



Efficiency of Three Extracts of *Carica papaya* as Molluscicidal and Anti-schistosomal Agents against *Biomphalaria alexandrina* and *Schistosoma mansoni* by Flow Cytometry

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Authors' contributions

This work was carried out in collaboration among all authors. Authors IA, MSG and SIG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript.

Authors MSG and HELS managed the analyses of the study. Author SMNA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Schistosomiasis is a prevalent parasitic disease in tropical and sub-tropical areas, which comes in the second place in terms of socioeconomic and public health burden. Around 600 million people in 74 countries are infected yearly, predominantly in the developing world. The aim of this work was to assess the efficiency of three extracts from *Carica papaya* (methanol, ethanol and butanol extracts)

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for their molluscicidal and anti-schistosomal activities. The LC₅₀ of methanol, ethanol and butanol extracts of *Carica papaya* against *B. alexandrina* were 180, 499.3 and 509.1 mg/L while the respective LC₉₀ values were 220.3, 700.6, 769.6 mg/L respectively. The effect of these extracts on *Biomphalaria alexandrina* snails and larval stages of *Schistosoma mansoni*, for miracidia the LC₅₀ of methanol, ethanol and butanol extracts of *Carica papaya* against miracidia were 3.4, 15.4 and 8.1 mg/L, respectively, while the respective LC90 values were 8.4, 38.2, 11.2 mg/L, and for cercariae the LC₅₀ of methanol, ethanol and butanol extracts of *Carica papaya* were 2, 20 and 4 mg/L, while the respective LC90 values were 13.5, 80.5, 18.5 mg/L respectively was evaluated, in addition to flowcytometric analysis of CD4, CD25, FOXP3 and TGF- β levels during *S. mansoni* infection in mice. The *in-vivo* results showed that the three extracts have variable potential against snails and miracidia and cercariae of *S. mansoni*. The mortality rate in *B. alexandrina* snails for methanol, ethanol and butanol extracts of *Carica papaya* were 86%, 45% and 64%, respectively. While it was 83%, 35% and 66%, respectively in miracidia and 92%, 40% and 70%, respectively in cercariae. The results indicated that methanol extract from *Carica papaya* recorded higher activity against snails, miracidia and cercariae. The levels of CD4, CD25, FOXP3 regulatory T (Treg) cells were decreased significantly ($p < 0.001$) in infected mice compared to healthy controls. However, there was a significant ($p < 0.001$) increase in levels of TGF- β . A significant increase in the levels of CD4, CD25, and FOXP3 Treg in *Carica papaya* treated group compared to infected control group, with a significant ($p < 0.001$) decrease in TGF- β level than infected group. In conclusion, methanol extract was more effective at concentration of LC₅₀ 180 and LC₉₀ 220.3 than ethanol and butanol extracts of *Carica papaya* therefore controlling *B. alexandrina* snails by methanol extract is a promising way as it is an eco-friendly strategy in rural areas of developing countries, where schistosomiasis is endemic. Moreover, the increased immune defense mechanism in treated group with the same extracts is a promising target for new immune modulatory strategies against schistosomiasis.

Keywords: *Biomphalaria alexandrina* snails; *Schistosoma mansoni*; *Carica papaya*; regulatory T cells.

1. INTRODUCTION

Schistosomiasis is a neglected tropical disease which ranks second only to malaria as the most common parasitic disease. It infects over 240 million people in about 78 countries, primarily in Africa [1,2]. Multiple studies have described major roles for immune responses with regard to both morbidity and resistance to reinfection in human schistosomiasis [3,4,5]. T Regulatory (Treg) lymphocytes are unique subpopulations of T cells involved in immune homeostasis and tolerance [6] and their elevation has been reported in human schistosomiasis [7,5]. Regulation of effector T cells during chronic antigenic exposure, such as in schistosomiasis, may protect the host from excessive pathology, but may also impair effective immune-mediated resistance to reinfection. However, Treg quantification and functionality during disease states remains controversial [8]. Several strategies have been used to control schistosomiasis through controlling the intermediate host to reduce the transmission [9] either by chemical or biological control. *Carica papaya* is one of the most important fruit crops grown in the tropical and sub-tropical regions worldwide, and its fruit is known to have many health benefits, such as reducing cardiovascular

disease risk, anti-inflammatory, antioxidant, anticancer, antimicrobial activities and serving as an immune-adjuvant for vaccine therapy [10]. Several plant species have been proved to have molluscicidal properties against different snail species. Several studies have showed that *Carica papaya* is a potential source of anti-parasitic agents including novel anti-schistosomal agents [11,12]. *Biomphalaria alexandrina* is a species of air-breathing freshwater snail an aquatic pulmonate gastropod mollusk in the family Planorbidae, This snail species is very closely related to several other African *Biomphalaria* species constituting a Nilotic species complex found in the Nile River basin [13]. *Biomphalaria* act as obligatory intermediate hosts for the transmission of *Schistosoma mansoni*, the parasite that causes the most widespread form of intestinal schistosomiasis which one of the neglected tropical diseases that affect more than 1.4 billion people worldwide [14]. The present study was designed to assess the role of *Carica papaya* extracts as molluscicidal and anti-schistosomal agents through evaluating the effects of different extracts (MeOH, EtOH, and BuOH) on snails, miracidia and cercariae as well as on the Treg activity in addition to TGF- β levels during *Schistosoma mansoni* infection in mice.

