



Effect of *Crocus sativus* aqueous extract (saffron) on *Schistosoma mansoni* worms in experimentally infected mice.

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ABSTRACT

The present study is designed to evaluate the therapeutic effect of *Crocus sativus*(saffron) aqueous extract (CSE) alone or in combination with praziquantel on *Schistosoma mansoni* infected mice. Parasitological, immunological, pathological and immunohistochemical parameters and surface topography of worms were determined. Treatment of *S. mansoni* infected mice with saffron or PZQ revealed a significant reduction in worm burden, hepatic and intestinal ova count with a decline in granuloma diameter. There was an increase in total IgG and IL-10 blood levels after treatment with PZQ and/or CSE. Superoxide dismutase (SOD) and catalase levels were improved in infected mice treated with CSE in comparison with infected mice. Male worm surface ultrastructure examination showed a swelling, rupture of numerous tubercles with marked decrease in spines and severe peeling of tegumental dorsal surfaces after treatment with saffron. In conclusion, administration of aqueous extract of *Crocus sativus* is effective treatment for *S. mansoni* infection.

INTRODUCTION

Schistosomiasis is a parasitic disease caused by blood helminthes of genus *Schistosoma* and still considered one of the major health problems which threaten human life in many countries. It is the third most devastating tropical disease in the world, after malaria and intestinal helminthiasis, being a major source of morbidity and mortality in the human population (WHO, 2014). It affected more than 250 million people, mainly in sub-Saharan Africa (Siqueira *et al.*, 2017). Previous study estimated that 4,400 to 200,000 people die from *Schistosoma* each year (Thétiot-Laurent *et al.*, 2013). Adult worm of the parasite are not the main cause of the injuries in the mammalian host body, but the massive egg production by the female worms, is the major stimulant of chronic disease (Gryseels *et al.*, 2006). The toxic egg material destroys the host tissue cells and the antigenic material stimulates the development of large inflammatory reactions (granuloma) around the egg (EL-Shenawy *et al.*, 2005).

There are many drugs which can be used on global scale for the treatment of schistosomiasis, including antimonials, oxamniquine, metrifonate and praziquantel (PZQ) while current treatment relies on PZQ (Zhang and Coultas, 2013). A single oral dose of 40mg/kg body weight is thought to be effective for treatment of *S. haematobium* and *S. mansoni* and can safely be used in pregnancy after the first trimester and two doses of 30 mg/kg body weight for *S. japonicum*, with cure rates of 75%–100% (Reich *et al.*, 1998).

PZQ mainly targets the adult worms, while the immature *Schistosoma* larva is less susceptible (Utzinger *et al.*, 2003). The benefits of PZQ are its high efficacy, ease of administration, relative safety, and mild to moderate side effects, including nausea, dizziness, rash, pruritus, headache, drowsiness, and abdominal pain were demonstrated (Ferrari *et al.*, 2003; Gray *et al.*, 2011). However, treatment with PZQ may sometimes fail, and this may be due to possible drug resistance. Therefore, there is a need for sustained research toward developing alternative chemotherapeutic compounds against schistosomiasis. Plants are possible sources of novel drugs and many plants have investigated for possible anti-schistosomal effects (de Melo *et al.*, 2015; Wakabayashi *et al.*, 2015).

Saffron is the dried red-orange stigmas of *Crocus sativus* L which belongs to the Iridaceae family. It apparently originated in the area of Iran, Turkey and Greece, but now it is also successfully cultivated in Europe, China, Morocco, Egypt, India, Pakistan, Australia and Japan (Abdullaev *et al.*, 2007). Saffron is the most expensive spice in the world. The main reason for its hefty price is that saffron is still cultivated and harvested by hand from the *Crocus sativus* flower (Chermahini *et al.*, 2010). From a long time ago, saffron can be used as natural food color, a flavoring agent and traditional herbal medicine for treatment of different types of illnesses, and it could enhance the absorption and bioavailability of other drugs (Srivastava *et al.*, 2010). Saffron contains more than 150 volatile, non-volatile and aroma-yielding. Also, saffron contain flavonoids, tannins and anthocyanins (Jalali-Heravi *et al.*, 2009; Bathaie and Mousavi, 2010).

Modern pharmacological investigations have indicated that saffron and its constituents has a lot of therapeutic roles including anti-tumor activity (Chermahini *et al.*, 2010), anti-inflammatory (Poma *et al.*, 2012), anti-depressant (Hosseinzadeh *et al.*, 2004), anti-genotoxic (Premkumar *et al.*, 2003) and anti-oxidant properties (Farahmand *et al.*, 2013). This study sheds the light of anti-parasitological, pathological and immunological activities of *Crocus sativus* aqueous extract alone or in combination with PZQ on *S. mansoni* infected mice.

MATERIALS AND METHODS

Experimental animals

The animals used were healthy male albino mice of (CD-1) strain weighting 20-25g obtained from *Schistosome* Biological Supply Unit, Theodor Bilharz Research Institute (TBRI), Imbaba, Giza, Egypt. Mice were transported to Zoology Department, Faculty of Science, Menoufia University, one week prior to initiation of the experiments for acclimatization to laboratory condition. Experimental procedures were carried out after the permission from the Institutional Animal Ethical Committee, Menoufia University, Egypt (approval ID: MUFS/S/IM/2/15).

