

Parasitological, pathological and immunological effects of hesperidin treatment on murine schistosomiasis *mansoni*

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Abstract: Hesperidin (HSP) is a natural plant extract which has various effective biological activities. HSP showed promising schistosomicidal properties against adult worms of *Schistosoma mansoni* (*S. mansoni*) both *in vivo* and *in vitro*. The aim of the present study was to evaluate the *in vivo* schistosomicidal effects of HSP on juvenile and adult stages of *S. mansoni* concerning parasitological, pathological and immunological parameters in murine model of infection. Swiss albino mice were used to achieve this aim. They were classified into 7 groups (10 mice in each group): group I which included normal control mice, group II which included infected untreated control mice (IU), group III given early HSP treatment, beginning on the first day of infection, group IV given early HSP+PZQ, group V given late HSP treatment, starting at the 4th week post-infection, group VI given late HSP+PZQ and group VII given PZQ alone. The highest reduction percentages of worm burden and tissue egg load were recorded in groups IV, VI and VII. The same groups showed the highest significant decrease in percentage of immature and mature ova and the highest significant increase in percentage of dead ova. Early HSP treatment gave the highest significant reduction in both granuloma number and diameter. There was significant increase in IgG level in response to *S. mansoni* soluble worm antigen protein in all treated mice groups. Also, early HSP treatment revealed the highest decrease in serum levels of IL-6, IL-12 and TNF- α and the highest increase in serum level of IL-10, while both early HSP and early HSP+PZQ treatments showed the highest decrease in serum level of IFN- γ . In conclusion, HSP treatment achieved promising schistosomicidal activities, especially when given early after exposure to *S. mansoni* infection, in addition it alleviated hepatic pathology by affecting some cytokines that are involved in granuloma formation. [Bahaa El Deen W. El Aswad and Gehan S. Sadek. **Parasitological, pathological and immunological effects of hesperidin treatment on murine schistosomiasis *mansoni***. *Life Sci J* 2014;11(7):840-855] (ISSN:1097-8135). <http://www.lifesciencesite.com>. 121

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1. Introduction

Schistosomiasis is a tropical parasitic disease caused by digenetic trematodes belonging to the genus *Schistosoma*. It is the second most frequent parasitic disease affecting man after malaria (Croft *et al.*, 2003 and King *et al.*, 2005). Recently, World Health Organization (WHO) estimated that 239 million people are infected with schistosomes (WHO, 2012), in addition, 600 million are at risk of infection (Chitsulo *et al.*, 2004 and Savioli *et al.*, 2004). In sub-Saharan Africa alone, 150,000 deaths per year are attributable to schistosomiasis (van der Werf *et al.*, 2003). Moreover, it has been reported that people infected with schistosomes may have increased susceptibility to other infectious diseases such as human immunodeficiency virus (Secor, 2012).

The pathology associated with *S. mansoni* is largely caused by the severe granulomatous inflammation and fibrosis elicited by the parasite eggs which become trapped in host organs such as liver and intestine. The formation of granuloma is dependent on CD4+T cell responses (Iacomini *et al.*, 1995 and Hernandez *et al.*, 1997). In murine schistosomiasis, Th1 type immune response predominates before the egg deposition and is characterized by the production

of IFN- γ , and then the immune response polarizes toward Th2 type mediated by the egg-derived antigens. Th2 type is characterized by predominance of IL-4, IL-5, IL-10 and IL-13 (Pearce *et al.*, 1991; Sher *et al.*, 1991; Vella and Pearce, 1992 and Hernandez *et al.*, 1997). Th2 activation appears to have a principal role in alleviating the lethal hepatic and intestinal damage of acute *Schistosoma* infection on the host (Fallon *et al.*, 2000). Also, it maintains the Th1 inflammatory immunopathology under control (Fallon and Dunne, 1999). However, some immunization studies showed that these Th2 responses might not achieve a considerable protective immunity, whereas Th1 immune response appeared to be more important in the induction of resistance against *S. mansoni* in the murine model (Fonseca *et al.*, 2004). Regards radiation-attenuated vaccine model of murine schistosomiasis, the protection in many studies was dependent on the Th1-associated humoral and cell-mediated immune responses (Wynn *et al.*, 1995 & 1996 and Mountford & Pearlman, 1998), but in the absence of Th1 responses, a reduction in worm burdens was detected suggesting that the Th2 type response might still possess a role in protection (Anderson *et al.*, 1998). Modulation of both Th1 and Th2 cytokines

could down regulate granulomatous formation and consequently reduce the morbidity of schistosomiasis (Hoffmann *et al.*, 2000 and La Flamme *et al.*, 2001).

WHO has recommended praziquantel (PZQ) as the drug of choice for either individual or mass schistosomiasis treatment and it has achieved a significant efficacy in most cases (Fenwick *et al.*, 2003 and Coura & Amaral, 2004). However, PZQ has reported a number of drawbacks. Juvenile stages are less susceptible to PZQ than adult *Schistosoma* worms (Silva *et al.*, 2003; Doenhoff *et al.*, 2008; Keiser *et al.*, 2009 and El-Lakkany & el-Din, 2013). It does not induce protective immunity against subsequent infection and therefore does not prevent re-infection (Bergquist *et al.*, 2002 and McManus & Dalton, 2006). Also, PZQ-resistant *Schistosoma* strains have been increasingly reported in the field (Fallon & Doenhoff, 1994; Botros & Bennett 2007 and Beckmann *et al.*, 2012). Moreover, PZQ was reported to induce abdominal pain and diarrhea (Kabatereine *et al.*, 2003) as well as hemorrhage in the lung tissue of the host (Flisser & McLaren, 1989). Therefore, the development of new anti-*Schistosoma* drugs are pursued until delivery of an effective anti-*Schistosoma* vaccine. There is a promising trend of using the natural compounds derived from plant extracts as drugs against *Schistosoma*, being safe and with less medical side effects (Molgaard *et al.*, 2001; Kayser *et al.*, 2003 and Parreira *et al.*, 2010).

Hesperidin (HSP) (5,7,3'-trihydroxy-4'-methoxy-flavanone-7-rhamnoglucoside) is a flavanone glycoside, comprising of an aglycone, hesperetin or methyl eriodictyol and an attached disaccharide, rutinose. It is the major flavonoid in the sweet orange and lemon (Barthe *et al.*, 1988). HSP has various effective biological activities. It possesses a significant anti-inflammatory effect, analgesic effect (Galati *et al.*, 1994; Boisseau, 2002 and Olszanecki *et al.*, 2002) and antioxidant properties (Jovanovic *et al.*, 1994 and Chen *et al.*, 2010). It has beneficial effects on abnormal capillary permeability and fragility (Versantvoort *et al.*, 1993). Many reports have shown that HSP has anticarcinogenic activities (Tian *et al.*, 2001; Winawer *et al.*, 2003 and Kamaraj *et al.*, 2010). Also, HSP and other flavonoids were found to inhibit the growth of many organisms such as some bacteria (Panasiak *et al.*, 1989 and Bae *et al.*, 1999), some fungi (Krolicki and Lamer-Zarawska, 1984) and some viruses (Wacker and Eilmes, 1975; Lee *et al.*, 1999 and Kim *et al.*, 2000). In addition, HSP has shown a molluscicidal activity against *Bulinus truncatus* snail; the molluscan intermediate host of urinary schistosomiasis (Lahlou, 2004).

