, it caused a highly significant reduction in granuloma count, diameter and ameliorated liver functions.

In conclusion, according to the parasitological, histopathological and biochemical criteria of the present study, PTX can be successfully introduced into antischistosomal therapy as a potent antifibrotic agent with immunomodulatory properties in combination with PZQ.

### BACKGROUND

Schistosomiasis is a poverty symptom that is prevalent in 78 countries with 92 % living in sub-Saharan Africa (World Health Organization, 2018). It is a widespread neglected tropical parasitic disease transmitted by snails (Ibrahim and Ghoname, 2018) caused by a digenian trematode of the genus *Schistosoma* and it is affected more than 261 million people worldwide (Feitosa *et al.*, 2018; Ibrahim and Sayed, 2019). *Schistosoma mansoni* infection resulted in severe histopathological changes and functional damage in the liver of the host (Leo and Peter, 1998). These alterations include the deposition of schistosome eggs and pigments, increase in organ size and formation of granulomas surrounding the deposited eggs which may result in scarring,

portal hypertension, haemorrhage and death (Gause *et al.*, 2003). Liver fibrosis is a wound-healing process that occurs when the liver is injured chronically (Friedman, 2003). Hepatic stellate cells (HSC) are responsible for the excess production of extracellular matrix (ECM) components (Han, 2018). The activation of HSC; a key issue in the pathogenesis of hepatic fibrosis (Bartley *et al.*, 2006); is mediated by various cytokines and reactive oxygen species released from the damaged hepatocytes and activated Kupffer cells (Iredale, 2003). Therefore, inhibition of HSC activation and its related subsequent events, such as increased production of ECM components and enhanced proliferation; are crucial goals for intervention in the hepatic fibrogenesis cascade (Bataller and Brenner, 2001).

Praziquantel is the cornerstone drug for the treatment of all species of *Schistosoma* (Vale *et al.*, 2017), which has low toxicity; great efficacy and is easy to administer (Utzinger and Keiser, 2004). PZQ has rapid action against adult schistosome worms with changes in the tegument and muscular activity, possibly resulting from calcium ion flux within the worm (Vale *et al.*, 2017). Although treatment with this drug was effective and inexpensive (Cioli and Pica-Mattocchia, 2003), frequent schistosome reinfection after treatment due to the relative resistance of the larval stages of *S. mansoni* to schistosomicide drugs (Silva *et al.*, 2003), occurred in endemic areas. Also, Abouel-dahab and Elhussieny (2016) found that PZQ has little or no effect on the developing larvae of 3 – 21 days post infection. Beshay *et al.*, (2019) stated that it was preferable to make combination of PZQ and anti-inflammatory drugs in the treatment of murine schistosomiasis *mansoni*.

Pentoxifylline (PTX) is a synthetic derivative of methylxanthine (El-Lakkany *et al.*, 2011) and had significant anti-fibrotic effects on experimentally induced schistosomal hepatic fibrosis (Khalifa and Nemenqani, 2014).

The main pharmacological action of PTX is to protect hepatocytes from excessive cytokines that are responsible for activation of HSC by inhibiting them. Li *et al.* (2016) confirmed that PTX prevents *in vitro* liver fibrosis induced by *Schistosoma japonicum* infection by inhibiting the Hedgehog (HH) signaling pathway. Also, El-Lakkany and Nosseir (2007) stated that PTX has limited toxic effects on schistosome worms and eggs and thus, can be used as an adjuvant therapeutic tool with anti-helminthic drugs in treatment of human schistosomiasis (Abdel aziz *et al.*, 2012). Rabia *et al.* (2010) and Mati *et al.* (2010) stated that the addition of antifibrotic drugs PTX or silymarin to PZQ potentiated an antipathological effect which minimized and ameliorated the liver fibrosis.

The aim of the present research is to investigate the effects of the administration of the antifibrotic agent PTX alone or associated with PZQ on the course of murine schistosomiasis *mansoni*, using different parasitological, histopathological and biochemical parameters.

## MATERIAL AND METHODS

### Animals:

Male C57BL/6 mice strain; 6–8 weeks (wk) old (18-20 g); were purchased from the Schistosome Biological Supply Program, Theodore Bilharz Research institute, Giza-Egypt (SBSP, TBRI) in accordance with international guidelines. Mice were maintained for 8 wk in plastic cages in an animal room, at temperature ranging between 20–25°C and were fed Purina chaw (20% protein) and given tap water.

