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Effects of *Callistemon citrinus* aqueous extract on prepatent and patent infections with *Schistosoma mansoni* in experimentally infected mice

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Research Paper

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Abstract

Schistosomiasis is a chronic debilitating parasitic disease that causes hepatic damage and is known to be endemic in developing countries. Recent control strategies for schistosomiasis depend exclusively on chemotherapeutic agents, specifically praziquantel. Unfortunately, praziquantel has low efficacy in the early phase of infection, and resistance to treatment is increasingly reported. The aim of this work was to find an alternative treatment by assessing the *in vivo* activity of aqueous extract of *Callistemon citrinus* against *Schistosoma mansoni* in both prepatent and patent phases in experimentally infected mice. The study was conducted on 80 male BALB/c albino mice divided into eight groups. *Callistemon* was administered at a dose of 200 mg/kg on days 14 and 45 post infection as a single therapy and in combination with praziquantel. Porto-mesenteric worm burden, hepatic and intestinal egg counts, hepatic granuloma number and diameter, and oogram pattern were assessed to evaluate the anti-schistosomal properties of *C. citrinus*. Liver enzymes and total bilirubin were tested to assess hepatoprotective effects. Results revealed that the use of *C. citrinus* was associated with a significant decrease in worm burden and tissue egg load together with an increased percentage of dead eggs. In addition, there was a significant reduction in granuloma formation. *Callistemon* also led to a significant improvement in liver function. The best results were obtained when *C. citrinus* was given in the prepatent phase of infection and when combined with praziquantel. Although the effects of *C. citrinus* are considered to be promising, further studies using different extracts, active ingredients and doses are needed.

Introduction

Schistosomiasis is considered a neglected tropical disease that affects millions of people worldwide (Neves *et al.*, 2015). Human infection is caused by flatworms of the genus *Schistosoma*: *Schistosoma mansoni*, *S. haematobium* and *S. japonicum*. The parasite usually causes a chronic debilitating disease that impairs human development and productivity (Colley *et al.*, 2014).

Schistosomiasis presents clinically in acute, subacute and chronic stages. Many cases present with late complications. The acute stage is species-independent and occurs early during invasion and migration of the parasite. The subacute stage occurs after parasitic maturity and settlement in the target organs. Granulomata are formed around eggs or around the dead worms in the colon and rectum in *S. mansoni* infection. The chronic stage occurs due to healing of the granulomas by fibrosis and calcification around trapped eggs. Malignancy may develop in the colon as a complication of the chronic stage, according to the infective species (Barsoum *et al.*, 2013).

Fibrosed granulomatous liver parenchyma in cases of *S. mansoni* infection causes compression and destruction of portal blood vessels, leading to portal hypertension with disabling sequelae. Unfortunately, praziquantel (PZQ) in therapeutic doses cannot reverse such damage. Thus, researchers were encouraged to develop new drugs that could protect the liver. Plant therapy played an important role in the research of schistosomiasis treatment (El Ridi & Tallima, 2013). Praziquantel is the only drug recommended by the World Health Organization (WHO) for treating and controlling human schistosomiasis. However, resistance to the drug has already been identified, indicating the need for new compounds to treat such disease (Neves *et al.*, 2015). In addition, PZQ has been ineffective in killing immature stages of the parasite during recent bilharzial infections, leading to reduced cure rates and failure to abort early infection (Gundamaraju, 2014). Schistosomiasis control over the past 40 years has depended mainly on PZQ, and there is an urgent need to develop new anthelmintics to be used in combination with PZQ in order to increase its efficacy and reduce resistance to PZQ (Bergquist *et al.*, 2017). Efforts are focused on natural plant extracts that may have

effective schistosomicidal activity. However, few studies have focused on isolation, identification and evaluation of these natural extracts (Ndjonka *et al.*, 2013).

Callistemon citrinus is a plant of great medicinal importance. Numerous bacterial, fungal, viral and parasitic diseases have traditionally been treated with this plant (Radulovića *et al.*, 2015). *Callistemon* species are widely encountered in the wet tropics, particularly in Australia, South America and tropical Asia, although they are now available worldwide (Shinde *et al.*, 2012). Previous studies and reports have shown that *C. citrinus* possesses a wide range of biological activities, including wound healing, hepatoprotective, cardioprotective, anti-inflammatory, antidiabetic, hypolipidemic, antioxidant and antithrombotic effects. In addition, it inhibits cholinesterase and elastase enzymes (Goyal *et al.*, 2012) and shows analgesic and antidiarrhoeal effects (Ahmed *et al.*, 2015). It also exerts antimicrobial, nematocidal, larvicidal, pupicidal and insecticidal activities (Ali *et al.*, 2011; Palanikumar *et al.*, 2017). The anthelmintic effects of various extracts of *C. citrinus* leaves on the adult Indian earthworm (*Pheretima posthuma*) were assessed by Pal & Pathak (2007). The study showed a dose-dependent lethal effect, with paralysis of *Pheretima posthuma* worms. Moreover, Ammar *et al.* (2016) recorded death of all *S. mansoni* cercariae and miracidia after 30 minutes of exposure to the methanolic extract of *C. citrinus* leaves. They also proved a direct marked lethal effect on adult *S. mansoni* worms *in vitro*. Aqueous extracts from *C. citrinus*, *Allium sativum* and *Moringa stenopetala* were also proven to have anti-leishmanial activities against both promastigotes and amastigotes of *Leishmania major* (Kinuthia *et al.*, 2013). The present study was designed to assess the effects of *C. citrinus* extract in mice experimentally infected with *S. mansoni* in both prepatent (juvenile worms) and patent phases (adult worms) of infection by parasitological, histopathological and biochemical studies.