Infection of animals

An Egyptian strain of *S. mansoni* cercariae were obtained from infected *Biomphalaria alexandrina* snails obtained from TBRI. The snails were kept four weeks after infection in de-chlorinated tap water and then exposed to artificial light at 28°C for two hours in order to induce the shedding of cercariae (Tekwu *et al.*, 2017). Each mouse was infected subcutaneously with 60 ± 5 freshly shed cercariae according to Liang *et al.* (1987).

Drugs

Praziquantel

Praziquantel (PZQ) was obtained from Egyptian International Pharmaceutical Industries Company (EIPICO), Egypt. Each tablet contains 600mg. A praziquantel 600 mg tablet was suspended in distilled water. PZQ was given orally to the infected mice at a total dosage of 600 mg/kg divided equally on 2 consecutive days (Chaiworaporn *et al.*, 2005).

Aqueous extract of Saffron

Saffron, the dried stigmas of *Crocus sativus* flower were obtained from Emirates. One gram of saffron was soaked in 100ml distilled water. After 2 hours it was homogenized in the same distilled water, stirred for 1 hour and filtered. The residue was re-extracted with fresh distilled water. This aqueous extract was lyophilized and stored at 4°C for further use. *Crocus sativus* extract (CSE) that dissolved in distilled water and 40 mg/kg body weight was orally administered to the experimental animals for two weeks (Premkumar *et al.*, 2003).

Experimental design

In the present study, thirty five male albino mice were used and classified into five groups (seven mice per each group) as the following:

Group 1: Normal mice were served as healthy control. Group 2: Mice infected with *S. mansoni* and sacrificed after 8 weeks post infection were served as infected control. Group 3: mice were infected (6 weeks post infection) and treated with PZQ (300mg/kg) given orally for two consecutive days. Group 4: Mice were infected (6 weeks post infection) and treated with CSE(40 mg/kg) given orally and daily for two weeks. Group 5: Mice were infected (6 weeks post infection) and treated with combination of PZQ and CSE.

All groups were sacrificed after 60 days of infection and worms were recovered from hepatic and mesenteric veins by perfusion technique as described by (Smithers and Terry, 1965). The worms were classified according to sex and counted. The oogram count was performed by microscopic examination and classified according to different developmental stages of eggs (Mati and Melo, 2013). The number of *S. mansoni* ova/g tissue (liver or intestine) was demonstrated according to (Herbert *et al.*, 2010). Eggs were counted after being spread on slides and number of ova/g tissues was calculated.

Histopathology

Specimens of both liver and intestine were removed from mice of the five groups and fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin blocks. Paraffin sections (5µm thickness) were stained with Ehrlich's haematoxylin and Eosin (H & E). After that diameters of granulomas in infected groups were measured using an ocular micrometer (Von Lichtenberg, 1962).

Scanning electron microscope (SEM) examination:

Adult male *S. mansoni* worms recovered from the treated and infected control mice were fixed in glutaraldehyde buffer solution (4%) with 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature. The samples were post-fixed with 1% osmium tetroxide for an hour and dehydrate by serial dilution of ethanol (30-100%). Then the samples were dried by using carbon dioxide liquid "critical point drying". Specimens coated by sputter coat of gold. The different parts of worms were examined using Joel JSM-5300, Japan scanning electron microscope at Faculty of Science, Alexandria University, Egypt (Matos-Rocha *et al.*, 2016).

Immunohistochemistry (IHC) examinations

Specimens of liver were removed from mice of the five groups and fixed as previously mentioned and reacted specifically with CD20 and CD3 monoclonal antibodies of rabbit according to the manufacturer's instructions (Cell Marque, CA, USA). Immunohistochemistry was done according to Panic *et al.*, (2017). All sections were quantified using Image J software (NIH, Bethesda, MD, USA).

Determination of the antioxidant enzymes in serum

Superoxide dismutase and catalase activities were determined by ZellBio kit (ZellBio GmbH, Germany). The enzyme assays were performed as described by the manufacturer's instructions.

Determination of immunoglobulins (total IgG), nitric oxide (NO) and IL-10

Total IgG turbidimetric was automatically measured using (COBAS, Integra 400/800 analyzer). NO level was measured using commercial kit (Bio-diagnostic Co., Egypt). IL-10 concentration in serum samples was measured using a sandwich ELISA Kit (Thermo-Scientific, USA).

Sub-typing of the immune cells

Mononuclear cells of blood (1×10^6 cells/ml) were prepared in RPMI-1640. CD4 $^{+}$ and CD8 $^{+}$ cell subtypes were counted using FACS flow cytometer (Becton Dickinson, Sunnyvale, CA) with Cell Quest Software for data acquisition and analysis using fluorescein isothiocyanate (FITC) mice anti-CD4 and anti-CD8 monoclonal antibodies (BD Biosciences, San Jose, CA) (Hassouna *et al.*, 2015).

Statistical analysis

Data were presented as mean \pm standard deviation (SD). The significant differences among values were analyzed using analysis of variance (one-way ANOVA) using Statistical Program of Social Sciences (SPSS) software for windows, version 22.0. coupled with post-hoc least significance difference (LSD). The data were considered significantly different if $P < 0.05$.

