



RESEARCH ARTICLE

Impact of *Allium sativum* ethanol extract on immuno-regulatory T cells and anti-inflammatory cytokine profile in murine schistosomiasis

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ABSTRACT

Parasite immune response against schistosomal antigens involves both the innate and adaptive immune response. Tregs have a suppressive effect and play a role on the parasite's immune evasion. This study aimed to evaluate active compounds of *Allium sativum* (AS) ethanol extract and the impact of AS extract alone or in combination with praziquantel on Tregs and anti-inflammatory cytokines TGF- β and IL-10 in mice infected with *S. mansoni*. Phytochemical screening of AS bulbs for various active constituents and qualitative and quantitative analysis of the flavonoids and phenolic acids were done using HPLC. Measurement of splenocytes Treg cell phenotypes and anti-inflammatory cytokines TGF- β and IL-10 was done by flow cytometric analysis. The data are expressed as mean \pm SD. Statistical significance was determined by one-way ANOVA utilizing the statistical package (SPSS version 17.0). HPLC of AS ethanol extract revealed presence of 22 and 18 compounds of flavonoids and phenolic acids, respectively. *S. mansoni* infection upregulated the Treg cells subsets (CD4, CD25, Foxp3) frequencies and the levels of TGF- β and IL-10 anti-inflammatory cytokines when compared to healthy control. AS ethanol extract alone or combined with PZQ decreases the production of Treg cells from spleen in addition to the reduction in anti-inflammatory cytokines IL-10 and TGF- β . This study recommends that the combination of AS ethanol extract and PZQ may play a role in maintaining the homeostasis of the immune system during schistosomiasis by decreasing Treg cells and anti-inflammatory cytokines IL-10 and TGF- β production.

Keywords: *Schistosoma mansoni*; *Allium sativum*; Tregs; TGF- β ; IL-10.

INTRODUCTION

Schistosomiasis, is a neglected tropical disease causing a public health problem in tropical and subtropical regions (Colley *et al.*, 2014). It infects nearly 300 million people in about 78 countries, primarily in Africa (Cioli *et al.*, 2014; Aula *et al.*, 2021). The parasite immune response against schistosomal antigens involves both the innate and adaptive immune response. In acute *S. mansoni* infection, antigen presenting cells (APC) such as macrophages and dendritic cells introduce the *schistosome* antigens on major histocompatibility complex-II (MHC-II) to T helper lymphocytes leading to the proliferation of Th1 responses through the production of proinflammatory cytokines including Tumor necrosis factor- α (TNF- α), Interleukin (IL)-1, IL-12 and interferon gamma (IFN- γ) in addition to the chemokine IL-8 (Pearce & MacDonald, 2002; El Ridi *et al.*, 2006). In the chronic phase of infection, this immune response is shifted, toward the much stronger Th2 response, reflecting a state of immune

suppression triggered by regulatory T cells (Treg) and a production of IL-10 and transforming growth factor- β (TGF- β) (Taylor *et al.*, 2006; Maizels & Smith, 2011; Taylor *et al.*, 2012). Treg cells is reported to play an important down-regulatory role in limiting protective immunity during chronic schistosome infections (Watanabe *et al.*, 2007; Tang *et al.*, 2011; Schmiedel *et al.*, 2015).

The schistosomiasis control is based principally on treatment with anthelmintic drug namely praziquantel (PZQ) (Doenhoff *et al.*, 2008; Cioli *et al.*, 2014). Many reports indicated a resistance to PZQ, through the induction of immune response by increasing IgE levels, decreasing IgG4 levels and production of IL-4, IL-5, IL-10, and IL-13 and IFN- γ responses (Black *et al.*, 2010; Ricciardi *et al.*, 2018); these responses lead to increase resistance to re-infection (Black *et al.*, 2010). Consequently, there is a strong need for discovering alternatives to that available drug. For this reason, extracts of natural products from medicinal plants were potentially used for controlling the disease.

Allium sativum (AS) L. (family: Amaryllidaceae) is an aromatic herbaceous annual aroma spice and commonly known as garlic. It is commonly used as a food ingredient due to its distinctive taste and possible health benefits in many countries and different cultures, consumption of garlic is used to promote health and well-being, in addition to liver diseases. Numerous studies indicated the role of garlic consumption in reduction of developing chronic diseases such as: cardiovascular disease, cancer, obesity, diabetes, high blood pressure, platelet aggregation and cholesterol reduction (Hussain et al., 1990; Lawson et al., 1992; Virginia, 2006). Additionally, garlic and its derivatives showed strong antioxidant, anti-inflammatory, and immunoregulatory properties (Arreola et al., 2015; Aly et al., 2017; Batiha et al., 2020). These health properties of garlic are derived from its polyphenols and organosulfur compounds (Lawson & Gardner, 2005; Shang et al., 2019). AS was successfully used against parasites such as *E. granulosus*, *Trypanosoma brucei*, *Schistosoma mansoni* and *Leishmania tarentolae* (Aly et al., 2017; Krstin et al., 2018; Mohammadi et al., 2018; Shirgholami et al., 2021).