Materials and methods

Experimental mice and S. mansoni *infection*

The study included 80 male BALB/c albino mice (10–12 weeks old and 20 ± 2 g). The mice were kept in an air-conditioned animal house at the Theodor Bilharz Research Institute (TBRI), Giza, Egypt, and fed a standard pellet diet and water *ad libitum*. Freshly shed cercariae were obtained from laboratory-bred *Biomphalaria alexandrina* snails infected with miracidia of the Egyptian strain of *S. mansoni*, reared and maintained at the Schistosome Biological Supply Program Unit, TBRI. The shed cercariae were counted using a stereomicroscope (Mohamed *et al.*, 2010). Infection was achieved by subcutaneous injection of mice with about 100 *Schistosoma* cercariae per mouse (Holanda *et al.*, 1974).

Drugs and doses

Praziquantel was provided by the Egyptian International Pharmaceutical Industries Company, Egypt (E.I.P.C.O.) and was suspended in 2% cremophore (Sigma Aldrich, USA). It was given orally at a dose of 500 mg/kg for two consecutive days, according to El-Lakkany *et al.* (2012), on the 14th day post infection to groups IV and V and on the 45th day post infection to groups VII and VIII.

Callistemon citrinus leaves were provided by a plant taxonomist from the Faculty of Agriculture, Menoufia University, Egypt. Leaves were washed with tap water, air dried and ground into fine particles. Aqueous extract of *C. citrinus* was prepared according to Kinuthia *et al.* (2015). Briefly, the obtained fine particles (100 g) were added to distilled water (600 ml) and placed in a 70°C water bath for 1.5 hours. The mixture was filtered using Whatman filter paper. Aqueous extract of *C. citrinus* was freshly prepared on the 13th and 44th days post infection, preserved at 4°C and given to the mice the next days as follows: 14th day post infection to groups III and V and 45th day post infection to groups IV and VIII. The extract was given orally at a dose of 200 mg/kg according to Ahmed *et al.* (2015).

Experiment design

The mice were divided into eight groups of 10 mice each:

Group I (GI): non-infected non-treated mice (negative control)
Group II (GII): infected non-treated mice (positive control)
Group III (GIII): infected mice treated with *C. citrinus* on the 14th day post infection
Group IV (GIV): infected mice treated with PZQ on the 14th day post infection
Group V (GV): infected mice treated with *C. citrinus* and PZQ on the 14th day post infection
Group VI (GVI): infected mice treated with *C. citrinus* on the 45th day post infection
Group VII (GVII): infected mice treated with PZQ on the 45th day post infection
Group VIII (GVIII): infected mice treated with *C. citrinus* and PZQ on the 45th day post infection

In the 7th week post infection, all mice were anaesthetized with ether and euthanized by decapitation. After worm counting, parts of liver and ileum were taken from each mouse to calculate egg load and oogram pattern. Liver specimens were taken for histopathological studies. Blood samples were collected and sera were separated for biochemical studies.

Porto-mesenteric worm burden and reduction %

Saline perfusion of *S. mansoni* adult worms from the portal vein and porto-mesenteric vessels was performed. Counting of males, females and couples was carried out according to Smithers & Terry (1965). The percentage reduction of adult worms after treatment was calculated according to Tendler *et al.* (1986) using the formula $R = C - V/C \times 100$, where R = reduction %, C = mean number of adult worms from infected non-treated mice, and V = mean number of parasites from treated mice.

Tissue egg load/g intestine and liver

The number of eggs per gram of tissue was calculated. Samples of ileum and liver were weighed and 0.5 g of each sample was placed in a test tube containing 5% KOH (5 ml) solution for 16 hours at 37°C. Eggs were counted under a light microscope at ×40 magnification (Herbert *et al.*, 2010).

Oogram pattern

Three fragments (1 cm each) were cut longitudinally from the ileum of each mouse, rinsed with saline and dried gently using Whatman filter paper. They were examined microscopically and

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the percentages of various developmental stages of eggs (immature, mature and dead) were determined (Pellegrino *et al.*, 1962).