2. MATERIALS AND METHODS

2.1 Extraction of *Carica papaya*

The *Carica papaya* leaves were washed and sun dried for 2 weeks to remove the residual moisture. The dried plant material was then grounded into fine powder by removing the stalk and woody part using mortar and pestle and stored in an air tight container away from moisture to use for further study. Extraction of compounds from papaya leaves was carried out by sequential Soxhlet extraction with different solvents of increasing polarity. Fifty grams of powdered leaves were sequentially extracted in a Soxhlet extractor using 250 ml of 99.5% methanol, 90% ethanol, and 90% butanol for three days at room temperature with occasional shaking. The extraction time was about 5-8 hrs for each solvent. At the end of extraction, the methanol, ethanol and butanol extracts were concentrated using vacuum evaporator at 40°C (BUCHI, Switzerland). The resulting extract was then filtered and the filtrate was stored at 4°C [15].

2.2 Water Suspension of Different Extracts of *Carica papaya*

Stock solution of 1000 ppm was freshly prepared on the bases of weight/volume in de-chlorinated tap water pH 7.5-7.7 from each extract. Series of concentrations expressed in terms of part per million (ppm) that would permit the computation of LC₅₀ and LC₉₀ were prepared. Standard procedures were followed through this study [16].

2.3 In-vitro Study

2.3.1 Screening for molluscicidal activity

A total of 10 snails were exposed per test to different concentrations of each extract prepared in dechlorinated tap water. To calculate LC₅₀ and LC₉₀, series of concentrations (0-400) ppm were prepared on the basis of volume/ volume as three extracts of the plants [17]. Snails were exposed to the extract for 24 hours under room temperature (25-27°C). After 24 hours, snails were removed from the container and were transferred to a new recipient filled with aged water. A group of ten snails were kept in aged water under the same experimental conditions as a negative control group. The experiment for each concentration was repeated three times. Mortality rate was recorded after 24 hours.

2.4 Miracidicidal Tests

Multi-well plates were used as test chambers to observe the viability and death of the miracidia under a dissecting microscope. Twenty miracidia were picked using 100 µl pipette and placed in each well. Serial concentrations (0-50) ppm of different extracts was added to each well. The miracidia was observed under a dissecting microscope for survival and mortality at a successive interval of 15 min., 30 min. and 45 min. Negative control was also set up using dechlorinated water. LC₅₀ and LC₉₀ were calculated.

2.5 Cercariacidal Activity Test of *Carica papaya* Extracts

A series of concentrations 0 - 40 ppm of different extracts were prepared in a Petri dish. An average of twenty freshly shed cercariae was transferred into each Petri dish using micro-pipette. The same number of cercariae was placed in Petri dishes containing aged water as a control group. Each dilution, as well as control group, was tested in duplicate. The cercariae were observed under a dissecting microscope for survival and mortality at a successive interval of 15 min, 30 min, 45 min, 1 hour, and 1 and half hours. They were considered to be dead when they stopped movement, sank down and detached their tail. At the end of each experiment, the total number of cercariae was counting. The LC₅₀ and LC₉₀ values of *Carica papaya* extracts on *Schistosoma mansoni* cercariae during 1 hour exposure were determined [18].

2.6 Infectivity of *S. mansoni* Cercariae

Infectivity of *S. mansoni* cercariae to mice was determined by pre-exposing the cercariae to sub-lethal concentrations (LC₅₀). Five test tubes were prepared for every concentration. One hundred fifty cercariae (the pre-determined infective dose) were transferred using micro-pipette into each test tube. The cercariae were then exposed to these sub-lethal concentrations for 2h at room temperature before being used to infect mice.

2.7 Animals and Parasites

2.7.1 Animals

Six to eight-weeks-old male albino mice of the CD1 mice (weight 24 ± 2 g) bred and kept at the

Schistosome biological supply center, Theodore Bilharz Research Institute Giza, Egypt (SBSP/TBRI). Mice were bred under environmentally controlled conditions, fed with a standard pellet diet and distilled water.

2.8 In-vivo Study

2.8.1 Infection

Forty-eight male albino mice with the age of 6–8 weeks were individually exposed for 2 hour using the tail immersion method in a total volume of 10 ml of 100 ± 10 cercarial suspension previously exposed to sub-lethal concentrations of different extracts. Mice were washed to remove cercariae that may remain in hairs. After 2 hour exposure to cercariae, mice were maintained under standard condition in the animal house. As a control, eight mice were individually exposed to the same number of cercariae untreated to the extract.