**Cercariae and infection of mice:**

Cercariae were obtained from the infected *Biomphalaria alexandrina* snails, from SBSP, TBRI. The number of cercariae was determined by using a dissecting microscope. Generally; three counts were made and the average was used to calculate the number of cercariae per 0.1 ml of the cercarial suspension (Moore *et al.*, 1977). Infection was done subcutaneously with 100 cercariae/mouse of the Egyptian strain of *S. mansoni*.

**Test drugs:**

Pentoxifylline (PTX) (Trental®, Aventis Pharma, Cairo, Egypt), was orally administered from day 30 post infection (PI) for 55 day (5 days/wk) at a dose of 360 mg / kg body weight.

Praziquantel (PZQ) (Distocide®, Epico Pharma, Cairo, Egypt) is a white powder insoluble in water. It was given orally, 7 wk PI, at a dose of 500 mg/kg b.wt for 2 consecutive days, using a blunt stainless steel oral cannula. It was freshly prepared before use as 2% suspension in Cremophor-El (Sigma Chemicals Co. St. Louis Missouri).

**Experimental design:**

30 mice were divided into 3 groups each of 10 mice as follows:

- 1- Normal untreated mice.
- 2- Normal mice treated with PTX (360 mg/kg/day) for 8 wk.
- 3- Normal mice treated with PZQ (500 mg/kg/day) for 2 consecutive days.

40 mice all infected with 100 *S. mansoni* cercariae/ mouse, were divided into 4 groups, each of 10 mice as follows:

- 1- Infected untreated control (positive controls).
- 2- Infected-PTX treated mice. Mice received PTX at 4 wk PI for 8 wk (360 mg/kg/day).
- 3- Infected-PZQ treated mice: Mice received PZQ (500mg/kg/day); 7 wk PI for 2 consecutive days.
- 4- Infected-PTX and PZQ treated mice. Mice received PTX at 4 wk PI for 8 wk, and were treated with PZQ; 7 wk PI for 2 consecutive days.

Mice of all experimental groups were sacrificed by cervical dislocation at 12 wk PI and were subjected to investigations.

**5- Parasitological study:**

5.1-Worm burden and distribution: Perfusion of hepatic and portomesenteric vessels of mice was carried out according to (Duvall and DeWitt; 1967).

Mice were sacrificed by cervical dislocation. No resort to general anesthesia or heparin was attempted, because of the hepatic shift they interfere with the interpretation of genuine drug effect.

**5.2- Oogram pattern:**

The percentage of eggs at different developmental stages in the small intestine was examined in three samples per animal and the mean of each stage per animal was obtained. Eggs were counted and classified into their stages of development according to (Pellegrino *et al.*, 1962).

5.3- Egg count in liver and intestine tissues: The number of ova per gram hepatic or intestinal tissue was counted (Kamel *et al.*, 1977).

**6-Histopathological investigation:**

Liver sample from each animal was preserved in 10% buffered formalin solution, till dehydrated; sectioned and stained with Ehrlich's hematoxylin and counter stained with eosin (Bancroft and Stevens, 1975).

### Granulomas counting and Measurement of granuloma diameter:

The number of granulomas in 5 successive fields (10x10) was counted and recorded. Measurement of granuloma diameter was done only for non-contiguous granulomas, each containing a single egg in the center using a calibrated ocular micrometer. The mean granuloma diameter (M.G.D) was calculated by measuring two diameters of the lesion at right angles to each other (Von Lichtenberg, 1962). The percent reduction in granuloma diameter/ treated group relative to the infected groups was calculated according to the following formula:

$$\% \text{ Reduction} = \frac{\text{M.G.D. of control group} - \text{M.G.D. of treated group}}{\text{M.G.D. of control group}} \times 100$$

### Serum preparation and biochemical investigation:

Mice were sacrificed by cervical dislocation and the blood was collected in plastic tubes not containing anticoagulant. Blood was allowed to stand at 37°C for 1 hr, then over night at 4°C, and centrifuged at 300 G for 30 minutes (min). Sera were separated and heat-inactivated at 56°C for 30 min and stored in aliquots at -20°C, until use. Measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined in serum according to the method described by (Reitman and Frankel, 1957).

### Statistical analysis:-

Data are expressed as mean± standard error of mean (X±SEM). Comparison between two means was done using student's t-test (Snedecor and Cochran, 1991).