RESULTS

Parasitological status

The results showed a significant decrease ($P < 0.05$) in the total worm burden, liver and intestinal egg load, with significant elevations in the percentages of dead ova in all treated groups (PZQ, CSE and CSE+PZQ) when compared with infected-mice group (Table 1 and Table 2). CSE administration alone to *S. mansoni*-infected mice showed a significant decrease in total worm burden 7.00 ± 1.00 , liver egg load 2998.0 ± 108 , intestinal egg load 3766.67 ± 305.51 , and a significant elevation in the count of dead ova 13.11 ± 1.68 and 45.99 ± 10.33 in the liver and intestine respectively, when compared to the infected-mice without any treatment. However, treatment with PZQ alone resulted in a significant higher results than those of CSE.

Table 1: Effect of *Crocus sativus* extracts and/or PZQ treatment on worm burden and ova count of *S. mansoni*-infected mice.

	Worm burden				Ova count	
	Total	Couple	Male	Female	Liver	Intestine
Infected control	10.6 ± 1.67	5.4 ± 1.14	5.2 ± 0.84	0.00	6973.86 ± 1184.32	10828.66 ± 644.72
PZQ	$0.00 \pm 0.00^*$	$0.00 \pm 0.00^*$	$0.00 \pm 0.00^*$	0.00	$1180.13 \pm 50.51^*$	$1544.53 \pm 161.12^*$
CSE	$7.00 \pm 1.00^{\#}$	$2.67 \pm 1.15^{\#}$	$4.33 \pm 0.58^{\#}$	0.00	$2998.0 \pm 108^{\#}$	$3766.67 \pm 305.51^{\#}$
CSE+PZQ	$0.00 \pm 0.00^*$	$0.00 \pm 0.00^*$	$0.00 \pm 0.00^*$	0.00	$1532.5 \pm 63.98^*$	$2633.33 \pm 115.47^{\#}$

Data are expressed as mean \pm standard deviation (SD), (n=7). *Indicates significant difference compared to infected control and # indicates significance against PZQ-treated group at ($P < 0.05$).

Table 2: Effect of *Crocus sativus* extract and/or PZQ treatment on oogram pattern and hepatic granuloma formation of *S. mansoni*-infected mice. (Oogram Pattern)

	Oogram pattern (Liver)			Oogram pattern (Intestine)			Granulomas diameter (μm)
	Mature Eggs	Immature eggs	Dead eggs	Mature eggs	Immature eggs	Dead Eggs	
Infected control	84.19±2.84	13.00±2.62	2.80±1.30	60.86±4.58	33.47±3.74	5.66±0.91	263.87 ±24.54
PZQ	7.27±3.90*	0.00±0.00*	92.89±4.07*	20.50±3.01*	0.00±0.00*	79.50±3.01*	118.12 ±10.80*
CSE	75.11±3.27**#	11.77±2.17#	13.11±1.68**#	53.99±10.33#	0.00±0.00*	45.99±10.33**#	108.04 ±12.34*
CSE + PZQ	37.16±1.37**#	0.00±0.00*	62.83±1.37**#	18.33±0.72 *	0.00±0.00*	81.66±0.72*	96.37 ±7.03*

Data are expressed as mean± standard deviation (SD), (n=7). * Indicates significant difference compared to infected control and # indicates significance against PZQ-treated group at ($P<0.05$).

Administration of CSE combined with PZQ showed the same result of reduction of worm burden, ova count/g tissue in the liver, and the count of dead ova in the intestine when compared to treatment with PZQ alone without any significant difference (Tables 1 and 2).

Oral administration of CSE alone showed no significant difference in mean numbers of mature eggs in intestine (53.99±10.33) when compared with infected mice group (60.86±4.58) and no significant difference in mean numbers of immature eggs in liver (11.77±2.17) when compared with infected control mice (13.00±2.62). Similar to PZQ, complete reduction in mean numbers of immature eggs in the intestine of the mice treated with CSE was detected.

Histopathological studies

Histopathological studies confirmed the parasitological results Figure (1). This examination of liver sections of the infected control group showed loss of the normal structure, disorganization of hepatic cords and the presence of large fibrocellular granulomas, with a mean diameter of 263.87±24.54 μm (Table 2 and Fig 1a).

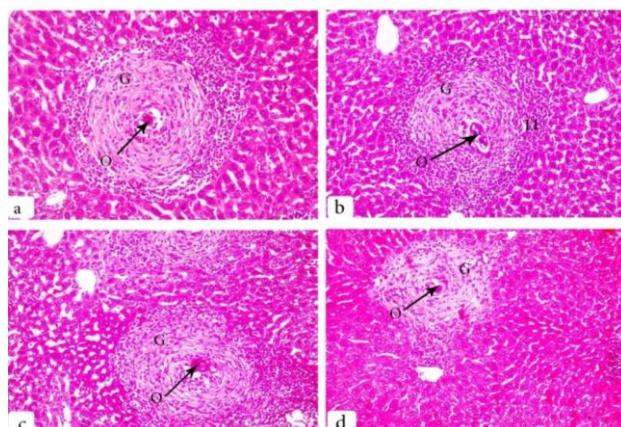


Fig. 1: Light photomicrographs of sections from liver of different mice groups (a-d), stained with hematoxylin and eosin and representing ; a: *S. mansoni*- infected mice (8 weeks post infection) showing large fibrocellular granuloma (G) formed of *Schistosoma*ova (O) surrounded by leukocytic inflammatory cells; b: Infected, PZQ-treated mice showing decrease of granuloma formed of *Schistosoma* ova (O) surrounded by leukocytic inflammatory cells (LI); c: Infected, CSE-treated mice showing decrease in diameter of granuloma formed of *Schistosoma*ova (O) surrounded by leukocytic inflammatory cells; d:Infected mice treated with PZQ and CSE showing markedly reduced granuloma (G) with regular contour formed of *Schistosoma* ova surrounding by leukocytic inflammatory cells, (200x).

