Original Article

Experimental *in vivo* assessment of immunomodulatory effect of Kalobin (*Pelargonium reinforme/sidoides* extract) on schistosomiasis *mansoni*

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ABSTRACT

Background: Schistosomiasis is a major tropical disease with significant morbidity and mortality in several developing countries. Hence, searching for a new immunomodulating supportive aid for the sole drug of choice, praziquantel (PZQ), is an important target.
Objective: The aim of the present work is to assess the immunomodulatory effect of Kalobin (*Pelargonium reinforme/sidoides* extract) on schistosomiasis *mansoni in vivo*.
Material and Methods: Swiss albino mice were infected with *S. mansoni* cercariae and were divided into two major categories, immunocompetent (IC) and immunosuppressed (IS). Immunosuppression was performed 14 successive days prior to infection. Kalobin and PZQ, either individually or combined, were given orally seven weeks post infection (wpi). Only in IC mice, both drugs were given in a combination of 100 mg/kg seven wpi for five consecutive days as a preliminary trial. Other groups were treated with PZQ (IC2, 50 mg/kg and IC1, 200 mg/kg) or Kalobin (IC3, 200 mg/kg) or combined PZQ-Kalobin treatment (IC4, 50 mg/kg each) for five consecutive days. This regimen was administered to both IC and IS subgroups and compared to the negative uninfected non-treated control subgroup and the two corresponding positive infected non-treated control CIC and CIS subgroups. All mice were sacrificed 9 wpi for assessment of parasitological parameters including total worm burden (TWB), tissue egg load, oogram pattern, and measurement of hepatic granuloma number and size. Immunohistochemical procedure was employed to assess the expression of vascular endothelial growth factor (VEGF) in both hepatocytes and sinusoids.
Results: Therapy with combined treatment (IC5, 100 mg/Kg each) proved to be superior to the sole PZQ treatment (IC1, 200 mg/kg and IC2, 50 mg/kg) as shown by its effect on TWB and oogram pattern and greater reduction in intestinal and hepatic egg counts in IS groups. Combined PZQ-Kalobin therapy (IC4, 50 mg/kg each) approached the higher individual PZQ dose (IC1, 200 mg/kg) in reducing the granuloma number. The highest reduction in the expression of VEGF in hepatocytes and sinusoids was recorded in the combined PZQ-Kalobin (IC4 and IS4, 50 mg/kg each) followed by subgroup IC3 (Kalobin 200 mg/kg) then IC1 (PZQ 200 mg/kg). Moreover, as regards the IS subgroups; IS4, PZQ-Kalobin 50 mg/kg combination and IS3, Kalobin 200 mg/kg showed better results than all IC subgroups. Individual Kalobin in (IS3) subgroup showed better response than in IC3 subgroup using the same dose of 200 mg/kg.
Conclusion: Our study highlighted the immunopotentiating outcome for Kalobin whether in combination with PZQ or individually on schistosomiasis *mansoni in vivo*.

Keywords: anti-schistosomal, experimental study, immunomodulation, kalobin, praziquantel, *S. mansoni*, VEGF.

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INTRODUCTION

Schistosomiasis remains one of the most prevalent parasitic diseases in the world with more than 240 million people infected in 78 countries[1]. It ranks as the second dangerous disease after malaria in terms of its socioeconomic and public health importance in developing countries, especially in Sub-Saharan Africa. Worldwide, at least one out of thirty individuals suffer schistosomiasis[2,3].

Treatment and morbidity control of schistosomiasis relies on a single drug, PZQ which increases the possibility of anti-helminthic resistance . This compromises the long-term effect of treatment and preventive therapeutic strategies and, increases possibilities of anti-helminthic resistance. Therefore, there is a pressing need to develop additional therapeutics against schistosomiasis[4], preferably from natural sources. Organically derived drugs may act directly as antimicrobial agents and/or indirectly

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by increasing cellular and humoral immunity against the organism[5].

Immunostimulants strengthen the natural resistance and activate different elements and mechanisms of the immune system of humans and animals[6]. Used as herbal medicine *Pelargonium* plants species are known to significantly promote the health care of populations in the Southern African region. The immunostimulating effect of *P. reinforme/sidoides* extract (Kalobin) was attributed to its bioactive ingredients: the tri- and tetraoxygenated cumarine, gallic acid and gallic acid methyl ester (polyphenols), various flavonoids, as well as significant levels of calcium and silica[5].

Kalobin showed effective immunopotentiator activity against *Prohemistomum vivax* infection in mice (a Cyathocotylidae trematoda of the fish-eating piscivorous animals) leading to a significant decrease in the worm burden in treated infected group[5]. Due to its content of significant amounts of phenolic compounds that have high antioxidant and free radical scavenging actions, *P. reinforme* roots reinstated liver enzymes, serum bilirubin, and protein to normal levels in alcohol induced liver damage[7]. In addition, it produced leishmanicidal effects on *L. major* in infected macrophages[8] and exhibited antimicrobial and antifungal properties[9]. Certain components of essential oils in *P. hortorum* that belongs to the same genus as *P. reiniforme*, possess anti-parasitic activity, and exhibit insecticidal, anti-bacterial and anti-fungal properties, among a range of other biological functions[10]. Recently, *in vitro* anti-parasitic activity of *P. x asperum* essential oil was demonstrated against *T. gondii*. It inhibited *T. gondii* growth in a dose-dependent manner, reduced tachyzoites burden, and caused roughness of tachyzoites' membrane. Subsequently, it reduced tachyzoites invasion due to its damaging effect on the capacity of tachyzoites to move[11].

Vascular endothelial growth factor (VEGF) plays an important role in chronic schistosomiasis. This sensitive marker increases significantly during liver fibrosis. It regulates several pro-fibrotic and immune cytokine genes in hepatic stellate cells (HSCs), including integrin, fibronectin, INF-γ, IL-6 and IL-10[12]. Thus, Kalobin presented a hopeful candidate for evaluation for the first time as an immunostimulant when combined to PZQ. Accordingly, the aim of the present study was to assess *in vivo* the immunomodulatory effect of Kalobin on schistosomiasis *mansoni* as regards disease progression in different groups of IC and IS infected mice.

MATERIAL AND METHODS

This case-control experimental study was conducted in the Animal House at Theodore Bilharz Research Institute (TBRI), Giza Governorate, Egypt; from September 2020 to July 2021.

Study design: The study started with 80 mice, 20 for the control group and 60 for the experimental groups after being successfully infected with *S. mansoni* cercaria. The control mice included positive control (infected non-treated), and negative control (uninfected non-treated). The positive control and experimental mice were divided into two major categories, IC, and IS. A preliminary study was conducted and showed promising results when IC mice were administered combined therapy of Kalobin and PZQ (IC5, 100 mg/kg each). Accordingly, five treatment regimens were settled for IC subgroups including 200 mg/kg PZQ (IC1); 50 mg/kg PZQ (IC2); 200 mg/kg Kalobin (IC3); 50 mg/kg combined PZQ-Kalobin therapy (IC4); and 100 mg/kg combined PZQ-Kalobin therapy (IC5). Settled regimens for IS subgroups included 200 mg/kg PZQ (IS1); 50 mg/kg PZQ (IS2); 200 mg/kg Kalobin (IS3); and 50 mg/kg combined PZQ-Kalobin therapy (IS4). All mice were sacrificed 9 wpi for parametric comparisons that included parasitological and immunohistochemical assessment.

Experimental animals: The study included 80 male Swiss albino mice ~6-8 weeks old, weighing 18-22 g, housed at the Animal House of TBRI under controlled temperature and light conditions, and provided with water and commercial chow ad libitum.