## RESULTS

The present study revealed that treatment of *S. mansoni* infected mice with PTX alone caused an insignificant reduction in the mean total worm number, while, when the infected mice treated with PZQ alone or in combination with PTX after 4 wk of infection caused a highly significant reduction (P<0.001) (table 1). Regarding the mean total number of ova in the intestinal vessels, there was a highly significant reduction in egg number (P<0.001) between the control group and the groups receiving PTX, PZQ or PTX+PZQ. Also, in the hepatic vessels, a highly significant egg reduction (P<0.001) was observed in groups taking PZQ or PTX+PZQ, while, there was no significant decrease was recorded between the control group and the group receiving PTX (table 1).

Table 1: The effect of PTX and PZQ administrations each alone or in combinations with each other; on total number of worms and the number of ova/gm tissue in mice infected with 100 *S. mansoni* cercariae; 4 wk PI and sacrificed 12 wk PI.

Animal groups	Mean total worm number	Mean total number of ova/gm tissue (X±SEM)	
	(X±SEM)	Intestine	Liver
a) Infected Control	31.5±2.070	64.7±2.582	51.5±4.562
b) Infected +PTX	24.6±2.500	48.6±4.728**	45.7±5.394
d) Infected + PZQ	0.375±0.374**	7.2±0.940 **	5.1±0.718 **
f) Infected +PTX+ PZQ	0.125±0.12**	9.1±1.187 **	4.3±0.1829 **

\*- Significant (P<0.01)

X=mean total number.

SEM=standard mean error.

The present results showed that there was no significant difference in the percentage of immature eggs between the control group and PTX group. While, a very

highly significant reduction ( $P<0.001$ ) in this percentage between the control group and groups receiving PZQ or combination of PTX+PZQ was reported. Regarding the mature eggs, a highly significant decrease ( $P<0.001$ ) was reported in all treated groups (PTX, PZQ or a combination of PTX+PZQ groups).

There was a significant increase ( $P<0.01$ ) in the mean percentage of dead eggs between the control group and the group treated with PTX, PZQ or a combination of PTX+PZQ was reported (Table 2).

Table 2: The effect of PTX and PZQ administrations; each alone or in combinations with each other, on % egg developmental stages (Oogram) in mice infected with 100 *S. mansoni* cercariae; 4 wk PI and sacrificed 12 wk PI.

Animal groups	% Egg developmental stages (X± SEM)		
	% Immature Ova	% Mature Ova	% Dead Ova
a) Infected Control	44.3±4.55	47.7±4.640	7.8±1.060
b) Infected +PTX	55.8±4.437	32.2±2.144**	11.8±1.381*
d) Infected + PZQ	0.0±0.0***	2.2±1.292***	97.7±8.293***
f) Infected +PTX+ PZQ	0.0±0.0***	1.6±1.224***	98.8±6.875***

\*\* Significant difference ( $P<0.001$ )

X=mean total number.

SEM=standard mean error.

The present results indicated that there was a highly significant reduction ( $P<0.001$ ) in the number of granuloma in the liver between groups that were taken PTX, PZQ and PTX+PZQ in comparison to the control group. Also, there was a very highly significant reduction ( $P<0.001$ ) in the mean granuloma diameter in the liver between groups that were taken PTX, PZQ and PTX+PZQ in comparison to the control group (table 3).

Table 3: The effect of PTX, PZQ and combination of PTX and PZQ administrations on number of hepatic granuloma (NG) [in 5 successive power fields (10x10)] and mean diameter of granuloma (DG) in mice infected with 100 *S. mansoni* cercariae; 4 wkPI and sacrificed 12 wkPI

Animal groups	NG X± SEM	% Reduction	Mean DG (µm) X± SEM	% Reduction
a) Infected control	13.4±0.927	---	342.047±7.208	---
b) Infected +PTX	9.2±0.374**	31.3	278.1±3.700***	18.6
d) Infected + PZQ	8.2±0.374***	38.8	264.3± 2.999 ***	22.7
f) Infected +PTX+ PZQ	8.0±0.447***	40.2	254.9± 5.24***	25.4

\* Significant difference between group (a) and the next groups.

X=mean total number.

SEM=standard mean error.

Liver sections of infected untreated control showed circumscribed round granuloma formation which consisted of centrally located ovum (O), surrounded by collagen fibers (C) and lymphoid cells (M), as well as, mononuclear leucocytes (arrow) (figure 1. B) as compared with the liver of normal mice (figure 1. A). In infected mice receiving PTX, around the granuloma there was Kupffer cells proliferation (arrow) in between the degenerated hepatocytes surrounding the central vein (CV) (figure 1.C).