To the best of our knowledge, there has been no study on the impact of AS extract administration on regulatory T cells immune response in the *S. mansoni* infected mice. The present work aimed to evaluate the active compounds of AS ethanol extract and its impact on the regulatory T cells and anti-inflammatory cytokines TGF- β and IL-10 in the *S. mansoni* infected mice, in comparison with the standard drug PZQ for treatment of *schistosomiasis*.

MATERIALS AND METHODS

Parasites and animals

Fifty male albino CD-1 mice weighing approximately 25 grams and age of 6 weeks, were obtained from Schistosome Biological Supply Program at Theodore Bilharz Research Institute (SBSP/TBRI), Giza, Egypt. Mice were maintained under a controlled condition and given access to water and standard commercial pellet diet. The animal experiments were carried out according to the animal ethics guidelines, after approval of the institutional ethical committee of TBRI.

Mice infection

Egyptian strain of *S. mansoni* cercariae were purchased from SBSP/TBRI, and used directly after shedding from *Biomphalaria alexandrina* snails. CD1 mice were infected by subcutaneous (SC) injection of 100 ± 2 *S. mansoni* cercariae/mouse (Peters & Warren, 1969).

Plant material

Allium sativum (AS) was collected from a private farm, El-Mansoura, Egypt, in the summer of 2019. The identification of the fresh plant was scientifically carried out in the herbarium of the Agricultural Museum, Giza (CAIM).

Preparation of powdered air-dried bulbs of AS

Fresh bulbs of AS were separated, cleaned, dried in shade then in oven at approximately 50°C until the weight became constant. Dried bulbs then grounded to fine powder and prepared to be used in further investigations.

AS bulbs extract preparation

Five hundred grams of the bulbs of AS was extracted with aqueous ethanol extract (70%) using the soxhlet apparatus at 80°C, and then filtration and evaporation of the alcoholic

extract was done under reduced pressure till dryness and the dried extract was kept at room temperature for analysis.

Phytochemical screening

The initial phytochemical screening of AS bulbs for various active constituents in 70% ethanol extract was done according to Lala (1993). Assessment of alkaloids presence was done using the Dragendorff test (Ezeonu & Ejikeme, 2016), while Molisch and Biuret tests were used for the determination of carbohydrates and proteins, respectively (Annapandian & Rajagopal, 2017). Cardiac glycosides were determined using concentrated H₂SO₄ test (Obianime & Uche, 2008), coumarin and flavonoids were determined by alcoholic sodium hydroxide and Pew's test, respectively. Saponin determined by the foam test, tannins were determined by ferric chloride test and Salkowski's test was used to determine terpenes (Ezeonu & Ejikeme, 2016). The oil distillation method was used to assess volatile oils.

Investigation of phenolic compounds using HPLC

The bulbs of AS were analyzed using HPLC. HP 1090M Series high performance liquid chromatography with an HP 1090M Series diode array and an eight-channel electrochemical coulometric array detector (EC; Esa Inc., USA) was used. 100-800 mV potentials were used to run the EC (100mV intervals). The detector array was placed at 35°C in a temperature-controlled compartment. The flavonoid and phenolic acids were separated using an ODS-3 (4.0 150 nm, 3m) column with a C-18 guard column at 35°C. The mobile phase flow rate was 0.7mL/min, and the injection volumes of the standards and sample extracts were 10L. The external standard procedure was used to measure both flavonoids and phenolic acids. Peak area (DAD) or peak height is used to quantify the animals (EC) s shown by Mattila et al. (2000).

Praziquantel (PZQ)

Purchased from SEDICO pharmaceutical Co. Giza, Egypt, in the form of tablets, each tablet contains 600 mg/kg. Tablets were crushed into powder and suspended in 4.8 ml distilled water according to the method of Muchirah et al. (2012). The freshly prepared drug was administered orally to the mice by stainless steel cannula.

Drugs and Doses

The dose of AS ethanol extract used were about 50 mg/kg of body weight (Riad et al., 2009). This dose was administered orally single dose per day for 40 days.

The dose of Praziquantel used were 500 mg (dissolved in 70% glycerin)/kg of body weight, and was administered orally 7 weeks post infection for two successive days (Panic et al., 2017).

Experimental groups

Mice were divided into five groups, with 10 mice each.

Group I: left uninfected and un-injected, represent the naïve group.

Group II: infected with 100 ± 2 *S. mansoni* cercariae/mouse; act as infected control group.