Drug preparation, dose, and administration: PZQ tablets (600 mg active substance each) (Epiquantel, Egyptian International Pharmaceutical Industries Company, EIPICO, Egypt) were ground into powder. It was prepared freshly and administered for each mouse in a dose of 200 mg/kg/d; *i.e.*, 40 mg ground PZQ were dissolved in 3 ml Cremophore-El to prepare the dose given to 10 mice (0.3 ml for each mouse). Kalobin (a dietary supplement) was obtained as oral drops solution (Marcyrl Pharmaceutical Industries El Obour City, Egypt). It was given as 200 mg/kg/d; i.e., each mouse received 1.5 ml and 0.75 ml (in 2:1 dilution of Kalobin in DW) for mice administration of 100 and 50 mg/kg/d, respectively. PZQ and Kalobin and their combinations were administered orally[5,13,14] in the scheduled doses seven wpi for five consecutive days[14].

Mice immunosuppression was attained by oral administration of synthetic corticosteroids of Dexasone 0.5 mg tablets (Cadila Pharmaceuticals Ltd, India). For preparation, a half tablet was dissolved in 15 ml DW for immunosuppression of 50 mice. Each mouse received 0.3 ml, i.e. a dose of 0.25 mg/kg/d. Mice were immunosuppressed for 14 successive days[15] prior to infection with *S. mansoni* cercariae. The success of immunosuppression was recorded when the mice started to suffer hair loss and decreased activity. All drugs were administered by oral-gavage using a stainless-steel oral cannula.

Cercariae collection: *S. mansoni* cercariae were recovered from laboratory-bred infected *B. alexandrina* snails 20-30 d after exposure to miracidia at the Medical Malacology Laboratory in TBRI. Infected snails were collected from the aquarium, washed in dechlorinated water by storage in an aerated place for one week before use. Snails were maintained under standard conditions, in 50 ml beakers, filled up to 1/3 with dechlorinated water and subsequently exposed to artificial light for two hours[16]. More than 30 snails were used to avoid unisexual infection. After two hours, the snails were removed and cercarial suspension was gently mixed. One ml was pipetted on a counting slide. To calculate infection load for each mouse, the cercariae on the slide were killed by addition of 10% formalin then stained with a drop of Lugol's iodine. For accuracy, 3 to 5 samples were counted to exclude the counts that showed deviation more than 15% from the mean[17].

Mice infection: Mice were infected by body immersion technique. First, they were placed in clean water to allow them to defecate and urinate to avoid damage to cercariae. Then, they were divided separately (one in each jar) with a small amount of DW containing 70±10 *S. mansoni* cercariae. They were left for 2 hours, and then transferred to their cages[18].

Study groups: According to the mortality rate observed (Table 1) due to mice immunosuppression, the study groups were formulated (Figure 1). Notably, the preliminary study using the combined PZQ-Kalobin therapy (IC5, 100 mg/kg each) for five consecutive days 7 wpi, showed the highest reduction for TWB (100%), intestinal (85.4%) and hepatic ova count (67%) and promising oogram patterns. Since these results were attributed to the PZQ high dose (IC1,100 mg/kg) but weaker effects of Kalobin (IC3, 200 mg/kg) in IC mice, a PZQ-Kalobin combination of 50 mg/kg of each drug (IC4) was compared to PZQ doses (IC1, 200 and IC2, 50 mg/kg).

Parasitological parameters

Total worm burden (TWB)[19]: The percentage of reduction of worm burden in all infected groups was calculated. Worm burden reduction % = [(mean worms from control group – mean worms from treated group)/ mean worms from control group] X 100.

Tissue egg count/gm intestine and liver[20]: The percentage of reduction of ova count in intestine and liver in all infected groups was calculated[21]. Ova count reduction% = [(mean ova from control group – mean ova from treated group)/mean ova from control group] X 100.

Oogram pattern[16]: Immature ova were classified into 4 stages (Figure 2); stage I: embryo one-third diameter of the ovum; stage II: embryo one-half diameter of the ovum; stage III: embryo two-third diameter of the ovum, and stage IV: embryo occupying the whole ovum shell. The accepted functional drug dose against *S. mansoni* was considered as that in which the oogram showed 50% or more mature ova, absence of one or more stages of immature ova or increase of dead ova.

Histopathological examination[22]: Around half of the liver was cut by scissors, and fixed in 10% neutral buffered formalin. Histological sections were

Table 1. Mortality rates in the control and experimental groups.

Groups	Total number of mice	Number of dead mice	Mortality rate
Control positive			
CIC	5	0	0%
CIS	9	4	44.4%
Experimental			
IC	30	4	13%
IS	30	8	26%

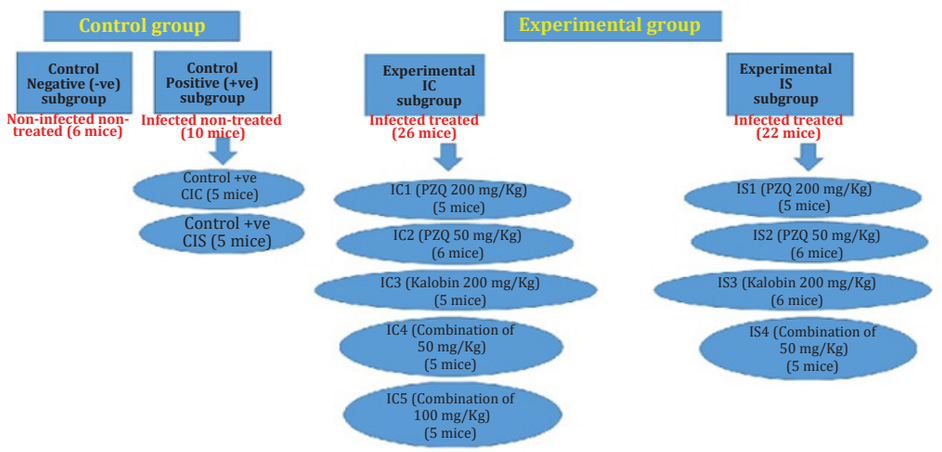

Fig. 1. Study groups.
IC: Immunocompetent;
IS: Immunosuppressed;
CIC: Control (+ve) infected non-treated IC subgroup;
CIS: Control (+ve) infected non-treated IS subgroup.

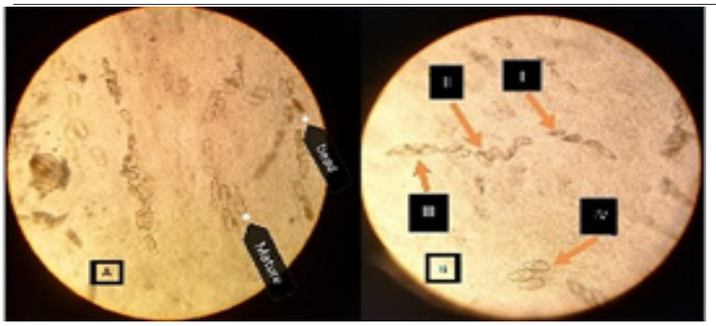

Fig. 2. Oogram pattern for *S. mansoni* ova (10X) showing **(A)** Mature and dead ova; **(B)** Immature viable ova. **Stage I:** Embryo one-third diameter of the ovum. **Stage II:** Embryo one-half diameter of the ovum. **Stage III:** Embryo two-third diameter of the ovum. **Stage IV:** Embryo occupying the whole ovum shell.

processed and stained with H&E. The histopathological changes and the diameters of hepatic granulomas were examined using an ocular micrometer. Only the granulomas around single eggs were measured and egg viability was assessed microscopically in the same sections. Granuloma diameters were calculated in 10 successive microscopic fields (x400)/section/animal. The greatest diameter and its perpendicular diameter were measured and the mean of both diameters was the registered granuloma diameter. The mean size of granulomas together with the mean number of granulomas/low power field (x10 objective), were calculated for each group.