Hepatic granuloma number and size

Liver tissues from the euthanized mice were fixed in 10% formalin, dehydrated in ascending grades of alcohol and paraffin embedded. Tissues were cut into 4 µm sections and stained with haematoxylin and eosin (H&E). Hepatic granulomas per section were counted and measured digitally at ×10 using a multi-head Olympus SC100 microscope and the analySIS getIT software (Olympus, Tokyo, Japan) at the Pathology Department, Faculty of Medicine, Menoufia Governorate. The mean number of granulomas per section was calculated for each group. To assess granuloma size, circular shaped granulomas with a single egg in the centre were measured. The diameters of the largest ten granulomas in each section were measured and the mean diameter was calculated for each studied group (El-kott *et al.*, 2011).

Estimation of liver function

Serum samples were separated by centrifugation at 3000 rpm for ten minutes. Liver function tests (aspartate transaminase (AST), alanine transaminase (ALT) and total bilirubin (TB)) were performed using an Integra 400 auto analyser (Roche, Germany). Quantitative determination of AST and ALT was carried out by the Warburg method with pyridoxal-5-phosphate at a wavelength of 340 nm according to the International Federation of Clinical Chemistry (IFCC) protocol (Bergmeyer *et al.*, 1986). Total bilirubin concentration (mg/dl) was measured by photometry according to a diazo method as described by Malloy & Evelyn (1937).

Statistical analysis

The data collected were tabulated and processed using SPSS version 22.0 (SPSS Inc., Chicago, Illinois, USA) on an IBM-compatible computer. Normality of data was assessed by visual methods as well as a Shapiro–Wilk test. Continuous parametric variables were presented as means ± SD. The difference between groups regarding parametric variables (tissue egg load, oogram pattern, mean hepatic granuloma pattern, AST, ALT and total bilirubin) was analysed by the one-way analysis of variance (ANOVA) test, and then the Tukey honest significant difference post-hoc test was used to identify the groups that were significantly different from each other. The Kruskal–Wallis test was applied to study the difference between the groups having non-parametric variables (worm burden).

Results

The highest reduction of total *S. mansoni* worm burden was seen in both groups that received *C. citrinus* either combined with PZQ (GV) or alone (GIII) early during the prepatent period of infection (89.3% and 85.2%, respectively). *Callistemon citrinus* administered on the 45th day post infection either alone (GVI) or combined with PZQ (GVIII) induced a higher reduction of total worm count (79.1%), with significant differences between all groups ($P < 0.0001$). PZQ administered early in the course of infection induced a negligible effect on the adult worm burden (6.63%) (table 1).

Callistemon citrinus administered early during the course of infection achieved the highest reduction in egg load in GV (combined therapy) (96.2% intestine and 93.1% liver) and in GIII (*C. citrinus* alone) (91.8% intestine and 90.6% liver), with no significant difference between the two groups (p8 = 0.9624 and 0.999 in intestine and liver, respectively) (table 2).

Callistemon citrinus administered late in the infection achieved 84.5% reduction in intestinal egg load and 78.9% reduction in hepatic egg load when given alone (GVI), and 87.6% intestinal reduction and 82.5% hepatic reduction when combined with PZQ (GVIII), with no significant difference between the two groups (p14 = 0.9940 and 0.995 in intestine and liver, respectively) (table 2).

The schistosomal oogram pattern showed a massive increase in the percentage of dead eggs in groups V (*C. citrinus* + PZQ 14 days post infection) and III (*C. citrinus* 14 days post infection), to 95.8% and 91.6%, respectively, with a highly significant difference in comparison to the control positive group (p3 and p1: 0.0000). This was followed by both groups that received *C. citrinus* late (45th day post infection) either combined with PZQ (GVIII) or alone (GV), where dead eggs increased to 84.8% and 82.3%, respectively, with a highly significant difference compared to the positive control group (p6 and p4: 0.0000) (table 3).

Haematoxylin and eosin sections of liver tissue showed that *C. citrinus* given early during prepatency reduced both the number and size of granulomas, either when combined with PZQ (GV; 90.7% and 93.4% for the number and size, respectively) or when administered as monotherapy (GIII; 87.2% and 90% for the number and size, respectively). *Callistemon citrinus* given on the 45th day post infection was associated with improvement in number and size of granulomas, although to a lesser extent, when combined with PZQ (GVIII; 81.3% and 64.2% for the number and size, respectively) or alone (GVI; 74.4% and 49% for the number and size, respectively). All showed a significant decrease compared to the control positive group (GII) (p3, p1, p6 and p4: 0.0000, respectively) (table 4 and fig. 1).

AST, ALT and TB serum levels were measured to assess liver function. The highest levels were detected in both the positive control group (GII) and the group administered solely PZQ early, on the 14th day post infection (GIV), with no significant difference between them. On the other hand, the highest improvement was detected in groups administered *C. citrinus* early, on the 14th day post infection, either alone (GIII) or combined with PZQ (GV), with no significant difference when compared to the negative control group (GI) (figs 2, 3 and 4).