In a recent report, HSP showed promising antischistosomal properties against adult worms of *S. mansoni* both *in vitro* and *in vivo* murine infection (Allam and Abuelsaad 2013a). Also, it modulated

production of some Th1, Th2 and Th17 cytokines, that could limit immunopathology of schistosomiasis, when it was incubated with the cultured splenocytes derived from *S. mansoni* infected mice in the presence of *Schistosoma* antigens (Allam and Abuelsaad, 2013b).

Owing to the above mentioned data and the recent promising trend of using natural safe plant extracts in the development of new drugs, the aims of the present study were to evaluate *in vivo* schistosomicidal effects of HSP on juvenile and adult stages of *S. mansoni* concerning different parasitological, pathological and immunological parameters in murine model of infection.

2. Materials and Methods

Mice

Eight-week old male Swiss albino mice of the CD-1 strain, weighing 20±2 grams were obtained from the *Schistosoma* Biologic Supply Center, Theodore Bilharz Research Institute (TBRI, Imbaba, Giza, Egypt). The mice were bred under environmentally controlled conditions, and fed with a standard pellet diet and water *ad libitum*. Handling and treatment of animals were conducted according to internationally valid guidelines and ethical conditions.

Parasite and infection

S. mansoni cercariae suspension (5 ml) was obtained from TBRI and placed drop-by-drop on a glass plate. One drop of 1% iodine was added to 0.1 ml of suspension in order to kill cercariae. With the aid of a dissecting microscope, the average number of cercariae in the 0.1 ml of suspension was determined by making five counts and taking their mean. Infection was then performed by subcutaneous injection of 100±10 *S. mansoni* cercariae into each mouse (Stirewalt & Dorsey, 1974).

Drugs and doses

a) PZQ (Distocide®, EIPICO, Egypt) was orally administered at a dose of 500 mg/kg body weight for 2 consecutive days (Piper *et al.*, 1990). It was freshly prepared before use as a 2% suspension in Cremophor-El (Sigma).

b) HSP (Sigma, USA) was used as a freshly prepared suspension in 7% Tween-80 and 3% ethanol before administration intraperitoneally (i.p.). It was given in two doses, each dose equals 600 mg/kg (divided into 100 mg/kg given three times per week for two weeks) which is 1/10 of LD50 (lethal dose, 50%) as previously calculated by Allam and Abuelsaad (2013a). So, treatment course covers four consecutive weeks.

Experimental design

The mice were divided into seven groups, each composed of 10 mice, as the following:

Group I: normal control mice (NC).

Group II: infected untreated control mice (IU).

Group III: infected and treated with 100 mg/kg HSP i.p. three times per week for four weeks,

beginning on the first day of infection (early HSP treatment).

Group IV: infected and received the early HSP treatment, in addition to treatment with 500 mg/kg body weight of PZQ for two consecutive days at the 6th week post- infection (p.i.) (early HSP + PZQ).

Group V: infected and treated with 100 mg/kg HSP i.p. three times per week for four weeks, beginning at the 4th week p.i. (late HSP treatment).

Group VI: infected and received the late HSP treatment, also received 500 mg/kg body weight of PZQ for two consecutive days at the 6th week p.i. (late HSP+ PZQ).

Group VII: infected and treated with PZQ alone in a dose of 500 mg/kg body weight for two consecutive days at the 6th week p.i. (PZQ).

At the end of the 8th week p.i., all the mice were sacrificed and the effects of the used drugs were evaluated concerning parasitological, pathological and immunological parameters.

Regarding the parasitological and immunological parameters, work was done in TBRI, while the pathological work was done in Department of Pathology, Faculty of Medicine, Menoufia University.

Parasitological parameters

Worm burden

After sacrifice of mice, worms were recovered from the hepatic portal system and mesenteric vessels using the perfusion technique described by Smithers and Terry (1965). Following perfusion, numbers and sex of the adult worms (males, females and couples) were determined. They were counted either by direct visualization or under dissecting microscope. The reduction percentage in worm numbers, after treatment, was calculated according to Tendler *et al.* (1986) as follows: reduction % = $(C - T/C) \times 100$, where C is the mean number of parasites recovered from infected untreated control animals and T is the mean number of parasites recovered from treated animals.

Tissue egg load

The number of eggs per gram of tissue was estimated by weighing a piece of liver or small intestine, which was then digested and incubated overnight in 5% KOH. The hepatic and intestinal tissue egg loads were determined by multiplying the average number of eggs in each 1 ml sample by the total volume of KOH and then dividing that value by the weight of the sample to yield the number of eggs per gram of tissue (Kloetzel, 1967).

Oogram pattern

After perfusion, the small intestine of each mouse was separated and transferred to a petri dish. Three fragments (each is 1 cm in length) of the small intestine were cut longitudinally, rinsed in saline and slightly dried on filter paper. Then, the fragments were

examined by the low-power microscopy and the percentages of immature, mature, and dead eggs were counted from a total of 100 eggs per each intestinal segment. They were classified according to the categories previously defined by Pellegrino *et al.* (1962). The mean percentage of each stage/animal, and then the mean percentage of each stage/each group of animals were calculated.

Pathological parameters

Hepatic granuloma studies

Approximately half of the liver of each sacrificed mouse was immediately fixed in 10% formalin and embedded in paraffin. Five paraffin liver sections (5 μ m in thickness) were prepared from each mouse and stained with hematoxylin and eosin (H& E). Measurement of granuloma diameter was done only for granulomas containing a single egg in the center with intact or degenerated miracidia using an ocular micrometer calibrated with millimetric reticle (Carl Zeiss, USA). The mean diameter of each granuloma was calculated by measuring two diameters of the lesion at right angles to each other (von Lichtenberg, 1962). The granulomas were counted in five successive fields of each examined slide. The mean number of granulomas and the mean value of granuloma diameter of each group were calculated from the mean values of each individual mouse of that group. Reduction % of number of granulomas = $(C - T/C) \times 100$, where C is the mean number of granulomas in infected untreated control animals and T is the mean number of granulomas in treated animals. Reduction % of size of granuloma = $(C - T/C) \times 100$, where C is the mean size of granuloma in infected untreated control animals and T is the mean size of granuloma in treated animals.

Immunological parameters

Blood was obtained from each mouse after sacrifice. Sera were collected from the clotted blood samples after centrifugation at 400 \times g for 15 minutes at 4°C, then divided into aliquots and stored at -80°C until use.