Infected mice subjected to PZQ showed hepatic granuloma formation with centrally located ova surrounded by fibrous tissue collagen and lymphocytes as well as mononuclear leucocytes, in an adhesive manner (figure 1. D). Kupffer cells proliferation with mononuclear leucocytes infiltration were observed in between the degenerated hepatocytes as well as in between the karyomegalic cells. In group receiving PTX+PZQ, mononuclear leucocytes inflammatory cells infiltration were observed in the portal area associated with dilatation in the central vein and collagen fibers were the most abundant component of the granuloma (figure 1.E and F).

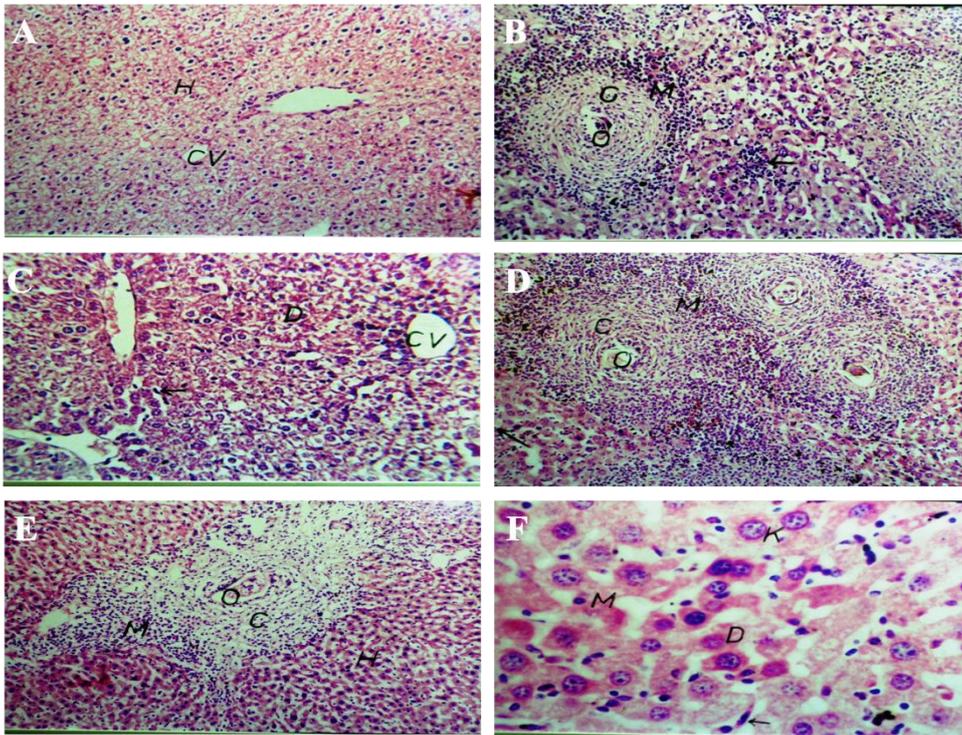


Fig. 1: Liver sections showing A) the normal histological structure of the central vein (CV) and surrounding hepatocytes (H) (H&E stain x 40). B) Showing circumscribed round granuloma formation of central ovum (O) surrounded by collagen (C) and macrophages as well as lymphocytes (M); while the other hepatic tissue showing focal as well as diffuse mononuclear leucocytes inflammatory cells infiltration (arrow) (H&E stain x 40). C) infected- PTX treated mice:-Showing Kupffer cells proliferation (D) in between the degenerated hepatocytes (D) surrounding the central vein (CV) (H&E stain x 64). D) infected-PZQ treated group:- Showing granuloma formation with central ovum (O) surrounded by collagen (C) and more lymphoid cells (M) and mononuclear leukocytes. Notice the multiple number of granuloma with peripheral zones adhesion and diffused kupffer cells (arrow) (H&E stain x 40). E) Liver sections of infected-PTX+PZQ treated group:- Showing a massive number of mononuclear leukocytes inflammatory cells infiltration (M) in the portal area and normal hepatocytes (H) (arrow) (H&E stain x 40) and F) Showing mononuclear leukocytes infiltration (M) with diffuse kupffer cells proliferation (arrow) in between the degenerated hepatocytes (D) and karyomegaly of some hepatocytes (K) (H&E stain x 160).