Discussion

With the increasing popularity of treatments based on natural medicinal plant extracts, the current study was designed to assess the anthelmintic effects of aqueous extract of *C. citrinus* in mice experimentally infected with *S. mansoni* in comparison with the currently used PZQ in prepatent and patent phases of infection. Aqueous extract of *Callistemon* (200 mg/kg) was selected because water is a safe, non-toxic universal solvent and avoids the high toxicity of organic solvents (such as methanol, acetone, chloroform and dichloromethane) to living cells. Previous results for aqueous extract of *C. citrinus* (Kinuthia *et al.*, 2015) are encouraging. Also, Bhushan *et al.* (2014) recorded the best yield of *C. citrinus* when water was used as a solvent in comparison to petroleum ether, ethyl acetate, chloroform and ethanol. The dose selected (200 mg/kg) is one fourth of the maximum therapeutic dose calculated by Bhushan *et al.* (2014), as recommended by toxicologists (Festing & Altman, 2014).

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Table 1. Comparison of mean *Schistosoma* worm burdens in the studied groups of experimentally infected mice.

Groups	Worm burden		
	Mean ± SD	% Reduction	Kruskal–Wallis test
Male			
GII: Positive control	4.1 ± 1.79	–	48.18 $P < 0.0001$
GIII: *Callistemon citrinus* 14 days post infection	1.2 ± 0.63	70.7	
GIV: PZQ 14 days post infection	3.9 ± 0.99	4.8	
GV: *C. citrinus* + PZQ 14 days post infection	0.7 ± 0.48	82.9	
GVI: *C. citrinus* 45 days post infection	1.7 ± 0.94	58.5	
GVII: PZQ 45 days post infection	2.1 ± 0.73	48.7	
GVIII: *C. citrinus* + PZQ 45 days post infection	1.5 ± 0.7	63.4	
Female			
GII: Positive control	1.1 ± 1.1	–	10.3 $P = 0.11$
GIII: *C. citrinus* 14 days post infection	0.5 ± 0.52	54.5	
GIV: PZQ 14 days post infection	1.0 ± 0.81	9	
GV: *C. citrinus* + PZQ 14 days post infection	0.4 ± 0.51	63.6	
GVI: *C. citrinus* 45 days post infection	0.8 ± 0.63	27.2	
GVII: PZQ 45 days post infection	1.0 ± 0.47	9	
GVIII: *C. citrinus* + PZQ 45 days post infection	0.8 ± 0.63	27.2	
Couple			
GII: Positive control	7.2 ± 1.31	–	48.4 $P < 0.0001$
GIII: *C. citrinus* 14 days post infection	0.6 ± 0.699	91.6	
GIV: PZQ 14 days post infection	6.7 ± 1.05	6.9	
GV: *C. citrinus* + PZQ 14 days post infection	0.5 ± 0.52	93	
GVI: *C. citrinus* 45 days post infection	0.9 ± 0.73	87.5	
GVII: PZQ 45 days post infection	1.3 ± 0.67	81.9	
GVIII: *C. citrinus* + PZQ 45 days post infection	0.9 ± 0.73	87.5	
Total			
GII: Positive control	19.6 ± 2.83	–	57.28 $P < 0.0001$
GIII: *C. citrinus* 14 days post infection	2.9 ± 0.87	85.2	
GIV: PZQ 14 days post infection	18.3 ± 2.26	6.63	
GV: *C. citrinus* + PZQ 14 days post infection	2.1 ± 1.19	89.3	
GVI: *C. citrinus* 45 days post infection	4.1 ± 1.197	79.1	
GVII: PZQ 45 days post infection	6.2 ± 1.398	68.3	
GVIII: *C. citrinus* + PZQ 45 days post infection	4.1 ± 1.197	79.1	

Vimieiro *et al.* (2013) described efficient treatment of schistosomiasis during the initial phase of infection as an important challenge. An ideal treatment would inhibit egg production and consequently granuloma formation, which is the main cause of the pathology of this disease (Coelho *et al.*, 2009).

All the anti-parasitic parameters assessed in this work, including the percentage reduction of the adult worm burden perfused from the portal vein and porto-mesenteric vessels, the percentage reduction of the ileal and hepatic tissue egg load and the ileal oogram pattern, indicated that early treatment with *C. citrinus* either alone or combined with PZQ was more effective compared to treatment received late during the course of infection. These results are supported by a number of studies on other anti-schistosomal therapeutic agents. When juvenile *S. mansoni* worms are treated early, they fail to produce eggs due to the inhibitory effect of anti-schistosomals on their sexual maturation, causing atrophy of their testes and ovaries, hence confirming the advantage of early treatment (Hamza *et al.*, 2012; Vimieiro *et al.*, 2013). In context, several studies have demonstrated that immature worms are less susceptible to PZQ (Botros *et al.*, 2005; Grandière-Pérez *et al.*, 2006; Vimieiro *et al.*, 2013).