Specific antibody responses to *S. mansoni* antigen

S. mansoni soluble worm antigen protein (SWAP) was purchased from TBRI and was suspended in 0.01M phosphate-buffered saline (PBS), pH 7.2. The protein content was estimated with Coomassie Plus™ (Bradford) Assay Kit (Pierce, USA), the antigen was aliquoted and stored at -20°C until use. Specific anti-SWAP IgM and IgG were measured using indirect ELISA based on the method of Engvall and Perlman (1971) with some modifications. ELISA microtitre plates (Nunc, Denmark) were coated with 100 μ l/well of 3 μ g/ml of SWAP antigen diluted in 0.05 M carbonate/bicarbonate coating buffer, pH 9.6 overnight at 4°C. On the next day, the plates were thoroughly washed with PBS and 0.05% Tween 20 (PBS/T), then they were blocked with 200 μ l/well of 0.1% bovine serum albumin (BSA) (Sigma) diluted in PBS/T for 2

hours (hrs) at 37 °C. After that, the plates were washed 3 times. Each individual mouse serum was diluted 1:100 in PBS and 100 µl of each diluted serum was added, then the plates were incubated for 1 hr at 37 °C. After thorough washing, 100µl/well of horseradish peroxidase-conjugated anti-mouse IgM and IgG (Sigma) diluted in PBS/T (1:1000 for IgM and 1:2000 for IgG) were added and the plates were incubated for 2 hrs at 37°C. The plates were washed and 100 µl/well of the substrate o-phenylenediamine dihydrochloride (Sigma) was added, then the plates were incubated for 30 minutes in the dark at room temperature. The enzyme reaction was stopped with 100 µl/well of 8N H₂SO₄, and the plates were read at optical density (OD) of 492 nm using a microplate ELISA reader (Bio-Rad, USA).

Determination of cytokines serum levels

Cytokines IL-10, IL-12, TNF- α , IFN- γ and IL-6, were measured in the sera of mice by using sandwich ELISAs with anti-cytokine antibodies according to the manufacturer's instructions (Phar Mingen, San Diego, USA). Recombinant cytokines were used as standards. Briefly, 96-well flat-bottom plates were coated with 100 µl/well of the capture antibodies diluted in carbonate/bicarbonate buffer, pH 9.6. After incubation overnight at 4°C, the plates were washed three times in washing buffer, PBS/T, and nonspecific binding sites were blocked using blocking buffer, PBS/T/1% low-fat dry milk powder. After washing three times, the serum samples or the recombinant cytokines were added in a volume of 100µl/well. The cytokine standards were serially diluted in the blocking buffer following manufacturer's instructions. The plates were incubated for 3 hrs at room temperature with gentle shaking. The plates were washed, then the appropriate biotinylated anti-cytokine detection antibody was added in a volume of 100µl/well. After incubation for 1 hr with gentle shaking at room temperature, the plates were washed three times. After that, the streptavidin-alkaline phosphatase conjugate was added and the plates were left for 30 min. Finally, the plates were washed for five times and the reaction was developed with *para*-nitrophenyl phosphate (Sigma), then the absorbance at 405 nm was measured using a microplate ELISA reader. The cytokine concentration was calculated from the standard curve using Microplate Manager Software (Bio-Rad).

Statistical Analysis

Data were expressed as mean \pm standard deviation (SD). Reduction % was calculated according to the equation: [(mean value of the control – mean value of treated group/mean value of the control) x 100]. Comparison relative to the infected untreated control group was performed using unpaired Student's *t*-test. SPSS computer program (version 12 windows) was used for data analysis. The data were considered significant if *P* value \leq 0.05.

3. Results

Parasitological parameters

Numbers of the recovered worms

The highest reduction percentages were recorded in groups IV (early HSP+PZQ), VI (late HSP+PZQ) and VII (PZQ). The reduction percentage in group IV were 89.3%, 80%, 75% and 100% in total number of worms, number of males, number of females and number of couples, respectively. The reduction percentages in group VI were 89.9%, 78%, 76.9% and 100% in the above mentioned worm groups, respectively. In group VII, the reduction percentages were 90.7%, 81%, 80.7% and 100% in the above mentioned worm groups, respectively. From the above mentioned results, it is noticed that the *Schistosoma* adult couples were not recovered (100% reduction). In these three groups, there were very high significant (*P*<0.001) differences between their results and the results of group II (IU) (Table 1).

Next to the above mentioned three groups, group III (early HSP) showed high reduction percentages; 44.1%, 36%, 42.3% and 50% in total number of worms, number of males, number of females and number of couples, respectively. There was a high significant statistical (*P*<0.01) difference between these results and results of group II (IU). The least reduction percentages were recorded in group V (late HSP) where they were 23.1%, 30%, 26.9% and 25% in the previously mentioned worm groups, respectively, but there was still a significant statistical (*P*<0.05) difference between results of this group and that of group II (IU) (Table 1).

Tissue egg load

The highest reduction percentages of ova in tissues were recorded in group IV (early HSP+PZQ), VI (late HSP+PZQ) and VII (PZQ). The reduction percentages in group IV were 90.5% and 80.7% in intestine and liver, respectively. In group VI, they were 89% and 82.8% in the same tissues, respectively, while in group VII, they were 93.2% and 86.2% in the above mentioned tissues, respectively. In these groups, there were very high significant (*P*<0.001) differences between their results and the result of group II (Table 2).

Next to the above mentioned three groups, group III (early HSP) gave high reduction percentages; 40.8% and 37% in intestine and liver, respectively. There was a high significant statistical (*P*<0.01) difference between these results and results of group II. The least reduction percentages were recorded in group V (late HSP) where they were 36.05% and 33% in the same tissues, respectively, but there was still a significant statistical (*P*<0.05) difference between results of this group and results of group II (Table 2).

Quality of the eggs (oogram)

Regarding group III (early HSP), the percentages of immature and mature eggs ($57.9 \pm 3.9\%$ and $29.9 \pm 3.1\%$, respectively) insignificantly ($P > 0.05$) decreased when compared to group II, while the percentage of dead eggs ($12.2 \pm 1.1\%$) significantly increased ($P < 0.05$). Concerning group IV (early HSP+PZQ), the percentages of immature and mature eggs ($4.9 \pm 5.4\%$ and $3.3 \pm 1.4\%$, respectively) very highly significantly ($P < 0.001$) decreased and the percentage of dead eggs ($91.8 \pm 7.4\%$) showed also very highly significantly ($P < 0.001$) increase (Table 3).

As regards group V, the percentage of immature eggs ($52.1 \pm 3.8\%$) significantly ($P < 0.05$) decreased when compared to group II, while the percentage of mature eggs ($30.8 \pm 3\%$) insignificantly ($P > 0.05$) decreased when compared to group II. On the other hand, the percentage of dead eggs ($17.1 \pm 2\%$) was of highly significantly ($P < 0.01$) increase (Table 3).

Group VI (late HSP+PZQ) showed very high significant statistical ($P < 0.001$) decrease in percentage of immature and mature eggs ($9.5 \pm 1.2\%$ and $8.8 \pm 1.1\%$, respectively), also it showed a very high significant statistical ($P < 0.001$) increase in the percentage of dead eggs ($81.7 \pm 4.1\%$) when compared to group II. Nearly similar findings were obtained from group VII (PZQ) where there was a very high significant statistical ($P < 0.001$) decrease in percentage of immature and mature eggs ($2.0 \pm 0.3\%$ and $1.7 \pm 0.2\%$, respectively), also it showed a very high significant statistical ($P < 0.001$) increase in the percentage of dead eggs ($96.3 \pm 4.9\%$) when compared to group II (Table 3).