The present investigation showed that a highly significant increase ( $P < 0.001$ ) between the normal groups and the infected untreated control group in the value of ALT and AST level was detected. Comparing between the infected control group and groups receiving PZQ or PTX+PZQ a very highly significant decrease ( $P < 0.001$ ) in the amount of ALT and AST was recorded. While, no significant decrease was observed in groups subjected to PTX (Figure 2).

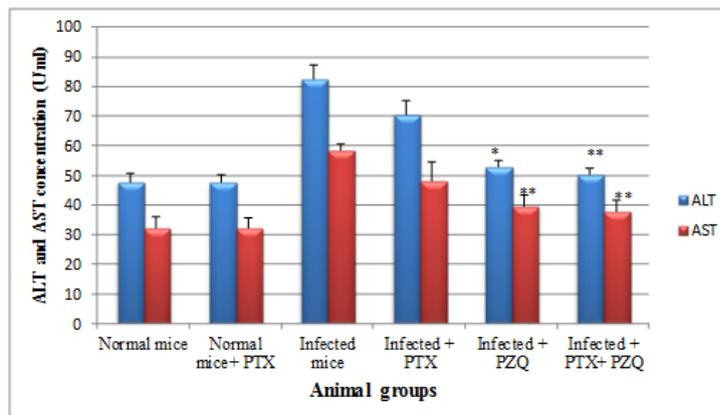


Fig. 2: The effect of PTX and PZQ administrations; each alone or in combinations with each other; on ALT and AST in mice infected with 100 *S. mansoni* cercariae; 4 wk PI and sacrificed 12 wk PI.

## DISCUSSION

The present study revealed that PTX treatment singly had a marked significant reduction in worm burden (21.8%); ova/gm tissue in intestinal and hepatic vessels (24.8 in intestinal tissue and 11.5% in hepatic tissue; respectively). This coincides with the result of El-Lakkany and Nosseir (2007) who reported 22.6% worm reduction. Also, the administration of PZQ alone or combined to PTX caused a high eradication percentage of worms, significant reduction in tissue egg load, in number and diameter of granulomas. The antischistosomal drug caused death of worms through making metabolic disorders, mechanical destruction and muscular contraction of the treated worms (García *et al.*, 2006). Moreover, Martins-Leite *et al.* (2008) concluded that the chemotherapy with PZQ is effective in reducing the morbidity of the disease. Guirguis (2012) reported that reduction in the egg tissue count of infected host after PZQ treatment could be through hindering the process of oviposition. The percent reduction in the egg count was found to be higher in the intestinal tissue than in the hepatic tissue. This variation was attributed to excretion of some ova from the intestine prior to digestion and to hepatic shift of worms after treatment (Ebeid *et al.*, 2008).

In the present work, PZQ either alone or combined with PTX caused the disappearance of immature egg stages; decrease in the number of mature eggs and increase in the number of dead eggs and this is in agreement with the findings of Botros *et al.* (1996). PTX alone caused an increase in immature and dead eggs and a decrease in the mature eggs. This was in accordance with the findings of El-Lakkany and Nosseir (2007) and Gouveia *et al.* (2019).

The manifestations of Schistosomiasis were attributable to granulomatous inflammation around parasite eggs (Abath *et al.*, 2006) in host liver and intestine. The formation of granulomas depends predominantly on CD4+ T helper (Th) cells, specific for antigens secreted from viable schistosome larvae within eggs trapped in host tissue (Garcia *et al.*, 2008); and represents a delayed-type hypersensitivity (Pearce, 2005). In the present study, the granuloma reached the maximal number ( $13.4 \pm 0.927$ ) and diameter ( $342.04 \pm 7.208$ ), at 8 wk PI, in the infected untreated mice. The liver of this group showed collagen fibers and lymphoid cells as well as mononuclear leucocytes and there were diffuse proliferation of kupffer cells, pigmented cells and mononuclear leucocytes inflammatory cells infiltration in between the degenerated hepatocytes in association with karyomegaly in the nuclei of the hepatocytes (Rojkind and Valadez, 1985; Ibrahim *et al.*, 2014).