Regarding late treatment during the patent period of infection, *C. citrinus* either alone or combined with PZQ exhibited considerable anti-schistosomal effects, with remarkable improvement of

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Table 2. Comparison of mean *S. mansoni* tissue egg load in the studied groups of experimentally infected mice.

	Tissue egg load (intestine)				
Groups	Mean ± SD	% Reduction	F test	Post-hoc value	
GII: Positive control	6678 ± 1414.2	–	187.7 <0.0001	p1: 0.0000	A
GIII: *Callistemon citrinus* 14 days post infection	544.6 ± 134.8	91.8		p2: 0.8654	B
GIV: PZQ 14 days post infection	5984.6 ± 1086.8	10.3		p3: 0.0001	A
GV: *C. citrinus* + PZQ 14 days post infection	248.8 ± 36.5	96.2		p4: 0.0000	B
GVI: *C. citrinus* 45 days post infection	1030 ± 336.8	84.5		p5: 0.0000	B
GVII: PZQ 45 days post infection	980.8 ± 133.4	85.3		p6: 0.0000	B
GVIII: *C. citrinus* + PZQ 45 days post infection	822.8 ± 238.6	87.6		p7: 0.0001	B
				p8: 0.9624	
				p9: 0.7077	
				p10: 0.0011	
				p11: 0.0000	
				p12: 0.5238	
				p13: 1.0000	
				p14: 0.9940	
				p15: 0.9987	
	Tissue egg load (liver)				
Groups	Mean ± SD	% Reduction	F test	Post-hoc value	
GII: Positive control	3895.6 ± 886.5	–	97.2 <0.0001	p1: 0.0000	A
GIII: *C. citrinus* 14 days post infection	364 ± 120.8	90.6		p2: 0.799	B
GIV: PZQ 14 days post infection	3584.4 ± 916.8	7.98		p3: 0.0000	A
GV: *C. citrinus* + PZQ 14 days post infection	266.7 ± 79.8	93.1		p4: 0.0000	B
GVI: *C. citrinus* 45 days post infection	820.0 ± 163.7	78.9		p5: 0.0000	B
GVII: PZQ 45 days post infection	908.0 ± 126.3	76.69		p6: 0.0000	B
GVIII: *C. citrinus* + PZQ 45 days post infection	680 ± 188.5	82.5		p7: 0.0000	B
				p8: 0.999	
				p9: 0.392	
				p10: 0.0000	
				p11: 0.0000	
				p12: 0.513	
				p13: 0.999	
				p14: 0.995	
				P15: 0.945	

p1: Group II vs Group III.
p2: Group II vs Group IV.
p3: Group II vs Group V.
p4: Group II vs Group VI.
p5: Group II vs Group VII.
p6: Group II vs Group VIII.
p7: Group III vs Group IV.
p8: Group III vs Group V.
p9: Group III vs Group VI.
p10: Group IV vs Group V.
p11: Group IV vs Group VII.
p12: Group V vs Group VIII.
p13: Group VI vs Group VII.
p14: Group VI vs Group VIII.
p15: Group VII vs Group VIII.
*Groups sharing the same letter are not statistically different.

all parasitological parameters, comparable to, if not better than, treatment solely by PZQ. To date, no data have been published regarding the *in vivo* anti-schistosomal effects of *C. citrinus*. However, many herbal and plant-based medicines have been used to treat *S. mansoni* infection and have achieved comparable results to those obtained with PZQ; e.g. Schitozim, *Tanacetum vulgare, Artemisia absinthium* and *Tanacetum parthenium* (Muya *et al.*, 2014; de Almeida *et al.*, 2016). Interestingly, previous studies have proved that *C. citrinus* fruits have calcium channel blocking effects (Ali *et al.*, 2011). Many studies have demonstrated that voltage-operated calcium channels of *Schistosoma* represent prime targets for chemotherapy (Redman *et al.*, 1996). These channels play an important role in regulating intracellular calcium levels and are essential for multiple parasite cellular events, muscular contractions, release of various neurotransmitters, as well as gene expression (Salvador-Recatalà *et al.*, 2008). Nifedipine (a calcium channel blocker) was tested for anti-schistosomal effects on both schistosomula and adult worms. It was found to be effective against *S. mansoni* schistosomula, as it significantly reduced their viability. It also caused impaired motility, several tegumental lesions and intense contractions in adult worms. Nifedipine also impaired egg production by *S. mansoni* females, decreasing *S. mansoni* fecundity (Silva-Moraes *et al.*, 2013).