Pathological parameters

Development of schistosomal hepatic granuloma

Group III (early HSP) gave the highest significant ($P < 0.01$) reduction in both granuloma number (56.6%) and granuloma diameter (60.3%) when compared to group II. Group IV (early HSP+PZQ) also gave a high significant ($P < 0.01$) reduction in granuloma diameter (59.3%) and only a significant ($P < 0.05$) reduction in granuloma number (29.54%) when compared to group II (Table 4). Concerning group V (late HSP), it showed a high significant ($P < 0.01$) reduction in granuloma diameter (49.9%), but insignificant ($P > 0.05$) reduction in granuloma number (22.72%) when compared to group II. As regards group VI (late HSP+PZQ), it gave a significant ($P < 0.05$) reduction in granuloma diameter (39.8%), but insignificant ($P > 0.05$) reduction in granuloma number (17.42%) when compared to group II. Regarding group VII (PZQ), it gave a significant ($P < 0.05$) reduction in both granuloma number (25.75%) and granuloma diameter (33.5%) (Table 4).

Hepatic granuloma morphometries

Microscopic examination of liver sections stained with H& E revealed intact liver architecture in all the studied mice groups. The liver parenchyma was

studded with *Schistosoma* granuloma surrounding newly laid eggs. The size of granuloma and the intensity of inflammatory infiltrate were evidently variable between the groups in this study (Figure 1, photomicrographs A-F).

Group II (IU) showed very large active granulomas formed centrally of *S. mansoni* ova surrounded by chronic inflammatory infiltrate in the form of lymphocytes, histiocytes and eosinophils (Figure 1, photomicrograph A).

In the other treated groups, there were either active and small granulomas or healed granulomas. The former consisted of a central *Schistosoma* ovum, surrounded by epithelioid cells, lymphocytes, eosinophils, and a few giant cells. Epithelioid cell is a large cell with abundant eosinophilic cytoplasm and central ovoid nucleus. However, in healed granuloma the inflammatory cellular infiltrate was replaced by fibrous tissue formed of fibroblasts and collagen bundles (Figure 1, photomicrographs B-F). It was observed that group III (early HSP) showed the best pathological improvement among all groups (Figure 1, photomicrograph B).

Immunological parameters

The effect of HSP on antibody response to worm antigen

Indirect ELISA showed that the anti-SWAP IgM levels in all treated mice groups were not significantly ($P > 0.05$) different when compared to that of infected untreated one (group II). In contrast, there was a significant ($P < 0.05$) increase in IgG levels in response to SWAP antigen of *S. mansoni* in all treated mice groups showing augmentation of the immune response when compared to the infected untreated one (Figure 2).

Effect of HSP on serum levels of cytokines

Group III (early HSP) showed a high significant ($P < 0.01$) decrease in serum levels of IL-6, IL-12, TNF- α and IFN- γ when compared to group II (IU). On the other, it showed a very high significant ($P < 0.001$) increase in serum level of IL-10 when compared to the same group (Figure 3). Concerning group IV (early HSP+PZQ), serum levels of IL-6, TNF- α and INF- γ showed a high significant ($P < 0.01$) decrease in comparison to group II (IU), while serum level of IL-12 showed only a significant ($P < 0.05$) decrease when compared to group II. However, serum level of IL-10 showed a high significant ($P < 0.01$) increase (Figure 3). Regarding group V (late HSP), there was a high significant ($P < 0.01$) decrease in serum levels of IL-6 and TNF- α , while only a significant ($P < 0.05$) decrease in serum levels of IL-12 and IFN- γ when compared to group II. In contrast, it showed a significant ($P < 0.05$) increase in serum level of IL-10 (Figure 3).

As regards group VI (late HSP+PZQ), there was a high significant ($P < 0.01$) decrease in serum level

of TNF- α , while there was only a significant ($P<0.05$) decrease in serum level of IL-6 and INF- γ . On the other hand, there was no significant ($P>0.05$) decrease in serum level of IL-12. The same group showed a significant ($P<0.05$) increase in IL-10 when compared to group II (Figure 3).

Analysis of results of group VII (PZQ) showed that there was a high significant ($P<0.01$) decrease in serum level of TNF- α , while there was only a significant ($P<0.05$) decrease in INF- γ and no significant ($P>0.05$) decrease in serum level of IL-6 or IL-12. On the other hand, there was an insignificant ($P>0.05$) increase in IL-10 (Figure 3).

Table 1: Effect of HSP on total worms, males, females and couples counts in mice infected with *S. mansoni*.

Animal groups	Total no. of worms	Reduction %	No. of males	Reduction %	No. of females	Reduction %	No. of couples	Reduction %
NC	-	-	-	-	-	-	-	-
IU	31.2 \pm 0.3	-	10 \pm 0.4	-	5.2 \pm 0.2	-	8 \pm 0.27	-
Early HSP	17.44 \pm 2.33**	44.1	6.4 \pm 0.2**	36	3 \pm 0.32**	42.3	4 \pm 1.2**	50
Early HSP+PZQ	3.33 \pm 1.08***	89.3	2 \pm 0.1***	80	1.3 \pm 0.6***	75	0.0***	100
Late HSP	23.99 \pm 0.5*	23.1	7 \pm 0.7*	30	3.8 \pm 0.9*	26.9	6 \pm 1.3*	25
Late HSP+PZQ	3.4 \pm 1.82***	89.9	2.2 \pm 0.8***	78	1.2 \pm 0.3***	76.9	0.0***	100
PZQ	2.9 \pm 1.11***	90.7	1.9 \pm 0.1***	81	1 \pm 0.5***	80.7	0.0***	100

Data are expressed as means \pm SD. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$ are of significant difference in comparison to the infected untreated mice group (IU).

Table 2: Effect of HSP on tissue egg load in mice infected with *S. mansoni*.

Animal groups	Mean number of ova count/g \pm SD			
	Intestine	Reduction %	Liver	Reduction %
NC	-	-	-	-
IU	18920 \pm 94.2	-	9270 \pm 83.0	-
Early HSP	11200 \pm 40.5**	40.8	5833 \pm 53.6**	37
Early HSP+PZQ	1789 \pm 50.5***	90.5	1789 \pm 70.0***	80.7
Late HSP	12100 \pm 27.9*	36.05	6212 \pm 9.0*	33
Late HSP+PZQ	2083 \pm 52.3***	89	1597 \pm 69***	82.8
PZQ	1293 \pm 52.3***	93.2	1279 \pm 68***	86.2

Data are expressed as means \pm SD. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$ are of significant difference in comparison to the infected untreated mice group (IU).

Table 3: Effect of HSP on the oogram pattern in mice infected with *S. mansoni*.

Animal groups	Oogram pattern (% ova)		
	Immature	Mature	Dead
NC	-	-	-
IU	67.2 \pm 5	33.1 \pm 2.6	1.7 \pm 0.3
Early HSP	57.9 \pm 3.9	29.9 \pm 3.1	12.2 \pm 1.1*
Early HSP+PZQ	4.9 \pm 5.4***	3.3 \pm 1.4***	91.8 \pm 7.4***
Late HSP	52.1 \pm 3.8*	30.8 \pm 3	17.1 \pm 2**
Late HSP+PZQ	9.5 \pm 1.2***	8.8 \pm 1.1***	81.7 \pm 4.1***
PZQ	2.0 \pm 0.3***	1.7 \pm 0.2 ***	96.3 \pm 4.9***

Data are expressed as means \pm SD. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$ are of significant difference in comparison to the infected untreated mice group (IU).