Group III: Mice were infected by *S. mansoni* cercariae/mouse and treated with AS aqueous ethanol extract;

Group IV: Mice were infected by *S. mansoni* cercariae/mouse and treated with PZQ.

Group V: Mice were infected by *S. mansoni* cercariae/mouse and received mixed treatment of PZQ and AS ethanol extract at the same doses and timeline as in group III and IV.

Mice were sacrificed eight weeks after infection with 100 ± 2 *S. mansoni* cercariae/mouse and the following testes were carried out.

Flow cytometric (FCM) analysis

Spleen cells isolation

Spleens from at least five mice per group were isolated and forced through 70 μ m cell strainer (BD Biosciences, San Jose, CA, USA) to obtain a single-cell suspension to analyze cell surface molecules and regulatory T cells using flow cytometry. Splenic red blood cells (RBCs) were lysed by RBCs lysis buffer (Sigma) and remaining splenic cells were washed twice by a pyrogenic saline. Trypan blue dye was used to evaluate the cell viability.

Flow cytometry

For the measurement of regulatory T-cell phenotypes, the splenocytes single-cell suspension from each mouse was adjusted to 1×10^6 cells/well, incubated with the following monoclonal antibodies (mAbs) for surface marker analysis using mouse regulatory T cell staining kit (BioLegend Inc, San Diego, CA) according to the manufacturer's protocol:

-fluorescein isothiocyanate (FITC) -conjugated anti-CD4 mAbs, FITC -conjugated anti- CD25 mAbs, phycoerythrin (PE)- anti-mouse FOXP3 Antibody, FITC anti-mouse IL-10 Antibody and FITC anti-mouse LAP (TGF- β 1) Antibody. Blocking of the nonspecific binding was done using 4 μ g rat IgG per 1×10^6 cells.

The flow cytometric analysis was performed using BD Accuri C6 Flow Cytometer (BD Biosciences). Data were analyzed using C6 Analysis software (BD Biosciences).

Statistical analyses

Data were first tested for normality using the Kolmogorov-Smirnov test, and after ensuring the normality of the data, one-way ANOVA was used to determine the statistical

significance by utilizing the statistical package (SPSS version 17.0). Data are expressed as mean \pm standard deviation of the mean (SD). $P < 0.05$ was considered as significant for all statistical analyses.

RESULTS

Phytochemical screening

The initial phytochemical screening of AS bulbs aqueous ethanol extract (70%) indicated the existence of flavonoids, carbohydrates, glycosides, tannins, coumarins, alkaloids, terpenoids, saponins and essential oils.

Investigation of phenolic compounds using HPLC

HPLC of AS bulb aqueous ethanol extract (70%) revealed the presence of 22 and 18 compounds of flavonoids and phenolic acids, respectively. Where the major flavonoid compounds were Rutin (34.156 mg/100g), Hesperidin (31.211mg/100g), Luteolin-6- arbinose-8- glucose (30.254 mg/100g), Quercetin (29.604 mg/100g) and Naringenin (27.954 mg/100g), while the major phenolic acid compounds were Ferulic acid (37.326 mg/100g), p-Coumaric acid (32.634 mg/100g) and Chlorogenic acid (32.634 mg/100g) (Table 1).

Effects of *S. mansoni* infection on host Regulatory T cell subsets (CD4, CD25, Foxp3) and anti-inflammatory cytokine profile

In each of two independent experiments, CD1 mice were infected with 100 ± 2 *S. mansoni* cercariae/mouse. The percentage of spleen Treg cell subsets (CD4, CD25, Foxp3) and the anti-inflammatory cytokine responses, were compared to uninfected mice. Flow cytometric analysis showed that, infection with *S. mansoni* led to significant ($p < 0.001$) increase in spleen Treg cell subsets (CD4, CD25, Foxp3) (Figure 1-3). These changes were associated with significant ($p < 0.001$) increase in IL-10 and TGF- β anti-inflammatory cytokines (Figure 4, 5).