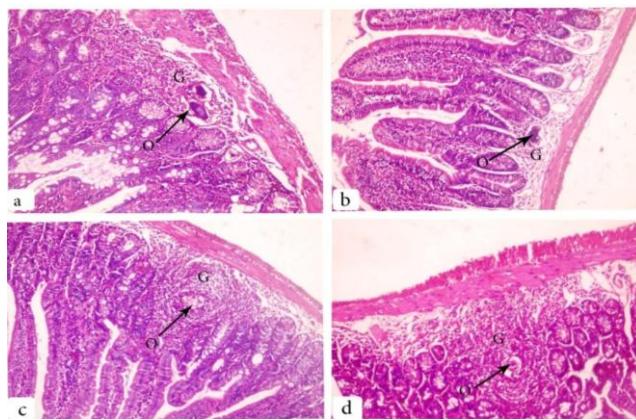


Fig. 2: Light photomicrographs of T.S from intestine of different mice groups (a-d), stained with hematoxylin and eosin and representing ; a: *S. mansoni*- infected mice (8 weeks post infection) showing multiple granulomas in mucosa and submucosa which formed of *Schistosoma* ova (O) surrounded by leukocytic inflammatory cells ; b: Infected, PZQ-treated mice showing decrease of granuloma (G) diameter with mild chronic inflammatory cells and little fibrous reaction around the degenerated schistosomal eggs; c: Infected, CSE- treated mice showing decrease of granuloma surrounded by leukocytic inflammatory cells ; d: Infected mice treated with PZQ and CSE showing decrease of granuloma (G) which consist of *Schistosoma* ova (O) surrounded by little amount of leukocytic inflammatory cells, (200x).

These granulomas formed of *Schistosoma* ova (O) surrounded by leukocytic inflammatory cells. Treatment of PZQ resulted in a significant reduction 118.12 ± 10.80 in granuloma diameters (Table 2); these fibrocellular granulomas were less defined, with degenerated eggs and less fibrosis (Fig. 1b). Administration of CSE alone or a combined with PZQ showing an obvious improvement and markedly reduced granuloma, with less leukocytic inflammatory cells near granuloma when compared to infected control group (Fig. 1c &1d). The same result was demonstrated in histopathological examination of intestine sections from studied groups (Fig. 2).

Ultrastructure morphological study

SEM examination of the adult male worms recovered from the infected control group showed normal dorsal tegument (Fig. 3a), with large, numerous, and spiny tubercles (Fig. 3b) normal oral sucker and ventral sucker (Fig. 4a); anterior region of oral sucker and ventral sucker which covered with numerous sharp spines (Fig. 4b and 4c, respectively).

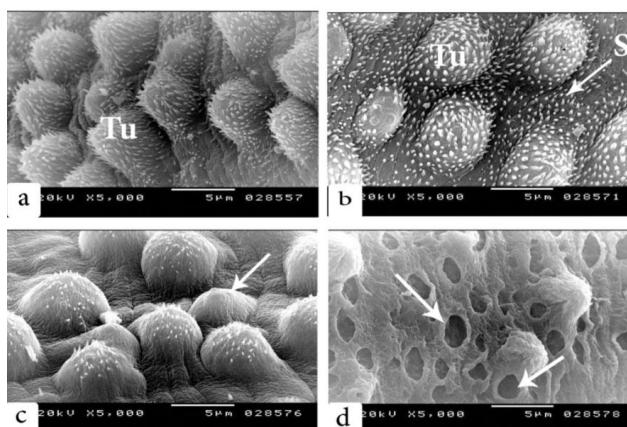


Fig. 3: Scanning electron micrographs of the dorsal surface of adult male *S. mansoni* worms collected from: (a-b): *S. mansoni* -infected control mouse showing dorsal tegument with large, numerous, and spiny tubercles (Tu). (c-d): An infected mouse treated with CSE showing rupture of numerous tubercles with marked decrease in spines and severe peeling of tegumental dorsal surfaces (arrow).

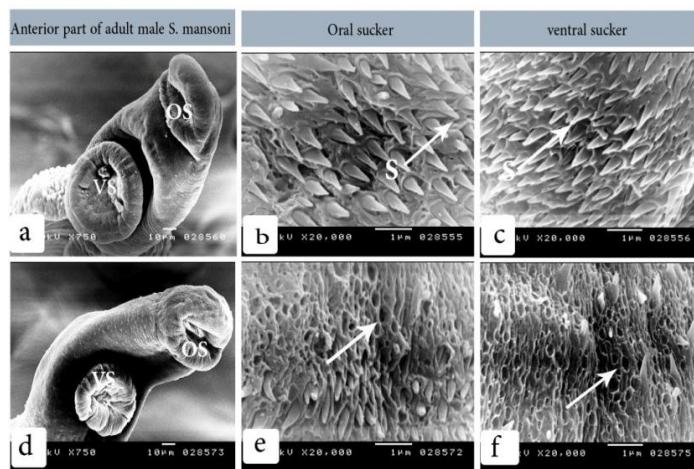


Fig. 4: Scanning electron micrographs showing oral sucker and ventral sucker of *S. mansoni* adult male worms collected from different group: a: *S. mansoni*-infected control showing oral sucker (OS) and ventral sucker (VS); b: An infected control mouse showing anterior region of oral sucker which covered with more numerous sharp spines (S); c: An infected control mouse showing anterior region of ventral sucker which covered with more numerous sharp spines (S); d: Adult male *S. mansoni* treated with CSE showing oral sucker (OS) and ventral sucker (VS) which loss their normal structure; e: Anterior region of oral sucker of adult male *S. mansoni* treated with CSE showing marked decrease in spines or even complete loss of spines (arrow); f: Anterior region of ventral sucker of adult male *S. mansoni* treated with CSE showing marked decrease in spines or even complete loss of spines.