Table 4: Effect of HSP on hepatic granuloma number and diameter in mice infected with *S. mansoni*.

Animal groups	Granuloma No. (Mean \pm SD)	Reduction %	Granuloma diameter (Mean \pm SD)	Reduction %
NC	-	-	-	-
IU	13.2 \pm 0.3	-	298.5 \pm 3.5	-
Early HSP	5.8 \pm 0.2**	56.06	118.5 \pm 2.9**	60.3
Early HSP+PZQ	9.3 \pm 0.12*	29.54	121.5 \pm 3.1**	59.3
Late HSP	10.2 \pm 0.09	22.72	149.5 \pm 2.7**	49.9
Late HSP+PZQ	10.9 \pm 0.11	17.42	179.5 \pm 2.2*	39.8
PZQ	9.8 \pm 0.27*	25.75	198.5 \pm 2.5*	33.5

Data are expressed as means \pm SD. * $P<0.05$ and ** $P<0.01$ are of significant difference in comparison to the infected untreated mice group (IU).

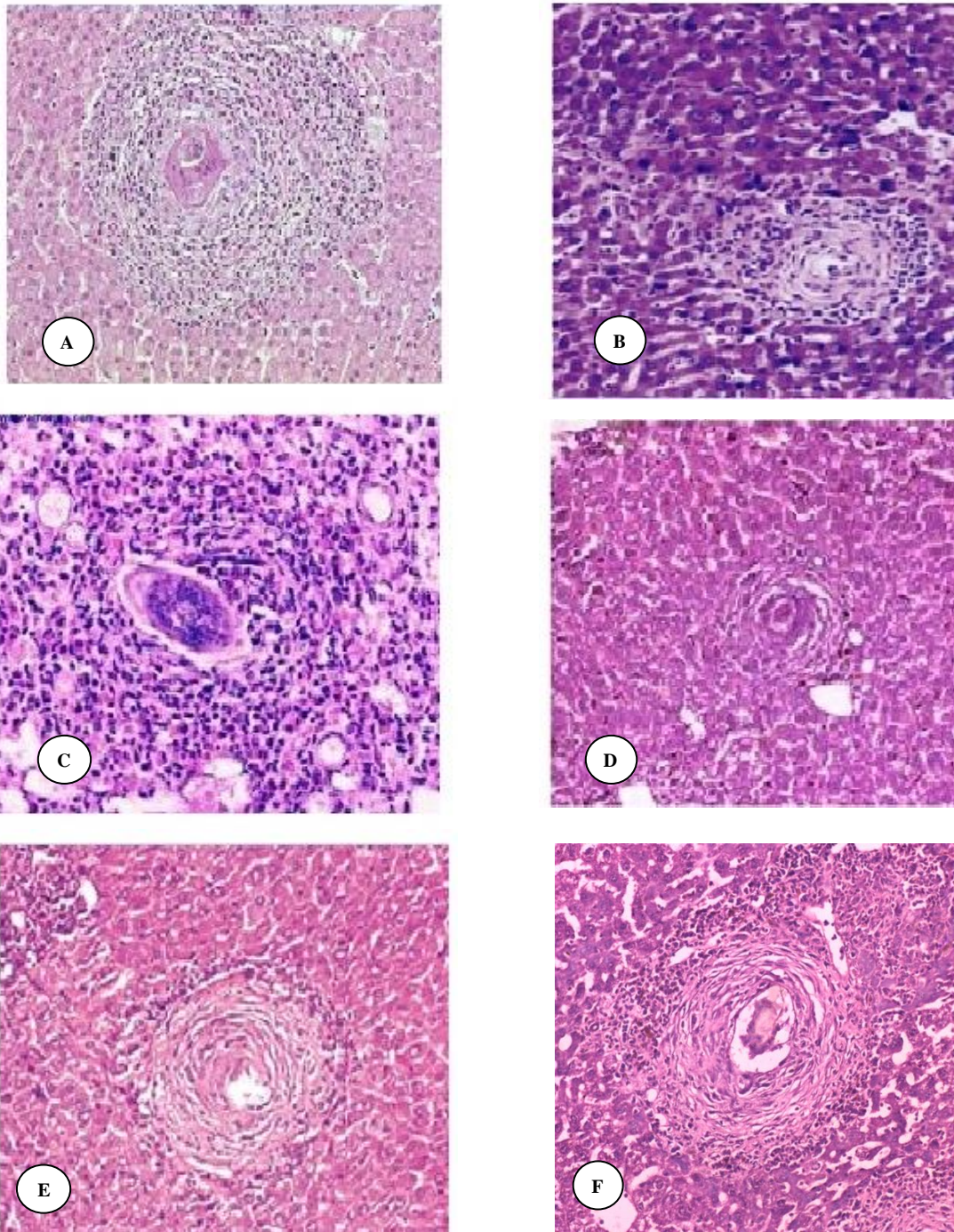


Figure 1: Photomicrographs of hepatic granulomas showing the effect of HSP on the hepatic granuloma diameter. A (infected untreated mice): exhibits an active granuloma formed centrally of *S. mansoni* ovum surrounded by chronic inflammatory infiltrate in the form of lymphocytes, histiocytes and eosinophils; B (early HSP treatment): shows a small sized granuloma formed of concentric layers of fibrous tissue and few inflammatory infiltrate, while the centrally located ovum is barely evident; C (early HSP+PZQ): shows an active granuloma surrounded by moderate infiltrate of lymphocytes, histiocytes and eosinophils; D (late HSP treatment): the size of the granuloma regressed to be formed mainly of the ovum, concentric layers of fibrous tissue and minimally surrounded by chronic inflammatory infiltrate; E (late HSP+PZQ): reveals healed granuloma formed of centrally located semi calcified ovum completely surrounded by concentric layers of fibrous tissue replacing entirely the inflammatory infiltrate and F (PZQ): exhibits a partly healed granuloma surrounding a centrally located ovum, where few cells of the inflammatory infiltrate are still evident (H&E, x200).

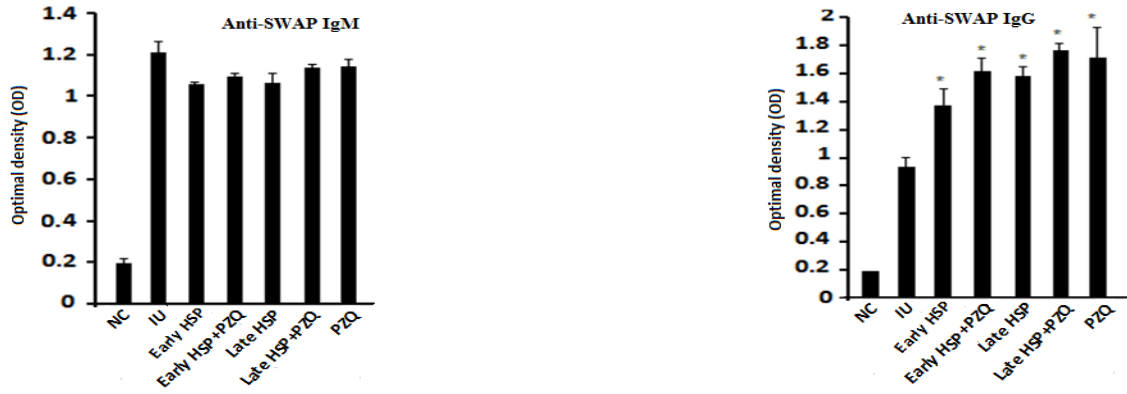


Figure 2: Effect of HSP on specific antibodies response against SWAP antigen of *S. mansoni*. Data are expressed as mean OD \pm SD. * $P < 0.05$ is of significant difference in comparison to infected untreated mice group.