Table 1. HPLC analysis of aqueous ethanol extracts (70%) of AS bulbs

No	Flavonoids	Mg/100g	Phenolic acids	Mg/100g
1	Luteolin-6- arbinose-8- glucose	30.254	Pyrogallol acid	13.210
2	Luteolin-6- glucose -8- arbinose	1.154	Gallic acid	20.352
3	Apigenin-6- arbinose -8-glactose	24.029	4-Amino-benzoic acid	2.120
4	Apignin-6- rhamnose -8- glucose	0.523	Catechin acid	3.254
5	Apignin-6- glucose -8- rhamnose	20.083	Catechol acid	14.015
6	Luteolin-7- glucose	4.189	Epicatchin acid	0.823
7	Narengin	0.764	p-OH-benzoic acid	0.531
8	Rutin	34.156	Chlorogenic acid	28.687
9	Hesperidin	30.537	Vanillic acid	0.231
10	Quercetin-3-O-glucoside	0.660	Caffeic acid	1.425
11	Rosmarinic	0.758	p-Coumaric acid	32.634
12	Apigenin-7-O- neohespiroside	0.298	Ferulic acid	37.326
13	Kampferol-3,7-dirhamoside	2.432	Iso-ferulic acid	2.919
14	apigenin-7- glucose	4.302	α -coumaric acid	10.446
15	Quercetrin	0.263	Benzoic acid	23.211
16	Quercetin	29.604	Ellagic acid	18.214
17	Naringenin	27.954	3,4,5-methoxy-cinnamic acid	3.125
18	Hespirin	25.811	Salicylic acid	0.238
19	Kampferol	23.128	—	—
20	Rhamnetin	0.468	—	—
21	Apignin	10.360	—	—
22	Acacetin	0.168	—	—

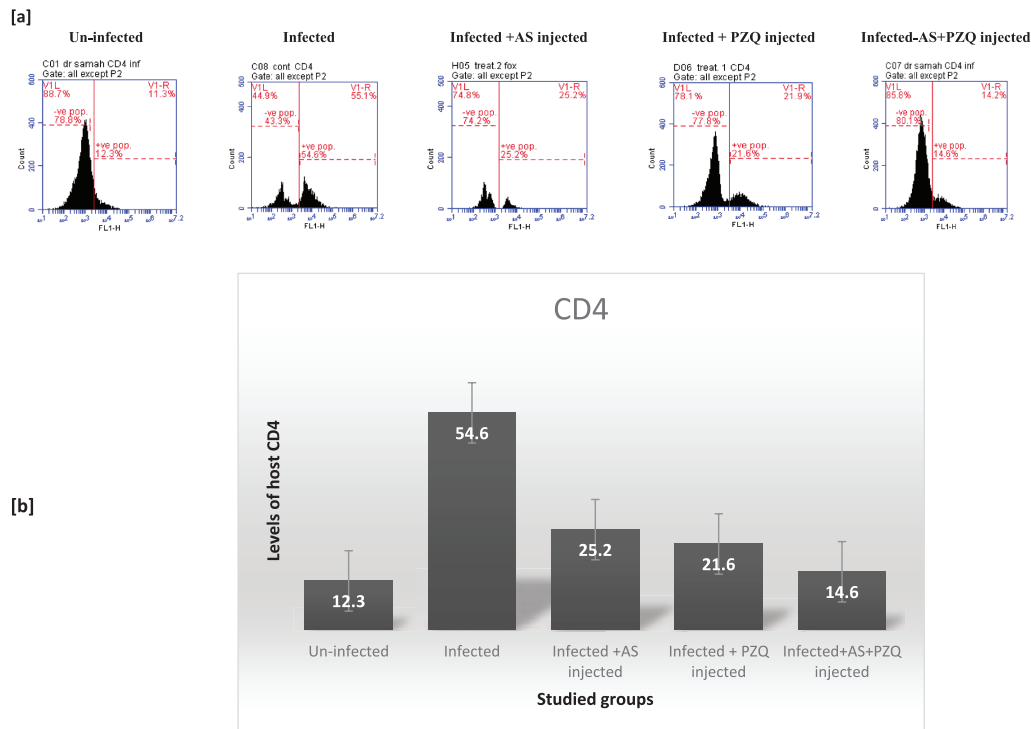


Figure 1. Effects of *S. mansoni* infection, AS ethanol extract and/or PZQ treatment on host splenic CD4 regulatory T cells. [A] Flow cytometric analysis of the spleen CD4 Treg cell subsets [B] The percentages numbers of CD4 Treg cell subsets. All data are measured by mean \pm SD and representative of two separate experiments five mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. AS: *Allium sativum*; PZQ: Praziquantel.