Immunohistochemical assessment: All chemicals were purchased from Sigma Chemicals Co., USA, unless otherwise mentioned. For detection of VEGF in hepatocytes and sinusoids, the normal avidin biotin immunoperoxidase method was used[22,23]. Paraffin sections (5 µm thick) were hydrated in ascending grades of ethanol and dewaxed in xylene. The sections were subjected to EDTA buffer (pH 8.0) for 20 min in a microwave at 700 W to allow good adherence of the tissues to the slides. Sections were covered with primary antibodies against VEGF diluted 1:20 and 1:40, in phosphate buffer solution (PBS), and incubated overnight at 4°C in a humid container. Then, the sections were incubated at room temperature for 15 min with biotinylated secondary anti-mouse antibody. A further washing in PBS was performed, then the slides were incubated with an avidin–biotin complex peroxidase solution. Sections were stained with Meyer© hematoxylin and dehydrated in ascending grades of ethanol prior to mounting in dpx. The liver sections were examined using a Zeiss light microscope. The sites of VEGF expression were identified in hepatocytes and sinusoids. The evaluation of immunoreactivity was estimated by counting the number of all positively brown stained cells. The highest record in 10 successive fields (x400) was counted semi-quantitativly in each section of each animal. The final result signified the mean of these readings per group.

Statistical analysis: Recorded data were analysed using the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA). Quantitative data were expressed as mean±SD. Qualitative data were expressed as frequency and percentage. ANOVA was used to compare between more than two means. Post Hoc test was used for multiple comparisons between different variables. Independent-samples *t*-test of significance was used to compare between two means. The confidence interval was set to 95% and the margin of error accepted was set to 5% (P<0.5).

Ethical consideration: The study was applied according to the Schistosome Biology Supply Center (SBSC) of TBRI in accordance with those of Higher Ministry of Education code of ethics. All experimental procedures were conducted according to the ethical standards approved by the institutional Animal Ethics Committee guidelines for animal care and use, Ain Shams University, Cairo, Egypt under registration number FWA 00006644.

RESULTS

Worm burden: Assessment of the TWB in the control positive mice, by independent sample t test revealed a statistical significance between control positive infected non-treated IC subgroup (CIC) and control positive infected non-treated IS subgroup (CIS) as regards the mean number of males in liver and portomesentric vessels and, also the total count in both. Regarding the mean number of females in liver and ova count/g liver tissue, the statistical relation was significant while non-significant results were noticed concerning the mean number of copula in liver, number of females and copula in portomesentric vessels, oogram pattern and ova count/gm intestine. This denoted that TWB was higher in IS subgroup which ensured that the mice became immunosuppressed under the effect of Dexasone (Table 2).

As regards the effect of Kalobin alone (IC3, 200 mg/dl) or with PZQ (IC4, 50 mg/kg and IC5, 100 mg/kg each) on TWB between IC1-5 subgroups (Table 3), ANOVA showed a statistical significance (P<0.001) for number of males in liver and number of males and copula in portomesentric vessels and also in the total count. The statistical relation of copula in liver was significant. However, statistically the number of females in liver and portomesentric vessels was non-significant. The effect of PZQ-Kalobin combination (IC5; 100 mg/kg each for five consecutive days) took

Table 2. Comparison between TWB, oogram, and ova count/g tissue among positive CIC and CIS control subgroups.

Total worm burden (TWB)	Control positive infected non-treated subgroups		Statistical analysis	
	CIC	CIS	*t* test	*P* value
Liver				
Male	2.00 ± 0.71	4.40 ± 0.55	-6.000	**<0.001***
Female	0.20 ± 0.15	2.60 ± 1.14	-4.382	**0.002***
Copula	0.60 ± 0.25	0.20 ± 0.15	1.265	0.242
Portomesentric				
Male	1.20 ± 1.10	5.60 ± 1.67	-4.919	**<0.001***
Female	0.40 ± 0.89	0.00 ± 0.00	1.000	0.347
Copula	7.00 ± 1.00	8.00 ± 1.00	-1.581	0.153
Total	19.00 ± 1.58	29.00 ± 1.00	-11.952	**<0.001***
Oogram				
Immature	56.20 ± 5.40	62.80 ± 5.26	-1.956	0.086
Mature	37.20 ± 5.07	31.20 ± 4.55	1.970	0.084
Dead	6.60 ± 1.14	6.00 ± 1.00	0.885	0.402
Egg count/gm tissue				
Liver	11441.20 ± 1396.46	15854.88 ± 3175.54	-2.845	**0.022***
Intestine	17818.20 ± 2566.22	22940.61 ± 5247.59	-1.961	0.086

CIC: Positive control (infected non-treated IC subgroup); **CIS:** Positive control (infected non-treated IS subgroup); ***:** Significant (*P*<0.05).

Table 3. Effect of Kalobin with or without PZQ on total worm burden between IC1-5 subgroups in comparison to CIC subgroup.

Total worm burden (TWB)	CIC	Experimental IC subgroups					Statistical analysis	
		IC1	IC2	IC3	IC4	IC5	ANOVA	*P* value
Liver								
Male		#		#@≠	≠†	#@≠†‡		
Mean ± SD	2.00±0.71	0.40±0.25	2.17±0.41	1.40±0.89	2.00±0.00	0.00±0.00	15.113	**<0.001***
Range	1-3	0-1	2-3	0-2	2-2	0-0		
Female			#					
Mean ± SD	0.20±0.15	0.60±0.49	0.00±0.00	0.20±0.15	0.40±0.25	0.00±0.00	1.183	0.346
Range	0-1	0-2	0-0	0-1	0-1	0-0		
Copula		#	#	≠	#≠†	#≠†		
Mean ± SD	0.60±0.25	0.00±0.00	0.00±0.00	0.60±0.25	0.00±0.00	0.00±0.00	5.081	**0.002***
Range	0-1	0-0	0-0	0-1	0-0	0-0		
Portomesentric								
Male		#	#	≠	#≠†	#@≠†		
Mean ± SD	1.20±1.10	0.20±0.15	0.33±0.52	1.40±0.55	0.00±0.00	0.00±0.00	5.907	**<0.001***
Range	0-2	0-1	0-1	1-2	0-0	0-0		
Female			#					
Mean ± SD	0.40±0.89	0.00±0.00	0.00±0.00	0.20±0.15	0.00±0.00	0.00±0.00	0.887	0.504
Range	0-2	0-0	0-0	0-1	0-0	0-0		
Copula		#	#	#@≠	#†	#†		
Mean ± SD	7.00±1.00	0.00±0.00	0.00±0.00	2.40±0.55	0.00±0.00	0.00±0.00	194.752	**<0.001***
Range	6-8	0-0	0-0	2-3	0-0	0-0		
Total		#	#	#@≠	#≠†	#@≠†‡		
Mean ± SD	19.00±1.58	1.20±0.84	2.50±0.55	9.20±1.92	2.40±0.55	0.00±0.00	218.656	**<0.001****
Range	17-21	0-2	2-3	7-12	2-3	0-0		

CIC: Positive control (infected non-treated IC subgroup); **IC:** Immunocompetent (infected and treated subgroups 1-5). **For ANOVA: *:** Significant (*P*<0.05). **For Post HOC: #:** Significant difference with **CIC**; **≠:** Significant difference with **IC1**; **@:** Significant difference with **IC2**, **†:** Significant difference with **IC3**; **‡:** Significant difference with **IC4**.

the upper hand followed by PZQ (IC1; 200 mg/kg for five consecutive days) then PZQ-Kalobin combination (IC4; 50 mg/kg each for five consecutive days) while the least effect was shown from the individual Kalobin (IC3; 200 mg/kg). Furthermore, the effect of PZQ-Kalobin combination (IC4; 50 mg/kg each) approached the individual PZQ (IC2; 50 mg/kg).