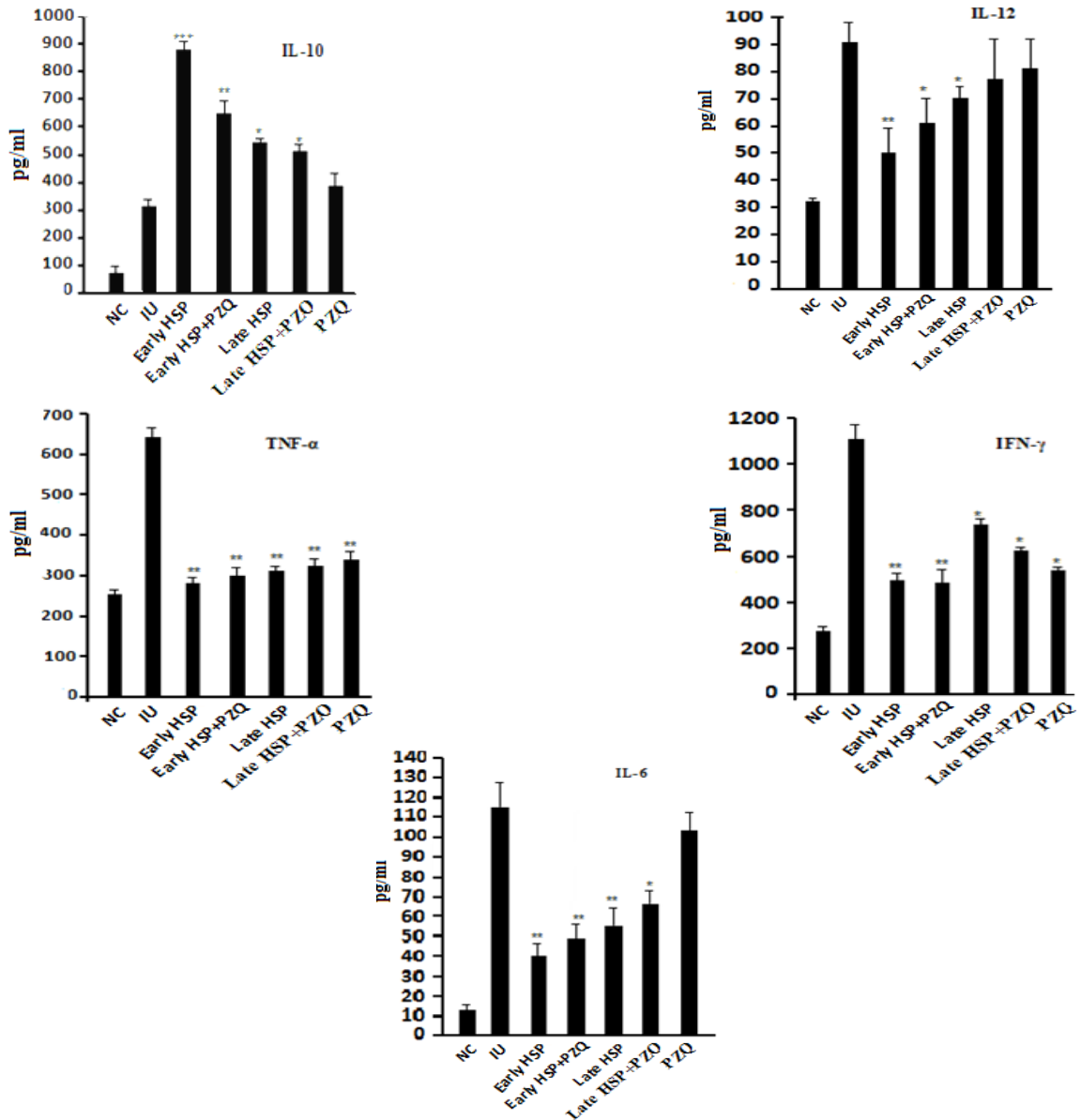


Figure 3: Effect of HSP treatment on serum levels of IL-10, IL-12, TNF- α , IFN- γ and IL-6 cytokines. Their levels were measured by sandwich ELISA. Data are represented as mean \pm SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ are of significant difference in comparison to infected untreated mice group (IU).

4. Discussion

Praziquantel is the only drug employed for treatment of infections caused by all *Schistosoma* species (Fenwick *et al.*, 2003 and Coura & Amaral, 2004). However, PZQ has many shortcomings (McManus & Dalton, 2006; Keiser *et al.*, 2009; Beckmann *et al.*, 2012 and El-Lakkany & el-Din, 2013) and dependence on a single drug to treat a disease that affects hundred millions of people is a real concern (Portela *et al.*, 2012). This raises an urgent need for effective and safe complementary or alternative drugs (Caffrey, 2007 and Lancelot *et al.*, 2013). Hesperidin has been reported to achieve promising results against adult stage of *S. mansoni* when it was tested *in vitro* and *in vivo* (Allam and Abuelsaad, 2013a).

Herein, *in vivo* schistosomicidal effects of HSP on both immature and adult stages of *S. mansoni* in mice infected by the parasite were evaluated concerning parasitological, pathological and immunological responses. HSP was administered intraperitoneally as absorption of this drug was proved to be higher by this route than after oral administration (das Neves *et al.*, 2004 and Hosseinimehr & Nemati, 2006) and each dose was divided over multiple settings to reduce any toxic effects of the compound (Allam and Abuelsaad, 2013a).

To test its *in vivo* anti-schistosomal activity against the juvenile stage of *S. mansoni*, HSP was given for 4 weeks starting from the first day of infection until the maturation of adults and before oviposition (group III) (early HSP). Early HSP treatment achieved a significant reduction in the perfused total adult worms (44.1%), males (36%), females (42.3%) and couples (50%) in comparison to the infected untreated mice group. To our knowledge, this is the first report which tests the efficacy of HSP on the juvenile stage of *S. mansoni*. These results were nearly similar to those of Allam and Abuelsaad (2013a) who reported that HSP, given at the 6th week p.i., significantly reduced total worm, male, female and possible worm pairs counts by 47.5%, 50%, 45.2% and 50%, respectively in comparison to the infected untreated mice. It appeared that HSP was active against both male and female worms and this was in agreement with Allam and Abuelsaad (2013a). This is a promising result concerning efficacy of HSP as the drug which acts on both sexes is preferred over those active against only one sex (Sanderson *et al.*, 2002).

Although late HSP treatment (group V) achieved less reduction percentages than the early treatment, but these were still of significant values. It decreased total worm burden, males, females and couples by 23.1%, 30%, 26.9% and 25%, respectively. These results were lower than those recorded by Allam

and Abuelsaad (2013a) and this could be attributed to the differences in the immunological responses between the mice strains used in both studies or may be due to difference in the techniques used for infecting the mice regarding either the number of infecting cercariae or the method of administration. Allam and his colleague used infectious dose of around 60 cercariae inoculated by tail immersion method, while in the current study, the infecting cercariae were around 100 and administered subcutaneously and this might lead to heavier parasitic load.