In CSE-treated group the worms showed a variety of changes in the tegumental surface in the form of rupture of numerous tubercles with marked decrease in spines and severe peeling of tegumental dorsal surfaces (Fig. 3c & 3d). The oral sucker and the ventral sucker loss of the normal shape (Fig. 4d), with obvious reduction in spines in the area of oral sucker and ventral sucker (Fig. 4e and 4f, respectively).

Total IgG, IL-10, NO, SOD and catalase alterations

In the current study, there was a significant elevation in the levels of total IgG, IL-10 and NO, accompanied by a significant reduction in SOD and catalase activities after infection with *S. mansoni* (140.98 ± 1.23 ; 46.27 ± 1.29 ; 8.84 ± 0.64 ; 87.95 ± 0.95 and 29.00 ± 1.58 , respectively) when compared with normal control (51.55 ± 1.18 ; 31.37 ± 1.70 ; 6.71 ± 0.37 ; 97.08 ± 0.35 and 37.40 ± 2.07 , respectively).

Results demonstrated a highly significant increase in total IgG in all treated groups compared with infected control group and the values of the combined PZQ and CSE-treated group were the highest. However, oral administration of PZQ or CSE alone showed a significant increase in the level of IL-10 in comparison with the infected-mice without treatment; while no significant difference was detected in PZQ and CSE-treated-mice. On the level of NO, oral administration of CSE showed no significant difference in the level of nitric oxide (8.74 ± 0.25) compared with infected control group (8.84 ± 0.64). Treatment with PZQ alone or a combined with saffron enhances the NO production with a significant increase in the mean values of nitric oxide (13.0 ± 0.16 and 11.8 ± 0.61 , respectively) compared with infected control group. Results demonstrated a highly significant increase in the activity of SOD and catalase in all treated groups compared with infected control group. But, oral administration of CSE showed no significant difference in the mean number of catalase (30.33 ± 2.08) compared with infected control group (29.00 ± 1.58).

Table 3: Effect of *Crocus sativus* extract (CSE) and/or PZQ treatment on total IgG, IL-10 levels, nitric oxide level, superoxide dismutase and catalase activities in serum samples of mice infected with *S. mansoni*.

	Immune parameters		Antioxidant parameter	
	Total IgG (mg/dL)	IL10 (pg/mL)	NO ($\mu\text{mol/L}$)	SOD (U/ml)
Normal control	51.55 \pm 1.18	31.37 \pm 1.70	6.71 \pm 0.37	97.08 \pm 0.35
Infected control	140.98 \pm 1.23 †	46.27 \pm 1.29 †	8.84 \pm 0.64†	87.95 \pm 0.95†
PZQ	155.33 \pm 1.92*	59.89 \pm 3.83*	13.0 \pm 0.16*	97.01 \pm 0.22*
CSE	164.70 \pm 0.30*#	56.86 \pm 2.67*	8.74 \pm 0.25#	94.30 \pm 0.19*#
CSE + PZQ	197.09 \pm 2.2*#	46.54 \pm 0.85 #	11.8 \pm 0.61*#	97.09 \pm 0.25*

Data are expressed as mean \pm standard deviation (SD), (n=7). * Indicates significance ($P<0.05$) against infected control, # indicates significance against PZQ-treated mice and † indicates significance against normal healthy control.

Cellular immune response changes

In the present study, the FACS flow cytometer was used to estimate the CD4 $^{+}$ and CD8 $^{+}$ cells in the blood and immunohistochemistry was utilized to demonstrate the CD3 $^{+}$ and CD20 $^{+}$ cells in the liver. We demonstrated a significant decrease in mean percentage values of CD4 $^{+}$ cells in the blood of infected mice group (Table 4), accompanied by a marked increase in the CD3 $^{+}$ cells and CD20 $^{+}$ cells in the examined tissue when compared with healthy control (Table 4, Figs. 5b, 6b).

Table 4: Effect of *Crocus sativus* extract and/or PZQ treatment on immune cell markers.

	Blood		Liver	
	CD4	CD8	CD3	CD20
Normal Control	59.50 \pm 2.89	37.94 \pm 2.08	0.37 \pm 0.06	5.68 \pm 0.87
Infected Control	45.99 \pm 2.29 †	36.26 \pm 1.53	30.57 \pm 0.85 †	22.19 \pm 0.82 †
PZQ	45.32 \pm 2.58	40.29 \pm 1.47 *	25.25 \pm 0.89 *	28.01 \pm 0.85 *
CSE	50.0 \pm 5.0	37.75 \pm 0.85#	25.08 \pm 0.98 *	37.24 \pm 1.02 *#
CSE+PZQ	41.0 \pm 1.70 *	33.25 \pm 0.55 *#	28.57 \pm 0.90 *#	12.02 \pm 0.99 *#

Data are expressed as mean \pm standard deviation (SD), (n = 7). * Indicates significance ($P<0.05$) against infected control, # indicates significance against PZQ-treated mice and † indicates significance against normal healthy control.