As regards the IS subgroups the effect of Kalobin alone (IS2; 200 mg/dl) or with PZQ (IS4; 50 mg/kg each) on TWB between IS1-4 subgroups (Table 4), there was a statistical significance (*P*<0.001) as regards the number of males and females in liver, protomesentric males and copula together with the total count number. Significant difference (*P*=0.018) among number of females in portomesentric vessels was observed. However, the number of copulas in liver showed a non-significant outcome (ANOVA). It was noticed that the female worms were more sensitive to treatment in most of the subgroups than males. Again, this denoted that PZQ (IS1; 200 mg/kg) action took the upper hand followed by Kalobin and PZQ combination (IS4; 50 mg/kg each) then Kalobin (IS; 200

Table 4. Effect of Kalobin with or without PZQ on total worm burden between IS1-4 subgroups in comparison to CIS subgroup.

Total worm burden (TWB)	CIS	Experimental IS subgroups				Statistical analysis	
		IS1	IS2	IS3	IS4	ANOVA	*P* value
Liver							
Male		#	#	≠	#≠†		
Mean ± SD	4.40±0.55	0.40±0.25	2.17±0.41	3.00±1.26	1.60±1.14	15.251	**<0.001***
Range	4-5	0-1	2-3	1-5	0-3		
Female		#	#	#≠	#≠†		
Mean ± SD	2.60±1.14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	29.130	**<0.001***
Range	1-4	0-0	0-0	0-0	0-0		
Copula							
Mean ± SD	0.20±0.15	0.00±0.00	0.00±0.00	0.33±0.22	0.00±0.00	1.375	0.309
Range	0-1	0-0	0-0	0-1	0-0		
Portomesentric							
Male		#	#	#≠	#†		
Mean ± SD	5.60±1.67	0.00±0.00	0.33±0.52	2.00±1.41	0.00±0.00	28.346	**<0.001***
Range		0-0	0-1	0-4	0-0		
Female				#≠	†		
Mean ± SD	0.00±0.00	0.00±0.00	0.00±0.00	0.50±0.35	0.00±0.00	4.278	**0.018***
Range	0-0	0-0	0-0	0-1	0-0		
Copula		#	#	≠@	#†		
Mean ± SD	8.00±1.00	0.00±0.00	0.00±0.00	2.50±0.84	0.00±0.00	180.006	**<0.001***
Range	7-9	0-0	0-0	2-4	0-0		
Total		#	#	#@≠	#≠†		
Mean ± SD	29.00±1.00	0.40±0.25	2.50±0.55	11.17±1.17	1.60±1.14	858.450	**<0.001***
Range	28-30	0-1	2-3	10-13	0-3		

CIS: Positive control (infected non-treated IS subgroup); **IS:** Immunosuppressed (infected and treated subgroups 1-4). **For ANOVA: *:** Significant ($P<0.05$). **For Post HOC: #:** Significant difference with **CIS**; **≠:** Significant difference with **IS1**; **@:** Significant difference with **IS2**, **†:** Significant difference with **IS3**.

mg/kg). The effect of Kalobin and PZQ combination (IS4; 50 mg/kg) was better than the individual PZQ (IS2; 50 mg/kg).

Egg burden: Assessment of tissue egg count/gm in liver and intestine (ANOVA) showed statistical significance ($P<0.001$) among the studied IC subgroups as regards egg count/gm liver and intestine (Table 5). All treated subgroups showed reduction in egg count in liver and intestine tissues when compared with the CIC control subgroup. The highest reduction was seen in subgroup IC5.

In the IS subgroups the effect of Kalobin alone (IS2; 200 mg/dl) or with PZQ (IS4; 50 mg/kg each) on ova count/g tissue showed statistically significant difference ($P<0.001$) in egg count/gm liver and intestine. All treated subgroups showed reduction in egg count in liver and intestine tissues when compared with the control CIS subgroup. The highest reduction was seen in subgroup IS4 (PZQ and Kalobin 50 mg/kg each) (Table 6).

Oogram pattern: Effect of Kalobin alone (IC3; 200 mg/dl) or with PZQ (IC4; 50 mg/kg and IC5; 100 mg/kg each) on oogram pattern between IC1-5 subgroups exhibited a statistical significance ($P<0.001$) according to immature, mature and dead egg counts. Disappearance of immature stages was observed in subgroups IC1, IC2, IC4 and IC5 together with a reduction in subgroup IC3 when compared to the control CIC subgroup. There was an increase in mature stages in subgroup IC3 when compared with control CIC subgroup or the other subgroups. Subgroup IC4 showed an increase in dead stages when compared with subgroup IC2. However, subgroup IC3 showed a little increase in dead stages when compared with control CIC

Table 5. Effect of Kalobin with or without PZQ on ova count/g tissue between IC1-5 subgroups in comparison to CIC subgroup.

Egg count/ gm tissue	CIC	Experimental IC subgroups					Statistical analysis	
		IC1	IC2	IC3	IC4	IC5	ANOVA	*P* value
Liver		#	#	#@≠	#≠†	#@†‡		
Mean±SD	11441.2±1396.5	4914.4±1620.3	6529.3±1189.3	8751.2±1517.4	7215.2±1044.0	3802.4±1638.0	18.936	**<0.001***
Range	9929-13250	2500-6513	5000-8100	7173-10714	5500-8100	1703-5523		
Intestine		#	#	#@≠	#≠†	#@≠†‡		
Mean±SD	17818.2±2566.2	3519.2±1267.8	5098.0±1548.9	10260.2±2160.1	5326.6±1448.9	2594.2±672.2	56.196	**<0.001***
Range	14090-20321	2557-5400	3200-7300	7520-12142	3588-7300	1429-3125		

CIC: Positive control (infected non-treated IC subgroup); **IC:** Immunocompetent (infected and treated subgroups 1-5). **For ANOVA: *:** Significant ($P<0.05$). **For Post HOC: #:** Significant difference with **CIC**; **≠:** Significant difference with **IC1**; **@:** Significant difference with **IC2**, **†:** Significant difference with **IC3**; **‡:** Significant difference with **IC4**.

Table 6. Effect of Kalobin with or without PZQ on ova count/g tissue between IS1-4 subgroups in comparison to CIS subgroup.

Egg count/ gm tissue	CIS	Experimental IS subgroups				Statistical analysis	
		IS1	IS2	IS3	IS4	ANOVA	*P* value
Liver		#	#	#@≠	#†		
Mean±SD	15854.9±3175.5	6750.6±2041.6	6529.3±1189.3	10298.0±1916.7	6151.2±1093.3	21.907	**<0.001***
Range	12645-20990	4210-9166	5000-8100	7100-12645	5000-7928		
Intestine		#	#	#@≠	#≠†		
Mean±SD	22940.6±5247.6	3951.8± 1762.9	5098.0±1548.9	8792.8±1485.0	5326.6±1448.9	51.563	**<0.001***
Range	17518.9-28820	2717-6875	3200-7300	7010-11428	3588-7300		

CIS: Positive control (infected non-treated IS subgroup); **IS:** Immunosuppressed (infected and treated subgroups 1-4). **For ANOVA: *:** Significant (*P*<0.05). **For Post HOC: #:** Significant difference with **CIS**; **≠:** Significant difference with **IS1**; **@:** Significant difference with **IS2**, **†:** Significant difference with **IS3**.

subgroup. This denoted that the effect of Kalobin and PZQ combination (IC5;100 and IC4; 50 mg/kg each respectively) and PZQ (IC1; 200 mg/kg) showed the best outcome followed by Kalobin (IC3; 200 mg/kg). Moreover, Kalobin and PZQ combination (IC4; 50 mg/kg each) was better than the individual PZQ (IC2; 50 mg/kg), as it caused a higher increase in dead stages (ANOVA) (Table 7).

Meanwhile in the IS subgroups the effect of Kalobin alone (IS3; 200 mg/kg) or with PZQ (IS4; 50 mg/kg each) on oogram pattern showed statistical significance (*P*<0.001) among the studied IS1-4 subgroups according to immature, mature and dead egg counts. The immature stages disappeared in subgroups IS1 (PZQ 200 mg/dl), IS2 (PZQ 50mg/dl) and IS4 (PZQ-Kalobin 50 mg/dl each).