Early HSP+PZQ, late HSP+PZQ and PZQ treatments achieved nearly similar results of very high significance regarding reduction percentages of total worm burden (89.3%, 89.9% and 90.7%, respectively), adult male count (80%, 78 % and 81%, respectively) and adult female count (75%, 76.9% and 80.7%, respectively) compared to IU mice. In addition, all these treatment regimens completely eradicated the couples of *Schistosoma*.

The current study pointed out that HSP has a higher killing effect on the juvenile (group III) than on the adult stage (group V) of *S. mansoni in vivo* (44.1% versus 23.1%). This result is of significant value as it is well documented that PZQ, whether *in vivo* or *in vitro* studies, has a limited effect on juvenile schistosomes (Shaw, 1990; Utzinger *et al.*, 2003; Aragona, *et al.*, 2009 and El-Lakkany and el-Din, 2013).

Overall, this reduction in worm load could be attributed to many explanations. First, HSP may possess direct schistosomicidal effects on the different developmental stages of *S. mansoni* especially the juvenile stage. Second, it may induce an effective immune response against *S. mansoni* infection and this was tested herein, where, HSP treatment augmented the mouse IgG response against *S. mansoni* whether given early or late. This was in accordance with Allam and Abuelsaad (2013a) who showed that anti-SWAP IgG level was significantly higher in HSP treated group than in the untreated one. Also, the present results were consistent with that of Ying *et al.* (2009) who found that HSP significantly increased the level of IgG in weaned piglets. On the other hand, it was found that anti-SWAP IgM levels were of insignificant difference, in all treated groups, when compared to those of IU mice and this also was in parallel with Allam and Abuelsaad (2013a) who stated the same observation.

The necessity of antibody response in protection against schistosomiasis has been well documented (Jankovic *et al.*, 1999; Dunne and Mountford 2001 and Torben *et al.*, 2011). The chemotherapeutic effect of PZQ against *S. mansoni* is dependent mainly on IgG response (Brindley and

Sher, 1987; Doenhoff *et al.*, 1987 and Pica-Mattoccia & Cioli, 2004). Such induced antibodies may impair worm viability by interfering with critical molecules found on the parasite membrane, such as the glucose transporter, the amino acid permease and some vital enzymes, also these antibodies may cause damage to tegument of the worm impairing its function, so the parasite could be easily attacked by the host's immune system (Khalife *et al.*, 1989; Grzych *et al.*, 1993; El Ridi, 2002 and Torben *et al.*, 2011). Besides, it has been shown that in rat, primate and human schistosomiasis, antibody-dependent cell-mediated cytotoxicity which mainly depends on IgG isotype is the main mechanism of killing the parasite larvae (Capron and Capron 1994 and Torben *et al.*, 2011). Moreover, it was recorded that HSP has inhibitory effects on some enzymes such as lipase of *Pseudomonas* (Kawaguchi *et al.*, 1997) and tyrosinase diphenolase (Zhang *et al.*, 2007) and it might show similar effects on some vital enzymes of the *Schistosoma* developmental stages hindering their development and/or survival but these need to be further studied.

Regarding the tissue egg load, early HSP treatment showed a high significant reduction in the egg count in the intestine and liver (40.8% and 37%, respectively). Reduction percentage in liver was nearly similar to that which was recorded by Allam and Abuelsaad (2013a) (41.5%). However, regarding intestine, the reduction percentage in tissue egg load was lower than that recorded by the same authors (63.7%). This discrepancy is probably attributable to the difference in distribution of eggs among tissues. Anyhow, the reduction in tissue egg load caused by HSP is probably due to reduction in worm load.

Late HSP treatment (group V) decreased the tissue egg load with lower percentage than early HSP treatment (36.05% in intestine and 33% in liver), but it was still of significant value. This lower reduction could be attributable to the lower reduction percentage of adult worms achieved by late HSP than early HSP treatment. On the other hand, early HSP+PZQ, late HSP+PZQ and PZQ treatments revealed the highest percentage of reduction in the intestinal egg (90.5%, 89% and 93.2%, respectively) or liver (80.7%, 82.8% and 86.2%, respectively). This result is not unexpected as these three groups gave the highest reduction percentages in worm counts.

Concerning oogram pattern, HSP taken early (group III) or taken at the 4th week p.i. (group V) significantly increased the percentage of dead eggs recovered from the intestinal segments ($12.2 \pm 1.1\%$ and $17.1 \pm 2\%$, respectively), also in group V, late HSP treatment significantly decreased the percentage of immature eggs by $52.1 \pm 3.8\%$. The oogram of other groups showed a very high significant increase in the

percentages of dead eggs. In early HSP+PZQ treatment, the percentage was $91.8 \pm 7.4\%$, in late HSP+PZQ treatment, the percentage was $81.7 \pm 4.1\%$ and in PZQ treatment, the percentage was $96.3 \pm 4.9\%$.

This oogram profile may refer that HSP could impair the egg development or maturation and interfere with egg laying. Also, the elicited immune response by HSP might kill the developing miracidia inside the eggs. Changes in the number and character of eggs (oogram) provide a reliable criterion that assesses the effects of a drug on oviposition of *S. mansoni*, as well as on the maturation and survival of eggs trapped in the intestinal mucosa (Pellegrino *et al.*, 1977 and Botros *et al.*, 2004).

The enormous number of eggs which are laid by mature *Schistosoma* worm will stay in liver, intestine and other tissues leading to severe pathology including granulomatous inflammation and tissue fibrosis. Therefore, reducing egg counts in the tissues can significantly relieve the symptoms of schistosomiasis (Bergquist, 2002; Huang *et al.*, 2005 and de Moraes *et al.*, 2014).

One of the most striking finding of this work was that early HSP treatment succeeded in achieving the highest significant reduction in the number (56.06%) and size (60.3%) of hepatic granuloma amongst all the treated mice groups. It exceeded the PZQ treatment which achieved only 25.75% reduction in number and 33.5% reduction in the size of hepatic granuloma. Late HSP treatment (group V) caused diminished diameter of granuloma by 49.9%, however, it decreased the number of granuloma insignificantly (22.72%) albeit it was nearly close to that revealed by PZQ. Also, when early HSP treatment was combined with PZQ, it kept high percentage of reduction (59.3%) of granuloma diameter although the number is reduced to only 29.54%. Late HSP+PZQ caused significant reduction in the diameter of the granuloma by 39.8% and insignificant reduction in number by 17.42%.

On histopathological examination of liver of treated mice groups, there were either active small granulomas or healed granulomas. Actually, no studies have examined the effect of HSP on *Schistosoma* granuloma before. However, this noticeable suppression in granuloma formation and diminution of histopathological changes indicated that HSP has a considerable effect on *Schistosoma* pathological changes in liver. This could be attributed partly to the reduction in number of eggs trapped in the hepatic tissues and the modulation of serum levels of some cytokines which are incriminated in the development of *Schistosoma* granuloma.