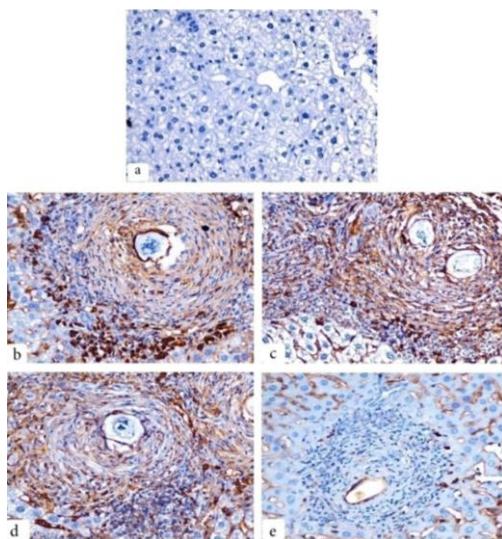


Fig. 5: Immunohistochemical staining of CD3 expression of liver sections of different mice groups (a-e); a: Non-infected-untreated mouse showing negative expression of CD3 in sinusoid and hepatocyte; b: *S. mansoni*-infected mice showing strong expression of CD3 in sinusoid and around granuloma; c: Infected, PZQ-treated mice showing slightly strong expression of CD3 around granuloma; d: Infected, CSE-treated mice showing slightly strong expression of CD3 around granuloma; e: Infected mice treated with combination of PZQ and CSE showing slightly strong expression of CD3 around granuloma, (400x).

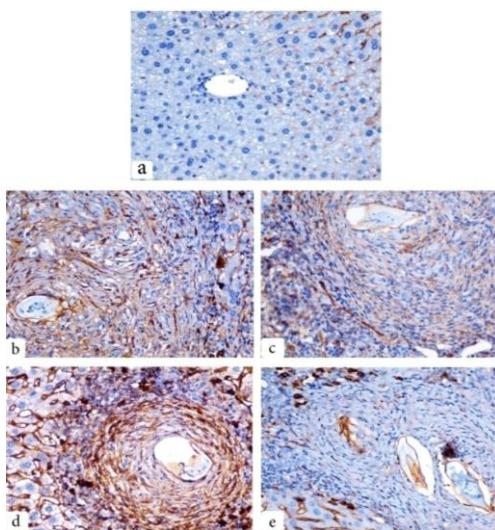


Fig. 6: Immunohistochemical staining of CD20 expression of liver sections of different mice groups (a-e); a: Non-infected-untreated mouse showing weak expression of CD20 in sinusoid and hepatocyte; b: *S. mansoni*-infected mice showing slightly strong expression of CD20 around granuloma; c: Infected, PZQ-treated mice showing strong expression of CD20 around granuloma; d: Infected, CSE-treated mice showing very strong expression of CD20 around granuloma; e: Infected mice treated with combination of PZQ and CSE showing mild expression of CD20 around granuloma, (400x).

Administration of PZQ alone led to a significant increase in the CD8⁺ cells percentage and marked increase in the expression of CD20⁺ cells when compared to infected control group (Table 4, Fig. 6c). On the other hand, mice treated with PZQ alone showed a significant decrease in the CD3⁺ cells when compared with the infected-mice group (Table 4, Fig. 5c). Treatment with CSE alone led to the elevation in CD4⁺ cells (Table 4), accompanied by a marked increase in the expression of CD20⁺ cells in the liver compared to infected-mice group. Combination of CSE with PZQ illustrated a significant decrease in the percentage of CD4⁺, CD8⁺ cells in the blood and CD20⁺ cells in the liver, associated with a marked increase in the CD3⁺ cells compared to PZQ-treated mice (Table 4, Figs. 5e, 6e).

DISCUSSION

Although PZQ is the drug of choice for the treatment of human schistosomiasis, sometimes treatment with PZQ may fail due to possible drug resistance (Melman *et al.*, 2009; Zhang and Coulas, 2013). So, there is a need for sustained research toward developing alternative chemotherapeutic compounds against schistosomiasis. Plants are possible sources of novel drugs and many plants have investigated for possible antiparasitic effects (de Melo *et al.*, 2015; El-Garawani *et al.*, 2016). In the current study, CSE was used in order to examine its potential role either alone or in combination with PZQ on treatment of *S. mansoni* infection. Data obtained in the present study, showed significant reduction in *S. mansoni* total worm count, intestinal and liver ova count, with a significant increase in the percentage of dead eggs in the oogram pattern on the level of the intestine and liver. The PZQ treatment was more effective than CSE alone. Treatment with PZQ gave complete reduction of total worm count of *S. mansoni* compared with infected control. This result comes in match with that reported by (Mahmoud *et al.*, 2002; Chaiworaporn *et al.*, 2005; Morsy, 2009). PZQ caused rapid damage to adult worms by causing paralysis and by shifting adult