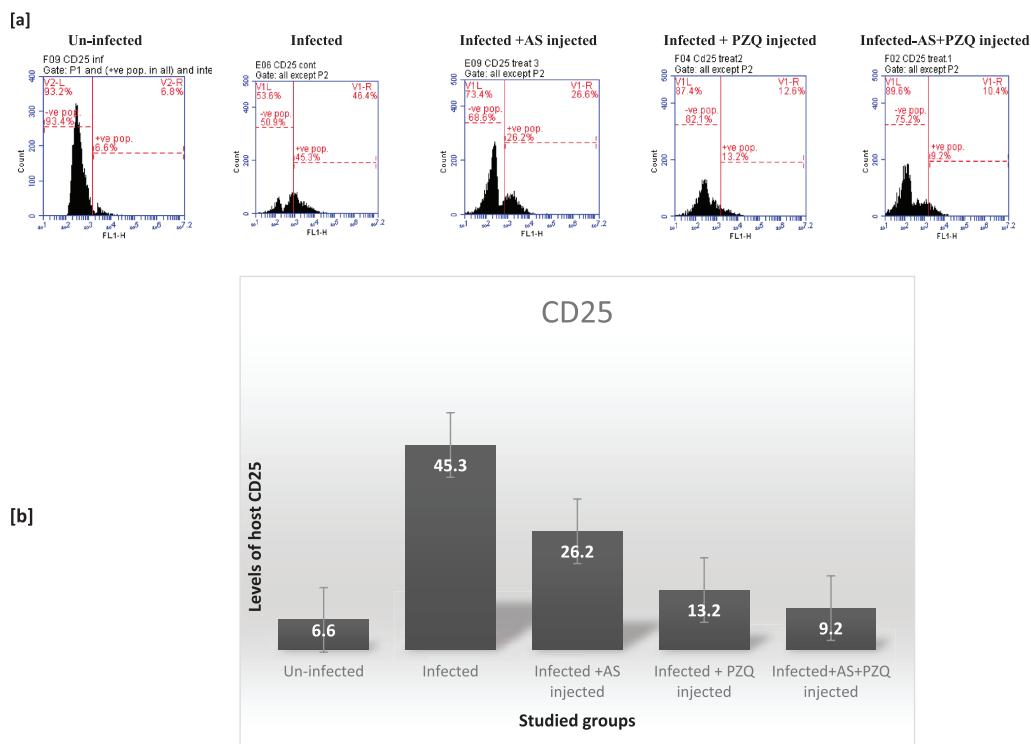


Figure 2. Effects of *S. mansoni* infection, AS ethanol extract and/or PZQ treatment on host splenic CD25 regulatory T cells. [A] Flow cytometric analysis of the spleen CD25 Treg cell subsets [B] The percentages numbers of CD25 Treg cell subsets. All data are measured by mean \pm SD and representative of two separate experiments five mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. AS: *Allium sativum*; PZQ: Praziquantel.