Indeed, granuloma formation is dependent on CD4+T cell responses, and is associated with an imbalance in Th1/Th2/Th17 cytokines (Hernandez *et*

al., 1997 and Yu *et al.*, 2012). A lot of studies have indicated that *Schistosoma* pathology development is affected by cytokines which regulate the granulomatous response especially IL-12 (Wynn *et al.*, 1995), IL-10 (Wynn *et al.*, 1998), and TNF- α (Leptak and McKerrow, 1997). In trying to explain the ameliorating effect of HSP on hepatic granuloma size, the serum levels of some cytokines which have been involved in *Schistosoma* granuloma formation were measured.

It was found that HSP treatment sharply increased the serum level of IL-10 when given early (group III) and this rise continued significantly in the other treated mice groups except PZQ which showed insignificant rise. This was in agreement with Allam and Abuelsaad (2013b) who reported that HSP increased the secretion of IL-10 by the stimulated splenocytes isolated from *S. mansoni* infected mice in the presence of *Schistosoma* antigens. This induced increase of serum level of IL-10 by HSP may be correlated with the reduction of the granuloma size, and this was in agreement with other workers who found that the increased level of IL-10 was associated with the reduction in the size of granuloma. For example, Flores-Villanueva *et al.* (1996) showed that administration of exogenous IL-10 resulted in reduction of granuloma size, while Wynn *et al.* (1998) stated that the opposite effect was observed in IL-10-deficient mice. Also, Pyrrho *et al.* (2002) and Aly *et al.* (2010) stated that the mean *Schistosoma* granuloma size in animals treated with dexamethasone showed a significant decrease probably due to the high serum level of IL-10 induced by treatment. Besides, it has been reported that IL-10 has a protective function during the course of schistosomiasis, where up to 30% mortality was observed in IL-10-deficient mice during acute stage of the infection (Hoffmann *et al.*, 2000). In the same context, Rezende *et al.* (1997) suggested that immunocomplexes, in the case of human chronic intestinal schistosomiasis, were able to modulate granulomatous reaction to *S. mansoni* eggs by inducing prostaglandin E production which in turn enhances IL-10 levels. The production of IL-10 is the key factor in preventing the polarization toward a Th1 or Th2 profiles hence controlling the excessive Th1 or Th2 responses, in addition to that, IL-10 suppresses macrophage and dendritic cell activation, and limits egg induced hepatotoxicity during the acute stage of *Schistosoma* infection (Hoffmann *et al.*, 2000; Asadullah *et al.*, 2003 and Hesse *et al.*, 2004).

In this work, the highest significant reduction of serum level of IL-12 was observed in group III (early HSP), followed by group IV (early HSP+PZQ) and then group V (late HSP) which revealed also significant reduction. On the other hand, the other groups showed insignificant decrease in the IL-12

serum level. This finding was in accordance with Allam and Abuelsaad (2013b) who found that HSP significantly decreased IL-12 delivered by splenocytes of *Schistosoma* infected mice that were stimulated by the *Schistosoma* antigens. Also, it was in agreement with a lot of reports which showed that decreasing IL-12 was associated with reduction in the granuloma size (Pyrrho, *et al.*, 2002, Allam, 2009 and Aly *et al.*, 2010). Moreover, it is well established that IL-12 is involved in reduction of extent of hepatic fibrosis caused by schistosomiasis *mansoni* (Wynn *et al.*, 1995 and Hoffmann, *et al.*, 1998).

In the current study, TNF- α was significantly reduced in all treated mice groups, however, the early HSP treatment reduced TNF- α to the lowest level. This was in agreement with the previous report which showed that HSP caused high significant decrease in TNF- α production from the stimulated cultured splenocytes of *S. mansoni* infected mice in a dose dependent manner on stimulation by the parasite antigen (Allam and Abuelsaad, 2013b). Also, it was suggested that curcumin treatment reduced *Schistosoma* granuloma size through reduction of TNF- α serum level (Allam, 2009). TNF- α has a central role in initiating *Schistosoma* granuloma formation. It has been found that administration of TNF- α restored the ability of T cell deficient mice to generate granuloma around *Schistosoma* eggs (Amiri *et al.*, 1992), and the treatment of immunocompetent mice with anti-TNF- α serum resulted in reduction of granuloma size (Joseph and Boros, 1993). TNF- α mediates its effect through the up-regulation of intracellular adhesion molecule 1 (ICAM-1) which mediates cell-cell interactions and migration across the endothelium, however other mediators play a role in the full expression of the lesion (Haseeb *et al.*, 2001 and Davies *et al.*, 2004).

In this study, serum level of IFN- γ reduced significantly in all treated mice groups and both early HSP and early HSP+PZQ treatments reduced IFN- γ to the lowest level. This was in agreement with previous reports which showed that decreasing IFN- γ serum level was associated with reduction in *Schistosoma* granuloma size (Pyrrho *et al.*, 2002 and Aly *et al.*, 2010). However, this result was contradictory to that of Allam and Abuelsaad (2013b) who found that HSP induced the cultured splenocytes of mice infected with *Schistosoma* to increase production of IFN- γ together with enhanced production of IL-4. IFN- γ appears to play an important role in the generation and maintenance of egg induced granuloma and the diminished focal and systemic production of IFN- γ resulted in down modulation of the granulomatous response (Hoffmann *et al.*, 1998 and Rakasz *et al.*, 1998).

Early HSP reduced the serum level of IL-6 to the lowest significant level. The other mice groups also showed a significant decrease in the cytokine serum level, however, PZQ did not cause a significant effect on the serum level of this cytokine. This reduction of IL-6 may have a role in diminution of granuloma formation. This was in parallel with Mantawy *et al.* (2011) who reported that IL-6 level decreased in *Schistosoma* infected mice after treatment with dry extract of onion or garlic, individually or mixed, with or without PZQ and this was associated with small sized, late fibrocellular granuloma. IL-6 has a confirmed role in the granuloma formation (Khalil *et al.*, 1996). It was found that the immune response against *S. mansoni* is strongly reduced in infected severe combined immune-deficient mice, but it is fully restored through the injection of these mice with TNF- α which is a major inducer of IL-6 (Amiri *et al.*, 1992 and Hirano, 1994). In addition to that, there is a strong evidence of the role of IL-6 in inducing hepatic fibrosis (Chen *et al.*, 2008).

In addition to cytokines changes caused by HSP which were detected in the current study, other properties of the compound such as anti-inflammatory (Galati *et al.*, 1994) and immuno-modulatory activities (Kim and Cho, 1991) may have regulatory effects on the granuloma and should be examined in details.

In conclusion, HSP treatment had effective schistosomicidal properties through reduction of worm burden and tissue egg load, especially when it was given early after exposure to *S. mansoni* infection suggesting that the juvenile stage is more susceptible to HSP than adult one. Moreover, HSP largely alleviated schistosomal hepatic pathology by modulating some important cytokines involved in granuloma formation. In addition to that, it augmented humoral immune response against *S. mansoni*. Therefore, HSP is a promising candidate that could be used for developing of new anti-*Schistosoma* drugs. It is recommended to perform further studies to examine the effect of HSP on *Schistosoma* worms of various ages both *in vitro* and *in vivo* and to test the prophylactic efficacy of HSP before exposure to the parasitic infection.

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