Histopathological assessment of the number and diameter of *S. mansoni* induced hepatic granulomas in this work showed that *C. citrinus*, either as sole therapy or combined with PZQ, significantly reduced the number of hepatic granulomas in all groups compared to both positive control and sole PZQ groups,

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Table 3. Comparison of oogram patterns in the studied groups of experimentally infected mice.

Groups	Oogram pattern			
	Mean ± SD	F test	Post-hoc value	
	Immature eggs (%)			
GII: Positive control	61.8 ± 13.63	89.08 <0.0001	p1: 0.0000	A
GIII: *Callistemon citrinus* 14 days post infection	5.0 ± 1.8		p2: 0.007	B
GIV: PZQ 14 days post infection	48.3 ± 13.6		p3: 0.0000	A
GV: *C. citrinus* + PZQ 14 days post infection	2.4 ± 0.83		p4: 0.0000	B
GVI: *C. citrinus* 45 days post infection	8.3 ± 2.65		p5: 0.0000	B
GVII: PZQ 45 days post infection	31.6 ± 8.44		p6: 0.0000	C
GVIII: *C. citrinus* + PZQ 45 days post infection	5.6 ± 1.9		p7: 0.0000	B
			p8: 0.990	
			p9: 0.969	
			p10: 0.0000	
			p11: 0.0004	
			p12: 0.973	
			p13: 0.000	
			p14: 0.9888	
			p15: 0.0000	
	Mature eggs (%)			
GII: Positive control	34.40 ± 9.6	56.37 <0.0001	p1: 0.0000	A
GIII: *C. citrinus* 14 days post infection	3.4 ± 1.3		p2: 0.225	B
GIV: PZQ 14 days post infection	41.3 ± 12.4		p3: 0.0000	A
GV: *C. citrinus* + PZQ 14 days post infection	1.8 ± 0.7		p4: 0.0000	B
GVI: *C. citrinus* 45 days post infection	9.4 ± 3.15		p5: 0.0000	B
GVII: PZQ 45 days post infection	14.9 ± 4.62		p6: 0.0000	B
GVIII: *C. citrinus* + PZQ 45 days post infection	9.6 ± 3.7		p7: 0.0000	B
			p8: 0.997	
			p9: 0.384	
			p10: 0.0000	
			p11: 0.0000	
			p12: 0.118	
			p13: 0.490	
			p14: 0.999	
			p15: 0.535	
	Dead eggs (%)			
GII: Positive control	3.8 ± 1.2	312.56 <0.0001	p1: 0.0000	A
GIII: *C. citrinus* 14 days post infection	91.6 ± 5.5		p2: 0.351	B
GIV: PZQ 14 days post infection	10.4 ± 3.35		p3: 0.0000	A
GV: *C. citrinus* + PZQ 14 days post infection	95.80 ± 4.2		p4: 0.0000	B
GVI: *C. citrinus* 45 days post infection	82.3 ± 8.68		p5: 0.0000	B
GVII: PZQ 45 days post infection	53.5 ± 8.75		p6: 0.0000	C
GVIII: *C. citrinus* + PZQ 45 days post infection	84.8 ± 11.2		p7: 0.0000	D
			p8: 0.824	
			p9: 0.057	
			p10: 0.0000	
			p11: 0.0000	
			p12: 0.012	
			p13: 0.0000	
			p14: 0.983	
			p15: 0.0000	

p1: Group II vs Group III.
p2: Group II vs Group IV.
p3: Group II vs Group V.
p4: Group II vs Group VI.
p5: Group II vs Group VII.
p6: Group II vs Group VIII.
p7: Group III vs Group IV.
p8: Group III vs Group V.
p9: Group III vs Group VI.
p10: Group IV vs Group V.
p11: Group IV vs Group VII.
p12: Group V vs Group VIII.
p13: Group VI vs Group VII.
p14: Group VI vs Group VIII.
p15: Group VII vs Group VIII.
*Groups sharing the same letter are not statistically different.

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Table 4. Comparison of mean number and diameter of hepatic granulomas in the studied groups of experimentally infected mice.