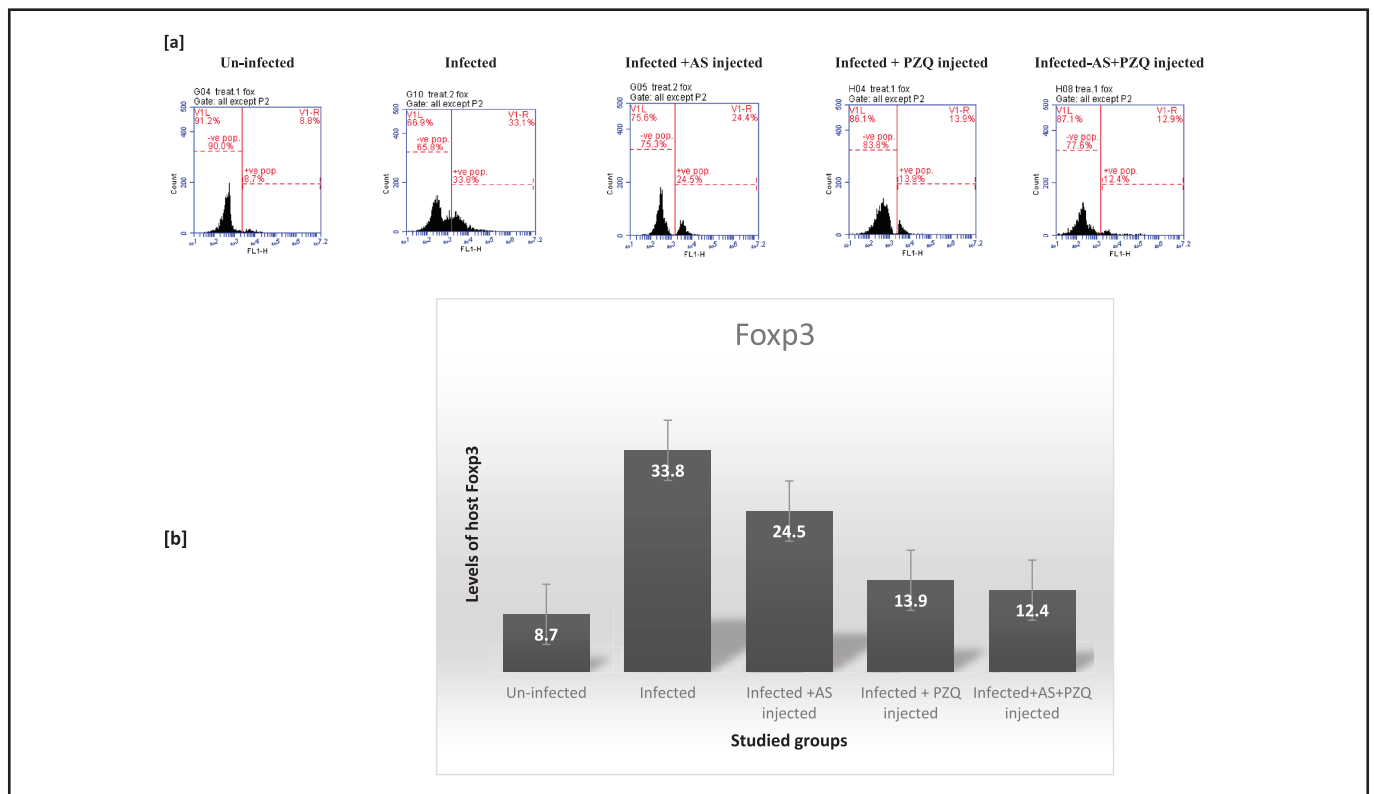


Figure 3. Effects of *S. mansoni* infection, AS ethanol extract and/or PZQ treatment on host splenic Foxp3 regulatory T cells. [A] Flow cytometric analysis of the spleen Foxp3 Treg cell subsets [B] The percentages numbers of Foxp3 Treg cell subsets. All data are measured by mean \pm SD and representative of two separate experiments five mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. AS: *Allium sativum*; PZQ: Praziquantel.

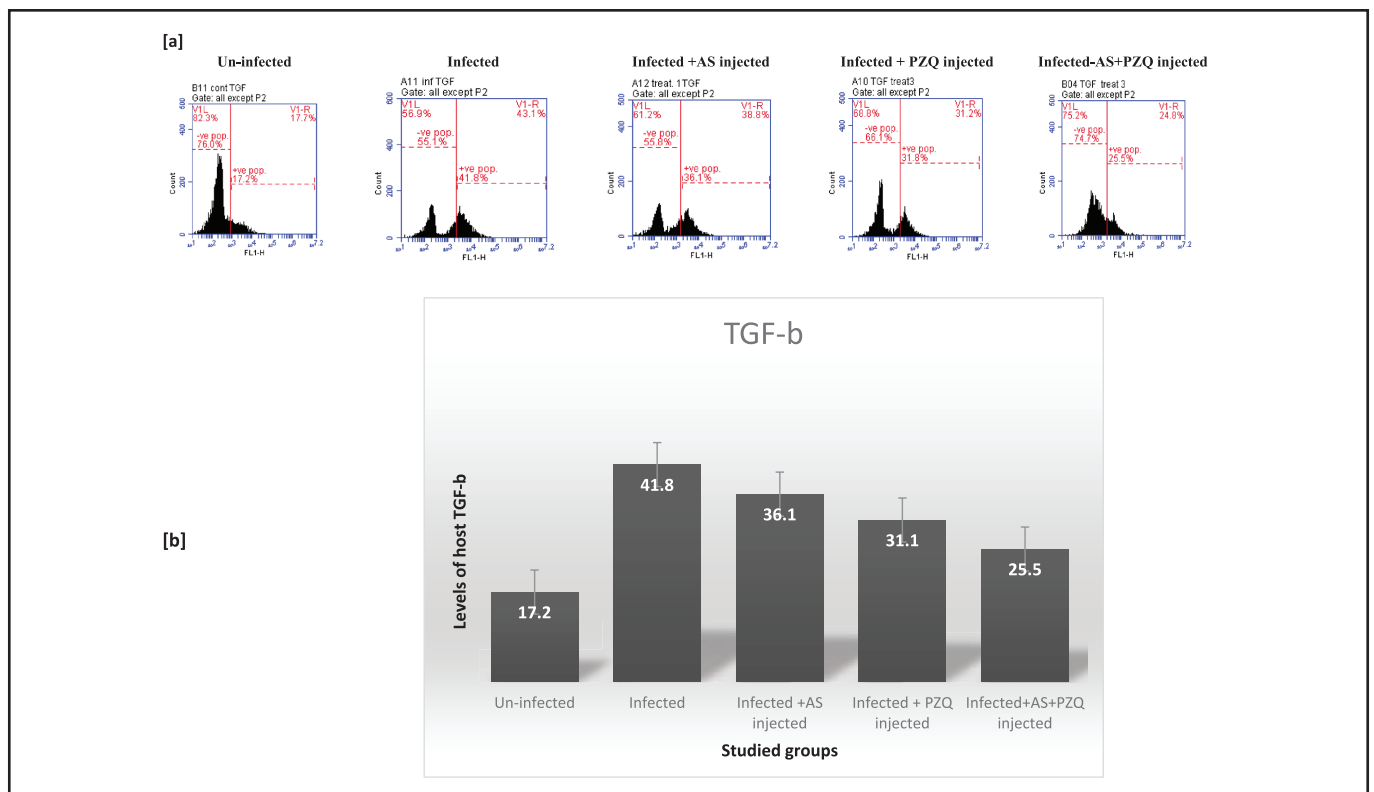


Figure 4. Effects of *S. mansoni* infection, AS ethanol extract and/or PZQ treatment on host TGF-b cytokine. [A] Flow cytometric analysis of the TGF-b cytokine. [B] The percentages numbers of TGF-b cytokine. All data are measured by mean \pm SD and representative of two separate experiments five mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. AS: *Allium sativum*; PZQ: Praziquantel.