worms from the mesenteric vein to the liver where they were finally destroyed by the phagocytic system. Also, it produced improvement of hepatic pathology and reduction in granuloma size. This may be due to mode of action of PZQ against *S. mansoni* which involves increasing the calcium uptake of the parasite, resulting in tegumental damage and death of the parasite as reported by (Gnanasekar *et al.*, 2009). In addition, Botros *et al.* (2004) found that in PZQ treated group, no immature eggs with marked reduction in mature eggs and marked increase in dead eggs when compared with infected control group. The anti-schistosomal properties of ethanol crude extracts of *Rauwolfia vomitoria* stem bark and root against two life stages of *S. mansoni*: cercariae and adult worms was reported (Tekwu *et al.*, 2017). The anti-schistosomal effect of *R. vomitoria* may be due to the different types of compounds present in the plant, such as, flavonoids, terpenoids, alkaloids, tannins, steroids and saponins (Ojo *et al.*, 2012). Moreover, previous study reported that methanolic extract of *Lannea schimperi* and *Searsia longipes* extracts have activity against different life stages of *Schistosoma mansoni* namely cercariae, schistosomula and adult stage (Mbugi *et al.*, 2019). The anti-schistosomal effect of CSE may be due to the different types of compounds that present in the plant, such as, flavonoids, terpene, crocin, tannins and safranal as an unsaturated monoterpenoids. Terpenoids are known to kill adult *S. mansoni* worms and cause complete separation of paired worms with tegumental disruption in worms (De Moraes, 2015). Oral administration of the alcoholic extract of saffron in adose of 100 mg/kg for 4 weeks to mice infected with *S. mansoni* caused significant reduction in egg count of *Schistosoma* when compared with infected control group (Badr *et al.*, 2017).

In the present study, the tegument of adult male worms treated with CSE alone exhibited severe peeling and rupture of tubercles with marked decrease in spines. Futhermore, extensive damage of the oral and ventral suckers included loss of the spines and loss the normal structure. The observed morphological alternations could be considered as a mechanism for the killing of the worms by CSE. The alternation in ventral and oral sucker could result in a loss of ability to adhere to blood vessels consequently the absorb nutrients from blood will be more difficult. The damage to the tegument along the worm's body would have impaired the functioning of the tegument and destroyed the defense system of the worm. Thus, it could be easily attacked by the host's immune system (Xiao *et al.*, 2000). Moreover, such tegumental alterations induced by CSE could probably be exerting a profound effect on the worm's metabolism and consequently resulting in their death (Lima *et al.*, 2011).

The basic pathological lesion in schistosomiasis is the granulomatous inflammation which develop around each ova in different tissues of the host could resulted in subsequent fibrosis (Alves-Oliverira *et al.*, 2006). This granuloma is formed of *Schistosoma* ova surrounded by leukocytic inflammatory cells. The major component of granuloma is eosinophils with some neutrophils, fibroblasts, plasma cells and macrophages (Mourra *et al.*, 2006). In the current study, the histopathological examination of hepatic and intestinal sections obtained from mice infected with *S. mansoni* and treated with PZQ showed significant reduction in granuloma diameter compared with the infected control group. The same results were reported in several studies carried out on the effect of PZQ on different experimental animals (Mahmoud *et al.*, 2002; Sheir *et al.*, 2015). These findings could be attributed to the elimination of adult worms by PZQ therapy and consequent stopping eggs deposition that lead to sustained diminution of eggs induced immunopathology where *Schistosoma* eggs have elevated to be highly immunogenic (Pearce, 2005). Treatment with CSE alone or combined with PZQ resulted in a significant reduction in

granuloma sizes compared to non-treated infected-mice. Badr *et al.* (2017) reported that administration of the alcoholic extract of saffron in *S. mansoni*-infected mice caused remarkable reduction in the number and size of *Schistosoma* granuloma in the liver section. Saffron and its main ingredient (safranal) are antioxidants. Therefore, the antifibrotic effect of CSE may be related to its antioxidant action (Farahmand *et al.*, 2013; Samarghandian *et al.*, 2017).

Furthermore, results of the present study showed a significant increase in the mean activity of SOD, without any significant increase in catalase activity in infected mice group treated with CSE as compared to infected mice group. Previous study, reported that saffron extract with dose (40 mg/kg/day) that treated diabetic rats had significantly increased serum SOD compared with the untreated diabetic rats without any significant difference on the activity of catalase (Samarghandian *et al.*, 2017). Another study of Farahmand *et al.* (2013) recorded that safranal elevated antioxidant-enzyme response through the elevation of the hepatic glutathione reductase, SOD, and catalase activities in the liver. Treatment of *S. mansoni* infected mice with PZQ alone or in combination with CSE showed significant increase in SOD and catalase levels. Several studies found that PZQ diminishes oxidative stress in schistosomiasis by increasing antioxidant enzyme activities (Abdel-Hafeez *et al.*, 2012; Rizk *et al.*, 2012).

Data in this study recorded significant increase in total IgG in *S. mansoni* infected mice group as compared to healthy control group. These results were in agreement with Boctor and Peter (1990). They showed that total IgG of the *S. mansoni* infected patients was elevated in the range of two to three times above normal. Also, mice infection with *S. mansoni* showed a significant ($P<0.001$) increase in serum IgG as compared to normal control (Kamel and El-Shinnawy, 2015). In addition, El-Sisi *et al.* (2011) reported that mice infected with *Schistosoma mansoni* live cercariae showed a time-dependent increase in serum IgE, IgG levels compared with the control group. In the current study, treatment of *S. mansoni* infected mice with CSE and/or PZQ showed a significant increase in the mean value of immunoglobulin IgG. This result comes in agreement with Vijayabhargava and Asad (2011), who reported that oral administration of saffron as suspension at doses of 50 and 100 mg/kg showed a significant increase in the level of serum immunoglobulins and circulating antibody titer. These findings indicate that saffron had been able to elevate the power of humoral mediated immunity. Saffron at low dose was effective in inducing a significant increase in the phagocytic activity. In the previous study, the authors concluded that *Crocus sativus* at low doses stimulate humoral and cell mediated immunity and it could have been considered as a potential immunostimulant and as anti-cancer agent (Vijayabhargava and Asad, 2011). Mantawy *et al.* (2011), proved that PZQ treatment in *S. mansoni* infected mice increase the level of IgG.