	Number of hepatic granulomas per section				
Groups	Mean ± SD	% Reduction	F test	Post-hoc value	
GII: Positive control	8.6 ± 2.1	–	76.6 <0.0001	p1: 0.0000	A
GIII: *Callistemon citrinus* 14 days post infection	1.1 ± 0.4	87.2		p2: 0.4638	B
GIV: PZQ 14 days post infection	7.6 ± 1.8	11.6		p3: 0.0000	A
GV: *C. citrinus* + PZQ 14 days post infection	0.8 ± 0.3	90.7		p4: 0.0000	B
GVI: *C. citrinus* 45 days post infection	2.2 ± 0.6	74.4		p5: 0.0000	B
GVII: PZQ 45 days post infection	4.1 ± 0.9	52.3		p6: 0.0000	C
GVIII: *C. citrinus* + PZQ 45 days post infection	1.6 ± 0.5	81.3		p7: 0.0000	B
				p8: 0.9971	
				p9: 0.3474	
				p10: 0.0000	
				p11: 0.0000	
				p12: 0.7136	
				p13: 0.0083	
				p14: 0.9053	
				p15: 0.0002	
	Diameter of hepatic granulomas (μm)				
Groups	Mean ± SD	% Reduction	F test	Post-hoc value	
GII: Positive control	288 ± 68	–	60.04 <0.0001	p1: 0.0000	A
GIII: *C. citrinus* 14 days post infection	27 ± 6.8	90.6		p2: 0.3086	B
GIV: PZQ 14 days post infection	247 ± 61	14.2		p3: 0.0000	A
GV: *C. citrinus* + PZQ 14 days post infection	19 ± 5	93.4		p4: 0.0000	B
GVI: *C. citrinus* 45 days post infection	118 ± 42	59.0		p5: 0.0000	C
GVII: PZQ 45 days post infection	132 ± 28	54.1		p6: 0.0000	C
GVIII: *C. citrinus* + PZQ 45 days post infection	103 ± 34	64.2		p7: 0.0000	C
				p8: 0.9995	
				p9: 0.0002	
				p10: 0.0000	
				p11: 0.0000	
				p12: 0.0006	
				p13: 0.9885	
				p14: 0.9835	
				p15: 0.7087	

p1: Group II vs Group III.
p2: Group II vs Group IV.
p3: Group II vs Group V.
p4: Group II vs Group VI.
p5: Group II vs Group VII.
p6: Group II vs Group VIII.
p7: Group III vs Group IV.
p8: Group III vs Group V.
p9: Group III vs Group VI.
p10: Group IV vs Group V.
p11: Group IV vs Group VII.
p12: Group V vs Group VIII.
p13: Group VI vs Group VII.
p14: Group VI vs Group VIII.
p15: Group VII vs Group VIII.
*Groups sharing the same letter are not statistically different.

indicating a marked effect of *C. citrinus*. As regards the size of granulomas, *C. citrinus* significantly decreased the granuloma diameter in all groups compared to the positive control group. It also showed comparable effect to that of PZQ, as it significantly reduced the size in all *C. citrinus* treated groups except for the sole late *C. citrinus* treated group, for which no significant difference was reported when compared to the sole PZQ late group. Improvement of both number and size of granulomas in *C. citrinus* treated groups can be explained by the calcium channel blocking effect of the extract, as reported previously for verapamil (a calcium channel blocker), which was found to interrupt egg production. This is valuable, as eggs are responsible for the development of granulomatous lesions (Bonn, 2004).

In this study, a hepatic shift phenomenon was observed, as few adult worms were found inside the hepatic parenchyma in some sections of the PZQ late monotherapy group. Hepatic shift was reported by Meister *et al.* (2014), who attributed it to racemate PZQ or its R-enantiomers treatment, which killed the majority of adult worms, while treatment with S-enantiomers of PZQ killed only a few adults. Worms migrate to the hepatic parenchyma mostly because of the loss of grip on the wall of the mesenteric blood vessels induced by the drug, allowing them to migrate back to the mesenteric veins. Meister *et al.* (2014) assumed that the hepatic shift phenomenon detected in their study in association with using S-enantiomers of PZQ was evidence of inefficacy.

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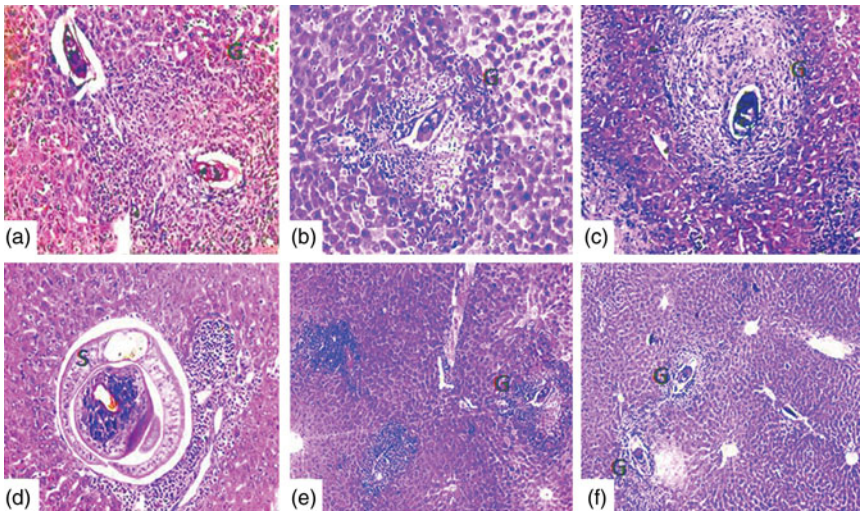