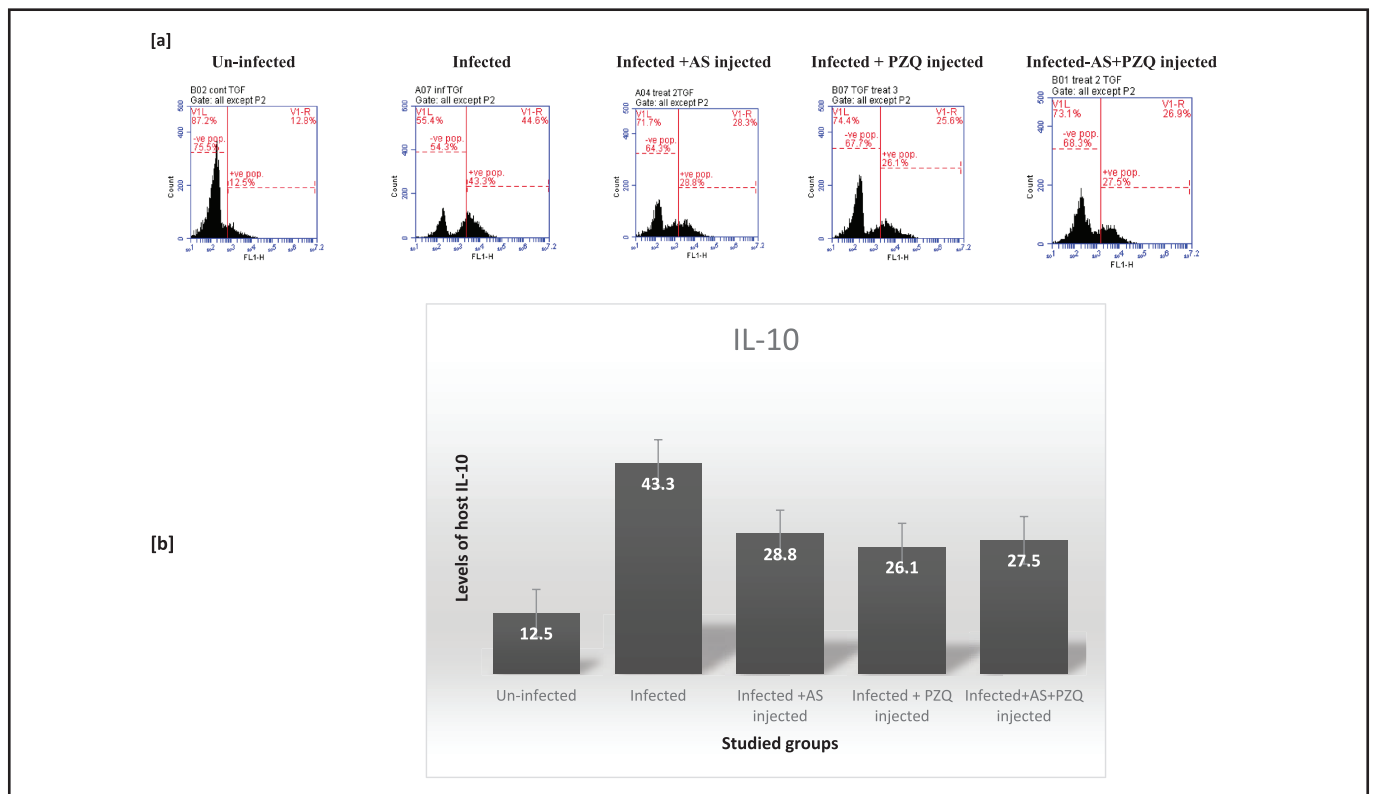


Figure 5. Effects of *S. mansoni* infection, AS ethanol extract and/or PZQ treatment on host IL-10 cytokine. [A] Flow cytometric analysis of the TGF- β cytokine. [B] The percentages numbers of IL-10 cytokine. All data are measured by mean \pm SD and representative of two separate experiments five mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. AS: *Allium sativum*; PZQ: Praziquantel.