Results of the present study demonstrated an increase in the level of IL-10 in infected mice group as compared with normal control group. Similar results were obtained previously by Hesse *et al.* (2004) who found that IL-10 was elevated after infection with *S. mansoni*. IL-10 undoubtedly plays a key role in controlling infection (Coutinho *et al.*, 2010). The same results were reported in several studies on different experimental animals (El-Sisi *et al.*, 2011; Zaia *et al.*, 2016). In the current study, infected mice treated with PZQ showed an increase in the level of IL-10. Wilson *et al.*, (2011) found an increase in IL-10 in PZQ treated humans. Also, the present study showed an increase in the level of IL-10 in infected mice treated with CSE compared with infected group. This result comes in agreement with Shahbazian *et al.* (2019) who found that administration of 15 mg of saffron capsules for three months resulted

in an increases in serum IL-10 levels in the diabitic patient without any significant difference to the control group.

The present study showed an increase in NO in *S. mansoni* infected mice as compared to healthy control. This result comes in agreement with Al-Olayan *et al.* (2016) who reported that Schistosomiasis caused a significant increase in the liver levels of LPO and NO compared with the control group. Also, the present study illustrated that *S. mansoni* infected mice treated with PZQ alone or combined with CSE recorded significant increase in NO level in comparison with infected control mice. Previous report found an increase in NO level in hepatic tissue of PZQ treated animal and attributed that to activation of the immune system which increase the level of IFN- γ that can activate macrophages to produce NO and other inflammatory mediators (Eid *et al.*, 2014). All used treatments stimulate NO production. This in turn may generate an antifibrogenic effect on the *S. mansoni* granuloma and decrease granuloma size.

Generally, the studies of protective mechanisms against schistosomiasis are concentrated on CD4 $^{+}$ T-cells and antibody responses (Bickle, 2009). CD4 $^{+}$ T-cell-produced IFN- γ is considered as the key factor for killing the worms (Mountford *et al.*, 1992; Wilson *et al.*, 1999). IFN- γ activates host macrophages to produce NO, which help in eliminating the parasites efficiently (Hewitson *et al.*, 2005). Previous study established that granuloma formation is a manifestation of CD4 cell-mediated immunity against soluble schistosomal egg antigen (Lundy and Lukacs, 2013). Granulomas are then gradually down-regulated, largely by CD8 $^{+}$ lymphocytes (Perrin and Phillips, 1988; Fidel and Boros, 1990). In the current study, CSE treatment showed a significant increase in the CD4 $^{+}$ cells. Also, result showed an increase in CD8 $^{+}$ cells without any significant difference when compared to infected control mice. This result comes in agreement with Zhang *et al.* (2018) who reported that crocin as a one constituent of saffron significantly promoted T cell proliferation and increase CD4/CD8 ratio of T subset of children with acute lymphoblastic leukemia. Also, PZQ exerted significant stimulatory effects on cell immune responses which increase the percentage of CD8 $^{+}$ and reduced the percentage of CD4 $^{+}$ T-cells without any significant difference compared to infected control mice. Cells resulting in a reduced hepatic granuloma size in schistosomiasis as reported by El-Ahwany *et al.* (2006). Probably, the increase in T-cell reactivity after chemotherapy can be explained by exposure of released antigens to the immune system following the destruction of worms by chemotherapy (Corrêa-Oliveira *et al.*, 2000).

The granuloma around *Schistosoma* eggs formed of collagen fibers and inflammatory cells of Th2 origins, including macrophages, eosinophils and CD4 $^{+}$ T-cells (Mourra *et al.*, 2006). Previous study reported that the *Schistosoma* granuloma contained mixture of CD68 $^{+}$ histiocytes, CD3 $^{+}$ cells and CD20 $^{+}$ cells (Hussein *et al.*, 2006). The immunohistochemical examination showed a significant decrease in granuloma diameters of mice treated with CSE or CSE plus PZQ accompanied by a reduction in CD3 $^{+}$ cells compared to the infected control mice. However, mice mono-treatment with PZQ or CSE showed a significant increase in CD20 $^{+}$ cells expression. This result comes in accordance with Bani *et al.* (2011) who reported that oral administration of alcoholic extract of *Crocus sativus* at 6.25 mg/kg resulted in a significant increase in CD19 $^{+}$ B-cells in normal Balb/c mice. The higher levels of IgG production in the current study is consistent with the high percentage of CD20 $^{+}$ cells and might support the stimulative abilities of CSE on the humoral immune response.

CONCLUSION

CSE alone or in combination with PZQ accelerated healing of the pathological granulomatous lesions of the liver architecture and improved the anti-oxidant and host immune status in the present experimental model.

CONFLICT OF INTEREST

The authors in the current study declare the absence of the conflict of interest.

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