However, in IS3 (Kalobin 200 mg/dl) there was reduction in ova due to increased dead stages when compared with the control (CIS) subgroup, and was associated with an increase in mature stages when compared with other treated subgroups. Moreover, subgroup IS4 (PZQ-Kalobin 50 mg/kg each) showed an increase in dead stages followed by subgroup IS1 (PZQ 200 mg/kg) when compared to control CIS subgroup. This denoted that the effect of Kalobin and PZQ combination (IS4; 50 mg/kg for each) and PZQ alone (IS1; 200 mg/kg) showed the best outcome followed by individual Kalobin (IS3; 200 mg/kg). In addition, Kalobin and PZQ combination (IS4; 50 mg/kg for each) was better than the individual PZQ (IS2; 50 mg/kg) as it caused a higher increase in the dead stages (ANOVA) (Table 8).

Hepatic granuloma: Histopathological examination of liver tissue aimed to determine granuloma formation and also to assess the effect of treatment on the number and size of granulomas. In uninfected non-treated negative control mice there was normal hepatic architecture that revealed hepatocytes arranged in thin plates with no granulomas or fibrosis. In infected non-treated positive control mice there were reactive changes in the liver cells with inflammatory cellular infiltration, degeneration of hepatocytes, dilated hepatic sinusoids and apparently contained more Kupffer cells. *S. mansoni* ova were seen in the centre of granulomas, where most of them were viable, fewer were dead. Histopathological examination of sections of liver of subgroups (IC1, IC3, IC4, IS1, IS3, IS4) revealed granulomas resembling the positive control subgroup. However, in the positive control subgroup, the percentage of cellular granulomas and viable eggs were higher than those of treated subgroups. The percentages of *S. mansoni* viable and degenerated eggs showed improvement among the combination subgroups (IC4, IS4) (Fig. 3).

The effect of Kalobin alone (IC3; 200 mg/dl) or with PZQ (IC4; 50 mg/kg and IC5; 100 mg/kg each) on hepatic granulomas diameter using post Hoc test revealed a statistically significant relations between all groups

Table 7. Effect of Kalobin with or without PZQ on oogram pattern between IC subgroups in comparison to CIC subgroup.

Oogramgm Pattern	CIC	Experimental IC subgroups					Statistical analysis	
		IC1	IC2	IC3	IC4	IC5	ANOVA	*P* value
Immature		#	#	#@≠	#†	#†		
Mean ± SD	56.20±5.4	0.0±0.0	0.0±0.0	29.0±7.42	0.0±0.0	0.0±0.0	209.963	**<0.001***
Range	48-62	0-0	0-0	20-40	0-0	0-0		
Mature		#	#	#@≠	#@≠†	#@≠†‡		
Mean ± SD	37.2±5.07	8.6±2.19	14.17±4.92	58.0±7.58	6.0±4.28	1.0±0.41	96.477	**<0.001***
Range	31-44	5-10	10-20	50-65	0-15	0-3		
Dead		#	#	#@≠	#†	#≠†‡		
Mean ± SD	6.6±1.14	91.4±2.19	85.83±4.92	13.0±4.47	94.0±6.28	99.0±1.41	595.985	**<0.001***
Range	5-8	90-95	80-90	10-20	85-100	97-100		

CIC: Positive control (infected non-treated IC subgroup); **IC:** Immunocompetent (infected and treated subgroups 1-5). **For ANOVA: *:** Significant (*P*<0.05). **For Post HOC: #:** Significant difference with **CIC**; **≠:** Significant difference with **IC1**; **@:** Significant difference with **IC2**, **†:** Significant difference with **IC3**; **‡:** Significant difference with **IC4**.

Table 8. Effect of Kalobin with or without PZQ on oogram pattern between IC subgroups in comparison to CIC subgroup.

Oogramgm Pattern	CIS	Experimental IS subgroups				Statistical analysis	
		IS1	IS2	IS3	IS4	ANOVA	*P* value
Immature		#	#	#@≠	#†		
Mean ± SD	62.8±5.26	0.0±0.0	0.0±0.0	53.33±6.06	0.0±0.0	409.482	**<0.001***
Range	56-70	0-0	0-0	45-60	0-0		
Mature		#	#	#@≠	#@≠†		
Mean ± SD	31.2±4.55	4.0±2.48	14.17±4.92	27.5±6.89	1.0±2.24	36.095	**<0.001***
Range	25-37	0-10	10-20	20-35	0-5		
Dead		#	#	#@≠	#@†		
Mean ± SD	6.0±1.00	96.00±5.48	85.83±4.92	19.17±9.17	99.0±2.24	342.233	**<0.001***
Range	5-7	90-100	80-90	10-30	95-100		

CIS: Positive control (infected non-treated IC subgroup); **IS:** Immunosuppressed (infected and treated subgroups 1-4). **For ANOVA: *:** Significant (*P*<0.05). **For Post HOC: #:** Significant difference with **CIS**; **≠:** Significant difference with **IS1**; **@:** Significant difference with **IS2**, **†:** Significant difference with **IS3**.

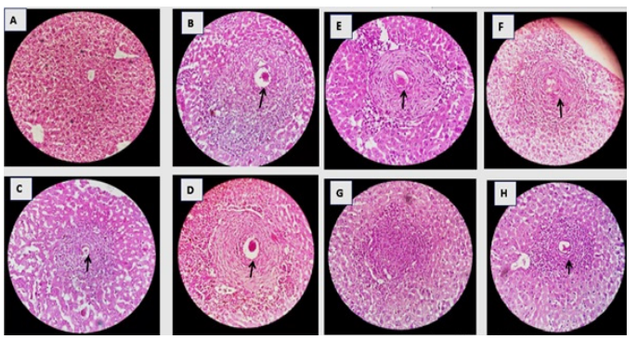

Fig. 3. Histopathological changes in hepatic tissue in different groups 9 wpi (HX&E x400). **(A):** Uninfected non-treated negative control mice showing normal hepatic architecture; **(B):** Infected non-treated positive control mice; **(C):** Immunocompetent, PZQ (IC1; 200 mg/kg) treated group. **(D):** Immunocompetent, Kalobin (IC3; 200 mg/kg) treated group; **(E):** Immunocompetent, PZQ-Kalobin (IC4; 50 mg/kg) treated group; **(F):** Immunosuppressed, PZQ (IS1; 200 mg/kg) treated group; **(G):** Immunosuppressed, Kalobin (IS3; 200 mg/kg) treated group; **(H):** Immunosuppressed, PZQ-Kalobin (IS4; 50 mg/kg) treated group. **(C-H)** Showed inflammatory response in the liver indicated by inflammatory cellular infiltration, cytoplasmic vacuolation, degeneration of hepatocytes, dilated hepatic sinusoids and more Kupffer cells with central intact ova (arrows) resembling the negative control mice **(B):** All treated groups showed a decrease in size of granuloma when compared with the positive control group.

according to the granuloma diameter. There was a reduction in granuloma diameter in groups IC1, IC3, IC4, IS1, IS3 and IS4 in comparison to the CIC and CIS control groups. Though the recorded effects were very close, PZQ (IC1; 200 mg/kg) showed the best results followed by Kalobin and PZQ combination (IC4; 50 mg/kg each) then the individual Kalobin in IC3 (200 mg/kg) group; while in IS subgroups, the effect of Kalobin and PZQ combination (IS4; 50 mg/kg each) was better than the individual PZQ (IS1; 200 mg/kg and IS2; 50 mg/dl) (Table 9, Fig. 4).