Effects of treatment with AS ethanol extract, PZQ individually or in combination with AS ethanol extract on host Regulatory T cell subsets (CD4, CD25, Foxp3) and anti-inflammatory cytokine profile In each of two independent experiments, treatment of *S. mansoni* infected CD1 mice with AS ethanol extract PZQ individually or in combination with AS ethanol extract elicited significant decrease ($p < 0.01$; $p < 0.001$; $p < 0.001$) respectively, in spleen Treg cell subsets (CD4, CD25, Foxp3), as compared to infected controls (Figure 1-3). These changes were associated with significant ($p < 0.05$) decrease in IL-10 and TGF- β anti-inflammatory cytokines (Figure 4, 5).

DISCUSSION

Tregs have a suppressive effect, and are involved in immune tolerance and induction, in addition to their central role on the parasite's immune evasion. Foxp3 is a nuclear transcription factor and is a characteristic marker of Tregs and the expression of Foxp3 determines their suppressive role (Iizuka-Koga et al., 2017). Treg cells secrete suppressive cytokines such as TGF- β and IL-10, therefore, the function of Treg cells is closely related with the effect of its secreted cytokines. Tang et al. (2011) reported that Tregs contributed to the escape of *S. japonicum* from the host immune responses. Consequently, Treg cells and its anti-inflammatory cytokines, TGF- β and IL-10, can be used as indicators to study immune response associated with schistosomiasis. AS is considered as a capable candidate for maintaining the homeostasis of the immune system. Thus, different studies have demonstrated interesting beneficial effects of garlic on the immunity and immune cells.

The present work aimed to evaluate the active compounds of AS ethanol extract and its impact on the regulatory T cells and anti-inflammatory cytokines TGF- β and IL-10 in the *S. mansoni* infected mice, in comparison with the standard drug PZQ for treatment of schistosomiasis.

The present study indicates that AS bulb extracts which is grown in Egypt contain several bioactive constituents (flavonoids, carbohydrates, glycosides, tannins, coumarins, alkaloids, terpenoids, saponins and essential oils) and showed similar results to the Phan et al. (2019). On the other hand, the AS bulb extracts contains the major compounds of the flavonoid glycosides (rutin, hesperidin and luteolin-6-arbinose-8- glucose), aglycone (quercetin and naringenin) and phenolic acids (Ferulic acid, p-Coumaric and Chlorogenic acid). In the present study, the major phenolic compounds found in garlic grown in Egypt differed marginally from the polyphenolics of nine commercial garlic varieties grown in different countries (Szychowski et al., 2018).

Our results showed that *S. mansoni* infection led to the upregulation of the Treg cells subsets (CD4, CD25, Foxp3) frequencies and the levels of both TGF- β and IL-10 anti-inflammatory cytokines when compared to healthy control. These results are in consistent with previous reports indicating increased in frequencies of Treg cells and the levels of TGF- β and IL-10 in parallel in schistosome-infected mice (Tang et al., 2011; Turner et al., 2011; Schmiedel et al., 2015; He et al., 2018). These data also go along with those found that the chronic phase of schistosome infection led to shifted immune response from Th1 toward Th2 response, causing a state of immune suppression through elevation of Treg cells and the production of IL-10 and TGF- β (Taylor et al., 2006; Maizels & Smith, 2011; Taylor et al., 2012).

Oral administration of *S. mansoni* infected CD1 mice with AS ethanol extract or PZQ individually elicited significant decrease, in spleen Treg cell subsets (CD4, CD25, Foxp3), as compared to infected controls. These changes were associated with significant ($p < 0.05$) decrease in IL-10 and TGF- β anti-inflammatory cytokines. Our observation of reduced immunosuppressive cells suggests that AS ethanol extract can maintain the homeostasis of immune functions. On the other hand, the combination treatment of AS ethanol extract with PZQ showed improved activity against the significant increase in spleen Treg cell subsets (CD4, CD25, Foxp3), as compared to infected controls, and IL-10 and TGF- β anti-inflammatory cytokines.

CONCLUSION

This study showed that AS ethanol extract decreases the production of Treg cells from spleen in addition to the reduction in anti-inflammatory cytokines IL-10 and TGF- β . Therefore, AS ethanol extract may be a suitable alternative herbal treatment for the prevention of schistosomiasis. However, this study recommends that the combination of AS ethanol extract and PZQ may play a role in maintaining the homeostasis of the immune system during schistosomiasis by decreasing Treg cells and anti-inflammatory cytokines IL-10 and TGF- β production. It is not yet fully understood the mechanism of how AS extract can cause reduction in Treg cells. However, it is notable that there are active compounds in AS bulb that may deactivate Treg cells proliferation and provide a protective pattern. Therefore, a further study on the impact of the AS active compounds on the Treg cells during schistosomiasis is highly recommended.

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Conflict of Interests

Authors declare that there is no Conflict of Interests.

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