Fig. 1. (a) Liver tissue of positive control group (GII), showing large granuloma (G) around viable *Schistosoma mansoni* egg surrounded by neutrophilic, lymphocytic and histiocytic cellular infiltration with moderate fibrosis (H&E, ×200). (b) Liver tissue of GIII that received *Callistemon citrinus* early, on the 14th day post infection, showing small granuloma (G) around viable *S. mansoni* egg surrounded by mild neutrophilic and histiocytic cellular infiltration with mild fibrosis (H&E, ×200). (c) Liver tissue of GVII that received PZQ on the 45th day post infection, showing medium-sized granuloma (G) around dead calcified bilharzial egg with chronic inflammatory cellular infiltrate and intervening fibrosis (H&E, ×200). (d) Liver tissue of the same group showing adult *S. mansoni* worm (S) inside the entire liver tissue surrounded by dense inflammatory infiltration (hepatic shift) (H&E, ×200). (e) Liver tissue of GVI that received *C. citrinus* on the 45th day post infection, showing small granuloma (G) around viable bilharzial egg surrounded by neutrophilic and histiocytic cellular infiltration with perigranulomatous fibrosis (H&E, ×100). (f) Liver tissue of GVIII that received late combined therapy, showing small granuloma around viable bilharzial ova surrounded by moderate inflammatory cellular infiltration (H&E, ×100).

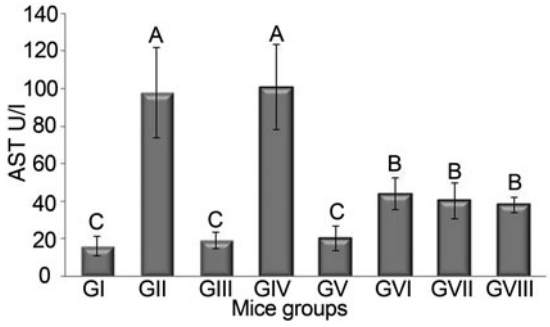

Fig. 2. Aspartate aminotransferase serum levels (mean ± SD) in the different groups studied. Groups with the same letter are not statistically different, whereas those with different letters are statistically different.

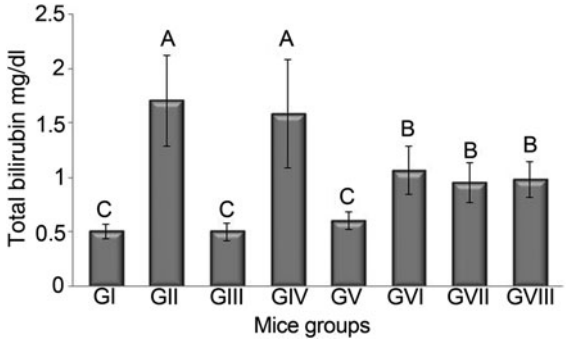

Fig. 4. Total bilirubin serum levels (mean ± SD) in the different groups studied. Groups with the same letter are not statistically different, whereas those with different letters are statistically different.

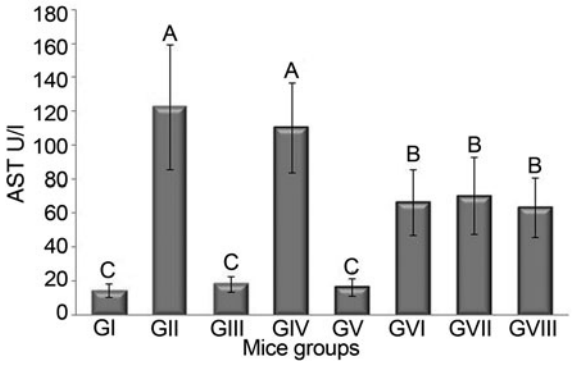

Fig. 3. Alanine aminotransferase serum levels (mean ± SD) in the different groups studied. Groups with the same letter are not statistically different, whereas those with different letters are statistically different.

Some of the multiple enzymatic systems in hepatocytes are markers for cell organelle activities. Hence, injury to such organelles is reflected by the serum levels of those enzymes, which can be used in assessing different therapies for *S. mansoni* infection (Hamed & Hetta, 2005; El-Kott *et al.*, 2011). In this study, AST, ALT and TB serum levels were measured as indicators of hepatic tissue injury. They were significantly increased in the positive control group, reflecting hepatic injury, as previously reported (El-Kott *et al.*, 2011). The highest improvement of hepatic functions was recorded in mice administered *C. citrinus* early in the prepatent phase of infection (alone or combined with PZQ). *Callistemon citrinus* administered late resulted in improved liver function, although to a lesser extent, which was significantly different from the positive control group. Hepatoprotective effects of dried leaf extract of *Callistemon lanceolatus* – the old synonym of *C. citrinus* – were reported by Jain *et al.* (2007), who detected significant improvement in AST, ALT, alkaline phosphatase and total bilirubin levels in *Callistemon*-treated mice after carbon tetrachloride induced hepatic damage.

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Conflict of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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