The effect of Kalobin alone (IC3; 200 mg/kg) or with PZQ (IC4; 50 mg/kg and IC5; 100 mg/kg each) on the numbers of hepatic granulomas in successive power fields (10x10) using post Hoc test revealed a statistical significance between the different groups according to the number of granulomas. There was a reduction in the granuloma number in groups IC1 (PZQ 200 mg/kg), IC3 (Kalobin 200 mg/kg), IC4 (PZQ-Kalobin 50 mg/kg each), IS1 (PZQ 200 mg/kg), IS3 (Kalobin 200 mg/kg) and IS4 (PZQ-Kalobin 50 mg/kg each) in comparison to the control CIC and CIS subgroups. Again, the effect of individual PZQ (IC1; 200 mg/kg) showed the best results followed by Kalobin and PZQ combination (IC4; 50 mg/kg each) then individual Kalobin (IC3; 200 mg/kg). The effect of the combination approached the individual PZQ (IC1; 200 mg/kg and IC2; 50 mg/kg) effect (Table 10, Fig 5).

Immunohistochemical assessment: The aim was to evaluate the VEGF expression in hepatocytes,

Table 9. Effect of Kalobin with or without PZQ on hepatic granulomas diameter.

Group/Subgroup		Granuloma diameter		Statistical analysis	
		Mean ± SD	Change (%)	*t* test	*P* value
Control positive SG		368.0±27.23	---		
IC	**IC1**	249.0±24.54	-32.3	7.259	**<0.001***
	IC3	318.0±25.12#	-13.6	3.018	**0.017***
	IC4	265.3±28.53≠	-27.9	5.823	**0.004***
IS	**IS1**	251.0±25.40	-31.8	7.026	**<0.001***
	IS3	312.45±24.0†	-15.1	3.422	**0.009***
	IS4	242.34±31.0‡	-34.1	6.810	**<0.001***

IC: Immunocompetent; **IS:** Immunosuppressed; ***:** Significant (*P*<0.05); **Control positive SG:** Granuloma diameter equivocal in both groups CIC and CIS.

endothelial cell lining of the blood vessels and sinusoids in different subgroups. There was statistically significant differences among all the subgroups in the expression of VEGF in hepatocytes and sinusoides determined by post Hoc. Examination of the healthy uninfected non-treated negative control mice revealed negative VEGF. Examination of the infected non-treated positive immunocompetent control (CIC) revealed positive VEGF expression in hepatocytes (34.5±2.4) and sinusoids (44.5±3.7).

Table 10. Effect of Kalobin with or without PZQ on the numbers of hepatic granulomas in IC and IS subgroups.

Group/Subgroup		Granuloma number		Statistical analysis	
		Mean ± SD	Change (%)	*t* test	*P* value
Control positive SG		11.51±2.29	---		
IC	**IC1**	5.2±2.4	-54.8	4.253	**<0.003***
	IC3	7.25±1.92	-37.0	3.188	**0.013***
	IC4	5.7±2.86#≠	-50.5	3.546	**0.007***
IS	**IS1**	251.0±25.40	-31.8	7.026	**<0.001***
	IS3	312.45±24.0†	-15.1	3.422	**0.017***
	IS4	242.34±31.0‡	-34.1	6.810	**<0.002***

IC: Immunocompetent; **IS:** Immunosuppressed; ***:** Significant (*P*<0.05); **Control positive SG:** Granuloma number equivocal in both groups CIC and CIS.

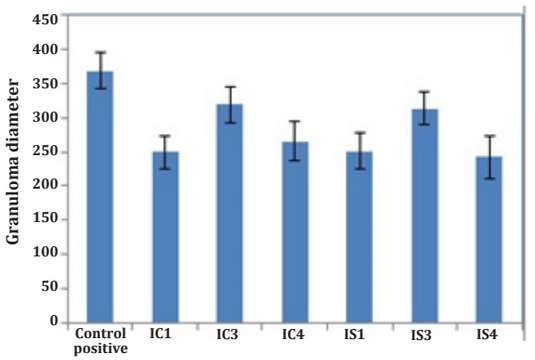

Fig. 4. Bar chart representing hepatic granulomas diameter among the studied subgroups.

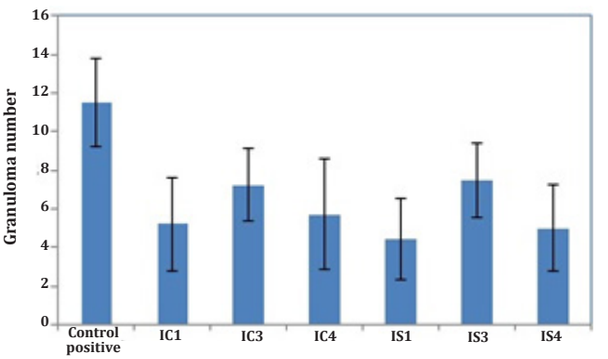

Fig. 5. Bar chart representing the number of granulomas in successive power fields (10x10) among the studied subgroups.

The expression of VEGF was reduced in both hepatocytes and sinusoids in all the treated subgroups compared to the control positive infected non-treated immunocompetent (CIC) and immunosuppressed (CIS). The highest reduction in the expression of VEGF was recorded in combination subgroups IC4 (PZQ-Kalobin 50 mg/kg) and IS4 (PZQ-Kalobin 50 mg/kg) with a reduction percentage in VEGF expression in hepatocytes (66.4% and 74.2%) and sinusoids (77.8% and 80.9%) compared to the rest of the subgroups. This denoted that the effect of Kalobin and PZQ combination (IC4; 50 mg/kg) showed the best results followed by Kalobin (IC3; 200 mg/kg) then PZQ (IC1; 200 mg/kg). Kalobin and PZQ combination (IC4; 50 mg/kg) and Kalobin (IC3; 200 mg/kg) showed better effect in IS subgroups than IC subgroups (Table 11; Fig. 6).

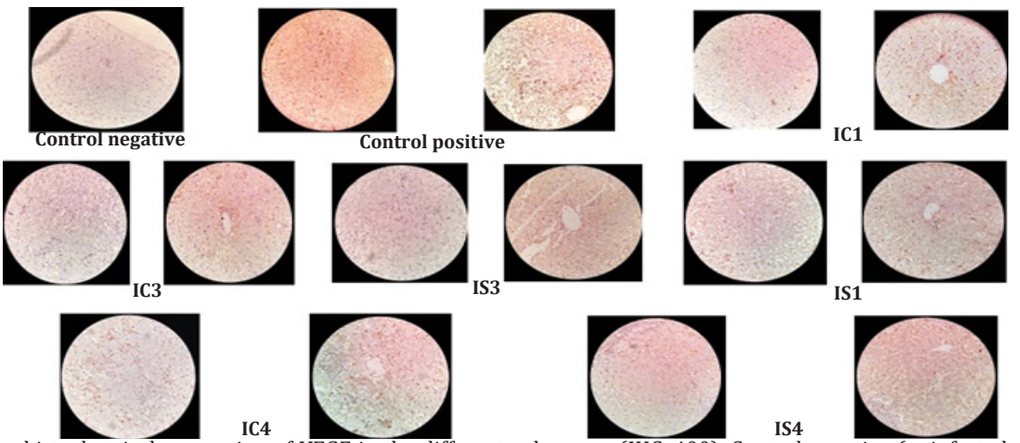

Fig. 6. Immunohistochemical expression of VEGF in the different subgroups (IHCx400). Control negative (uninfected non-treated) with negative expression. Control positive (infected non-treated): strong positive brown stained hepatocytes, sinusoids with anti-VEGF antibody. IC1 (PZQ 200 mg/kg), IS1 (PZQ 200 mg/kg): marked positive brown stained hepatocytes, sinusoids with anti-VEGF antibody. IC3 (Kalobin 200 mg/kg), IS3 (Kalobin 200 mg/kg): moderately positive brown stained hepatocytes, sinusoids with anti-VEGF antibody. IC4 (PZQ-Kalobin 50 mg/kg each), IS4 (PZQ-Kalobin 50 mg/kg each): mildly positive brown stained hepatocytes, sinusoids with anti-VEGF antibody. **IC:** Immunocompetent; **IS:** Immunosuppressed.