In the present work; a great improvement in the histopathological aspects in infected mice treated with PTX which caused a reduction in granuloma count and in granuloma diameter. The improvement of the histopathological situation of the liver was evidenced by the decreased number and size of granuloma and the disappearance of pathological changes in hepatocytes (Kamel *et al.*, 2000). There was circumscribed round granuloma formation in the hepatic tissue with centrally located ova surrounded by abundant collagen, less cellular lymphoid cells and mononuclear and leucocytes diffuse kupffer cells proliferation in between the degenerated hepatocytes with normal histological collagen fibers. This agrees with the observation of El-Lakkany and Nosseir (2007). Khalifa and Nemenqani (2014) concluded that pentoxifylline (PTX) caused significant reductions in granuloma sizes, hepatic hydroxyproline and serum levels of leptin and transforming growth factor-  $\beta$ 1. PTX decreased the

intragranulomatous eosinophil accumulation possibly due to its immunomodulatory capability; interfering in cellular recruitment and/or differentiation (Conceição *et al.*, 2005).

The group treated with PZQ had a granuloma with centrally located ova surrounded by fibrous tissue collagen and lymphoid as well as mononuclear leucocytes were detected in the hepatic tissue in an adhesive manner. Kupffer cells proliferation with mononuclear leucocytes infiltration were observed in between the degenerated hepatocytes; as well as; in between the karyomegalic cells. This agrees with the finding of El-Banhawey *et al.* (2007) who concluded that PZQ was effective in reducing granuloma size in infected mice. The treatment of *S. mansoni*-infected mice with PZQ showed a minimal decrease in the number of granulomas two wk PI with diffused kupffer cells proliferation in between the degenerated hepatocytes (Kamel *et al.*, 2000). Marked improvements of the histopathological changes were observed in group treated with PTX+PZQ, where, data recorded reduction in granuloma count and diameter. The histopathological change showed that the granuloma formation had mostly collagen content associated with dilatation in central vein and proliferated kupffer cells in between the hepatocytes (Farrag *et al.*, 2015). El-Lakkany and Nosseir (2007) stated that treatments with PTX and/or PZQ ameliorated the liver functions. Also, Rabia *et al.* (2010) stated that the addition of antifibrotic drugs PTX or silymarin to PZQ ameliorated the liver status.

Serum enzymes (AST and ALT) are a helpful screening way for the detection of liver damage (Badrack and Turner, 2016). In the present work; the infected control mice showed a significant increase in serum ALT and AST than normal controls. Similarly, raised activities of these enzymes have been recorded by (Ezzat *et al.*, 2011). This alteration seemed to be due to hepatic cell damage and impaired cell membrane permeability (Ahmed, 1995) or due to heavy schistosome egg deposition (Panic *et al.*, 2017). The administration of PTX decreased the concentration of ALT and AST but insignificantly; compared to the infected untreated control group. Massart *et al.* (2012) stated that long-term PTX therapy achieves effectively sustained biochemical improvement. This correlates well with histological resolution of the disease. A tendency for the normalization of serum AST and ALT in this study; was noticed in group treated with PZQ, where, it caused a highly significant reduction in the concentration of both ALT and AST than infected group. This agrees with the findings of Utzinger *et al.* (2001). These decreased levels after PZQ administration might be due to loss of hepatic tissue proteins that follows hepatic necrosis (Badawy *et al.*, 1996).

In this work, combined treatment of PTX with PZQ caused a highly significant decrease in liver enzymes as compared to the infected control group. This can be explained by the dual action of the antifibrotic action of PTX, in addition to its antioxidative properties, through inhibiting the free oxygen radicals of toxins released by eggs and worms or acting as a hydroxyl radical scavenger (Shukla and Gude, 2003) and the anti-helminthic drug.

## CONCLUSION

According to the parasitological, histopathological and biochemical criteria, the present findings indicate that PTX treatment for 8 wk has a partial toxic effect on worms, eggs and it ameliorates the liver functions. The combination of PZQ and PTX can successfully be introduced into antischistosomal therapy as a potent antifibrotic agent with immunomodulatory properties in combination with PZQ. Other studies on the effect of PTX on substances that regulate fibrosis or participate in the generation of the liver extracellular matrix were necessary for the complementary evaluation and monitoring of the effect of PTX on the progression of schistosomal hepatic fibrosis.

### Declarations:

### Ethics approval and consent to participate:

Ethical approval had been granted approval by the Ethics Committee of Theodor Bilharz Research Institute (TBRI).

### Availability of data and materials:

All the data obtained during the study are presented in this manuscript. Any further enquiries for additional information are available upon request from the corresponding author.

### Competing interests

The authors declare that they have no competing interest.

### Authors' contributions

AMI conceived and designed the study, performed the experiments, analyzed the data and wrote the first draft. AMI and TMH made the infection of the mice. AMI and HA revised and edited the manuscript. All authors read and approved the final manuscript.

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