Table 11. Effect of Kalobin with or without PZQ on VEGF expression in hepatocytes and sinusoids.

Group/Subgroup		VEGF		Statistical analysis	
		Mean ± SD	Change (%)	*t* test	*P* value
Hepatocytes					
Control positive SG		34.5±2.4	---		
IC	**IC1**	20.65±2.9	-39.9	8.227	**<0.001***
	IC3	17.15±3.6	-50.3	8.967	
	IC4	11.6±3.1$^{\#\neq}$	-66.4	13.061	
IS	**IS1**	22.3±3.5	-35.4	6.429	
	IS3	10.85±2.3†	-68.6	15.909	
	IS4	8.9±1.9†	-74.2	18.701	
Sinysoids					
Control positive SG		44.5±3.7	---		
IC	**IC1**	23.4±2.4	-47.4	10.698	**<0.001***
	IC3	18.35±2.3$^{\#}$	-58.8	13.422	
	IC4	9.9±2.3$^{\#\neq}$	-77.8	17.759	
IS	**IS1**	16.6±3.3	-62.7	12.588	
	IS3	11.7±2.3†	-73.7	16.835	
	IS4	8.5±1.6†‡	-80.9	19.969	

IC: Immunocompetent; **IS:** Immunosuppressed; **VEGF:** Vascular endothelial growth factor; ***:** Significant (P<0.05). **Control positive SG:** VEGF in both hepatocytes and sinusoids equivocal in both groups CIC and CIS. **Post HOC: #:** Significant difference with subgroup **IC1; ≠:** Significant difference with subgroup **IC3; †:** significant difference with subgroup **IS1; ‡:** Significant difference with subgroup **IS3.**

DISCUSSION

Schistosomiasis remains a major health problem with significant socioeconomic impact[24]. The estimated number of disability adjusted life years (DALYs) has actually increased[25]. Expected serious co-morbidities and complications occur especially if neglected[26,27]. For several decades, PZQ has been the only commercially available drug for the treatment of all human schistosome species[28]. Depending on a single drug is inadvisable for any infectious condition, especially those with high prevalence as schistosomiasis[4]. It might lead to the appearance of drug resistance[29]. Praziquantel resistance was first recorded in *S. mansoni* in the mid-1990s[30]. A number of studies suggesting reduced PZQ efficacy in response to mass drug administration (MDA) were previously reported[31-33]. Observed low cure rates can be caused by a range of factors other than resistance, like high infection intensities, high rates of reinfection, low drug absorption and minimal immunological response to previous exposure to schistosomes[34]. Currently no effective alternative treatments are documented[35]. On the other hand, re-purposing of already approved drugs may offer a safe, rapid and cost-effective alternative[36].

Combined treatment with other drugs having different modes of action such as immunostimulatory agents, might be useful. Alone or combined with other drugs, immunostimulatory agents might be valuable adjuvants to reduce morbidity and mortality of schistosomiasis[36]. Kalobin (*P. reinforme*/*sidoides* extract) proved to exert an immunostimulatory effect against mice infected with *Prohemistomum vivax*[5]. It has protective and curative effects on alcohol-induced liver damage with high antioxidant and free radical scavenging activities[7]. Thus, the present study was conducted to assess the immunostimulatory effect of Kalobin on schistosomiasis *mansoni in vivo* regarding disease progression in a comparative experimental study to PZQ in different subgroups of IC and IS infected mice.

In IC subgroups, Kalobin and PZQ in the combination of 100 mg/kg each, for five consecutive days, 7 wpi showed the highest reduction as regards TWB, ova count in intestine and liver and promising oogram patterns. That was consistent with El-Lakkany *et al*.[37] who proved that Mefloquine in combination with PZQ in a dose interval ranging from 1000 descending to 200 mg/kg divided equally and administered for two consecutive days, 7 wpi produced no noticeable differences in the therapeutic efficacy between the reduced and high doses of these combinations[37]. Our results were exceptional compared to these findings, as we used a lower scale of combination dosage which supported the proposed potential of Kalobin.

The current results indicated a better effect of the combined 50 mg/kg dose of PZQ-Kalobin than the individual PZQ (50 mg/kg) as regards all the parasitological parameters. This suggests a better alternative for lowering the dose and decreasing resistance to PZQ. An additional advantage is the

compensation of decreased PZQ effect by the combination with the lower dose (50 mg/kg) of Kalobin. Hence, we preferred to apply this low dose in our study, targeting decreased side effects and toxicity. This low dose (50 mg/kg for five consecutive days) was recommended previously[18,38,39].

Regarding the TWB, unlike the IS subgroup where the 50 mg/kg combination (IS4) dose surpassed the PZQ 50 mg/kg (IS2), in the IC subgroup, the effect of the 50 mg/kg combination (IC4) was near to the sole 50 mg/kg PZQ (IC2). This indicated that Kalobin had a decreasing effect on TWB when used as a low dose combination. This suggests an important option for the proposed dose of this combination. In our study the recorded decreased effect of Kalobin was consistent with the report by Xiao *et al*.[13] who tested Mefloquine, Artesuante and Artemether at different doses combined to PZQ at a single dose of 50 mg/kg or 100 mg/kg, 35 days post infection in S. *japonicum* infected mice. Previous studies confirmed the synergistic potential of drug combinations. In one of those, the combination of Silymarin and PZQ at a dose of 500 mg/kg for two consecutive days in the 7th wpi caused complete eradication of worms[40]. Also, Artemisinin, Arachidonic acid or Nifedipine combined to PZQ at a single dose of 300 mg/kg on day 42 after infection showed a better effect on TWB[41]. Moreover, Turmeric-PZQ combination at a dose of 500 mg/kg for two consecutive days at the 4th wpi caused complete absence of adult worms in mice and so enhanced the individual effect of PZQ[42]. Furthermore, a maximum reduction in TWB was found when using a combination subgroup of PZQ at a single oral dose of 500 mg/kg, 6-wpi, with Artesunate[43]. Our study emphasized the hopeful potential for Kalobin in the applied lower dose (50 mg /kg for five consecutive days).

In IC and IS subgroups, the effect of the 50 mg/kg combination (IC4, IS4) was again confirmed by the oogram pattern. The pattern showed a higher increase in dead stages, and the highest reduction in egg count in intestine and liver in IS4 subgroup. While in IC2 subgroup the effect of the individual 50 mg/kg PZQ surpassed the effect of the 50 mg/kg combination (IC4).This supports the use of Kalobin in IS state as it may have a better impact on immune system and on the progression of the disease. Similarly, El-Lakkany *et al*.[40] demonstrated that the use of Silymarin with PZQ at a dose of 500 mg/kg, caused absence of viable eggs with decrease in the hepatic tissue egg load. Also, Hegazy *et al*.[43] showed the highest reduction in the immature egg count in intestine and liver, and recorded the highest dead egg count in their combination subgroup of Artesunate and PZQ at a single oral dose of 500 mg/kg.

As regards granuloma number in IC and IS subgroups, the effect of the combination (50 mg/kg each in IC4 and IS4) approached the PZQ (200 mg/kg dose in IC1). In other studies, this high PZQ dose was the mostly applied one for granuloma assessment[22,42,44]. While, as regards granuloma diameter, in IS subgroups the combination 50 mg/kg (IS4) [34.1%] surpassed the PZQ 200 mg/kg (IS1) [31.8%] which may be attributed to the immunostimulatory effect of Kalobin, as previously mentioned[5]. That again highlighted the potential effect of Kalobin in IS state. The effect of the combination (50 mg/kg each; IC4) was superior to PZQ in its highest dose of 200 mg/kg (IC1) in decreasing the *S. mansoni* viable eggs and increasing the degenerated eggs as assessed histopathologically.

Formerly, El-Lakkany *et al*.[40] demonstrated that the use of Silymarin with PZQ (500 mg/kg) caused better healing of hepatic granulomatous lesions. Also, Abdel-Ghaffar *et al*.[41] showed that Artemisinin, Arachidonic acid or Nifedipine combined to PZQ at a single dose of 300 mg/kg showed the least granuloma number and diameter better than PZQ at a single dose of 300 mg/kg. Moreover, the combination of both Turmeric and PZQ (500 mg/kg for two consecutive days) reduced granuloma number and diameter than the monotherapy with PZQ (500 mg/kg at the 4th week post infection[42].

The potential of immunostimulatory agents was reported in another study by Zumla *et al*.[45] who highlighted Vitamin D3, Zileuton and Carbamazepine as adjunct host-directed therapies for multidrug-resistant tuberculosis. Also, Richardson *et al*.[46] studied the potent immunostimulatory derivative of Thalidomide (IMiD) CC-5013 to overcome drug resistance in relapsed patients with multiple myeloma. With this concept in mind the effect of Kalobin may help to overcome the feared resistance to PZQ. The PZG-Kalobin combination may provide benefits such as reducing the dose of PZQ and thus its side effects, besides the benefit of adding another safe drug that reinforces the immune system.

Using Kalobin alone showed an unexpected moderate anti-schistosomal potency[37]. In our study it led to a reduction in TWB in all IC (1-5) subgroups and IS (1-4) subgroups. Formerly, using Kalobin on *Prohemistomum vivax* showed a decrease in TWB[5]. Studies using other immunostimulatory agents such as *Curcuma longa* extract[21], Silymarin[40], garlic extract and *Nigella sativa* oil[47], Curcumin[48], the crude extract and the essential oil from *Tanacetum vulgare*[49], garlic oil extract[50], Artemisinin, Arachidonic acid and Nifedipine[41] and Turmeric[42] on *S. mansoni*, showed TWB in various degrees of reduction. Notably, TWB reduction has always been associated with reduced pathology with no relapse because of the fact that schistosome parasites do not multiply in the host[51].

Kalobin caused a reduction in egg count in intestine and liver in IC and IS subgroups, due to a decrease

of total immature ova, indicating the cessation of oviposition[10,52] in addition to increase in dead ova in both IC and IS subgroups. These results were consistent with several authors who evaluated the effect of other immunostimulatory agents on *S. mansoni* and attributed this to the direct effect on female numbers, which resulted in a reduction in egg output and in tissue egg load and also to the inhibition of coupling[21,40,43,47,48,50,53]. Egg reduction decreases egg shedding and the potential for parasite and disease progression[51]. Absence of immature egg stages and the increase of dead eggs have always been considered good indicators for testing the definitive action of medications on *S. mansoni*[13].

Kalobin individually led to uncoupling which has always been used as an indicator of drug potency[42,49,51,54,55]. In our study Kalobin led to a reduction in granuloma diameter and number in sections of liver in IC and IS subgroups with better consequence in IS subgroups, which adds to its potential. That was consistent with other researchers who tested other immunostimulatory agents such as Silymarin[40], Curcumin[48], *Clerodendrum umbellatum*[56], and garlic oil extract[50] on *S. mansoni*. This could be due to the reduction of egg output, tissue egg load and deposition[40,57]. The researchers attributed this to the antioxidative properties that eliminate the products of oxidative reactions and assist in the immune-mediated destruction of worms and eggs, proving their direct impact as anti-schistosomals[40,58]. Female worms were more sensitive than males in most of our treated subgroups which was consistent with reports by other authors[51,59,60]. Any drug is considered of great potential when it selectively targets female fecundity[51].

Supporting the immunostimulatory effect, the PZQ-Kalobin combination (50 mg/kg each) was better than PZQ at a dose of 200 mg/kg in reducing VEGF expression in hepatocytes and sinusoids in IC and IS subgroups with a better effect in IS subgroups. In agreement Botros *et al*.[22] demonstrated that the combination of Artemether and PZQ at a dose of 200 mg/kg (for five consecutive days, 6 wpi) was associated with the highest reduction in VEGF expression in hepatocytes and sinusoids. Kalobin individually at 200 mg/kg, led to a reduction in the expression of VEGF in both hepatocytes and sinusoids in IC and IS subgroups, superior to PZQ at 200 mg/kg. Superiority was more evident in IS subgroups, which again supports its immunostimulatory effect. Its effect on VEGF could explain the reduction of granuloma diameter in sections of liver due to its effect on HSCs. It increases HSCs activation, proliferation and collagen production which play a role in fibrotic pathogenesis[12].

Our study revealed the different effects of Kalobin in IC and IS subgroups as regards granuloma diameter and VEGF expression in hepatocytes and sinusoids. The enhanced effect of Kalobin in IS subgroups supports its use in the IS state, even if used for a long duration since the long-term use of immunostimulatory agents potentiates their effect, reducing and controlling the relapses of the diseases[61].

The role of immunostimulatory agents against *S. mansoni* is through modulation of the immune system. Curcumin was found to modulate granuloma formation through regulation of cytokines expression like TNF-α and inhibition of the release of IL-2 and IL-4 that play a role in granuloma formation[48]. Arachidonic acid plays an important role as precursor of prostaglandins, leukotrienes and thromboxanes[62]. Silymarin has antioxidative properties assisting the immune-mediated destruction of worms and eggs[40,63]. Resiquimod (R848), a low molecular weight imidazoquinolinamine compound, causes a significantly higher level of IL-10, IFN-γ and significantly lower level of IL-4[64]. Ginger extract plays a crucial role through its effect on IgE, IgG and IgM and IL-4, 10 and 12[65]. The combined effect of Turmeric and PZQ was potent due to the special different impact of both drugs on the interleukins as PZQ increases TNF-α, IL-6, IL-8, IFN-γ, IL-12 and IL-23 levels, while Turmeric inhibits TNF-α, IL-6 and IL-8[42].

The precise mechanism of action of Kalobin as an anti-schistosomal was broached by Amer *et al*.[5] who demonstrated that *P. reinforme/sidoides* extract, the main active ingredient of Kalobin, may activate antigen presenting cells in addition to antibody production and augment anti-*Prohemistomum vivax* IgG and IgA production in mice. Moreover, Adewusi and Afolayan[7] explained that the role of *P. reinforme/sidoides* extract in injured liver is the removal of acetaldehyde and the wiping of noxious toxic metabolites' of reactrive oxygen species (ROS). This may protect the liver cells from damage and improve the function after damage. Unsal *et al*.[66] revealed that the antioxidant and detoxification properties of *P. reinforme/sidoides* extract against mushroom poisoning is through reduction of malondialdehyde, increasing Paraoxonase levels and regulating purine metabolism thus reducing ROS. The extract of *P. reinforme/sidoides* also significantly decreased levels of liver enzyme markers (AST, ALT, ALP and GGT) indicating that the extract possessed a curative effect. The increased levels of these enzymes are linked to increased permeability, damage, and necrosis of hepatocytes. *P. reinforme/sidoides* extract caused decrease in serum bilirubin indicating the effectiveness of this drug in the maintenance of normal functional status of the liver. Stabilization of serum protein levels in the post-treatment subgroups administered *P. reinforme/sidoides* extract is further indication of the improvement of the liver cells[7]. Moreover, other studies showed that it contains citronellol, geraniol, 3, 7-dimethyl formate and selinene, which are associated with its antimicrobial activities[9,67]. Gallic acid (the main constituent of *P. reinforme/sidoides*) showed an anti-leishmanial effect promoting macrophage activation through the induction of TNF-α, inorganic nitric oxide and INF-γ production[68].

In conclusion, Kalobin showed synergistic potential with PZQ. Further studies are recommended to assess the cytokine titre and ILs to evaluate its specific immunomodulatory effect against *S. mansoni*.

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