2.9 Experimental Groups

Total of 48 mice were divided randomly into four groups as follow:

Group 1: Normal healthy control group of 10 mice.

Group 2: Infected control group of 10 mice each infected with 100 *S. mansoni* cercariae / mouse.

Group 3: Infected treated group (10 mice). Mice were infected with 100 *S. mansoni* cercariae / mouse and treated orally with (200 mg/kg) Me OH extract starting from 7th day p.i. till the end of experiment.

Group 4: Infected treated group (10 mice). Mice were infected with 100 *S. mansoni* cercariae / mouse and treated orally with (2000 mg/kg) Et OH extract starting from 7th day p.i. till the end of experiment.

Group 5: Infected treated group (10 mice). Mice were infected with 100 *S. mansoni* cercariae / mouse and treated orally with (400 mg/kg) Bu OH extract starting from 7th day p.i. till the end of experiment.

- Mice were sacrificed of done 8 weeks post-infection then the following parameters were examined.

2.10 Cell Isolation and Purification

Eight weeks post-infection mice were sacrificed and Peripheral blood samples were collected on an individual basis into heparinized (100 U/ml

heparin, Biochrom, Berlin, Germany), sterile Eppendorf tubes and over layered carefully over Ficoll-paque (Amersham Biosciences, Uppsala, Sweden), and centrifuged at 1800 rpm for 25 min at room temperature. The peripheral blood mononuclear cells (PBMC) in the buffy coat were collected carefully, washed twice with sterile phosphate-buffered saline, pH 7.4 (PBS), and used in flow cytometric analysis.

2.11 Antibodies and Flow Cytometry

Flow cytometric analysis using Accuri C6 flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). Equipped with a compact air-cooled low power 15 mW Argon Ion Laser beam (488 nm). The average number of evaluated nuclei per specimen was 10.000 and the number of nuclei scanned was 120/s. Histogram derived from flow cytometry was obtained with a computer program AccuriC6 software. The following antibodies were used FITC -conjugated anti-CD4 anti human antibody (Bio Legend Cat. No. 300505), FITC-conjugated anti- FOXP3 anti mouse/rat/human antibody (Bio Legend Cat. No.320007), FITC -conjugated anti- CD25 anti human antibody (Bio Legend Cat. No. 302605), and FITC- conjugated anti-TGF- β antibody. The staining method that was performed on the entire antibody was the direct technique in one stain step and after incubation the samples were washed twice with 2%BSA/PBS and spin down at 1800 rpm. The supernatant were discarded and then fixed the stained cells with 4% paraformaldehyde until acquire the sample on flow cytometry.

2.12 Statistical Analysis

Data were expressed as the mean \pm SD. LC50 and LC90 were calculated using probit analysis (2nd Edition). Comparison between the mean values of different parameters in the studied groups was performed using 1-way ANOVA test. The data were considered significant if *P*-value was ≤ 0.05 .

3. RESULTS

3.1 In-vitro Study

3.1.1 Screening for molluscicidal activity

The potency of the tested extracts of *Carica papaya* as molluscicidal and larvicidal against *B. alexandrina* and *S. mansoni* larvae was concentration dependent. Mortality rate

increased with the increase in concentration of the extracts. The mortality rate of *B. alexandrina* snails was 86%, 45% and 64% for methanol, ethanol and butanol extracts from *Carica papaya*, respectively. The LC₅₀ of methanol, ethanol and butanol extracts of *Carica papaya* against *B. alexandrina* were 180, 499.3 and 509.1 mg/L while the respective LC₉₀ values were 220.3, 700.6, 769.6 mg/L respectively after 24 hours from exposure to different plant extracts. The results indicated that methanol extract was more effective than ethanol and butanol extracts of *Carica papaya* (Fig. 1).

3.2 Miracidicidal Activity

After being incubated without extracts of *Carica papaya* on miracidia of *S. mansoni* showed normal motor activity and viability throughout the incubation. While after incubation with extracts of *Carica papaya* at different concentrations, the methanol extract showed highest effectiveness and reduced motor activity with abnormal shape attitude. The mortality rate for *Carica papaya* against miracidia was 83%, 35% and 66%, respectively, for methanol, ethanol and butanol (Fig. 2). The LC₅₀ of methanol, ethanol and butanol extracts of *Carica papaya* against miracidia were 3.4, 15.4 and 8.1 mg/L, respectively, while the respective LC₉₀ values were 8.4, 38.2, 11.2 mg/L, after 1/2 hour from exposure to different plant extracts.

3.3 Cercariacidal Activity

Different extracts of *Carica papaya* showed varying cercariacidal potency against *S. mansoni* cercariae and this activity was more pronounced at higher concentrations of the extracts. The effect of incubation with different concentrations of *Carica papaya* extracts on the viability of cercariae for up to 1 h is depicted in Fig. 3. The exposure of *S. mansoni* cercariae to the methanol extract of *Carica papaya* showed an increase in the mortality rate of cercariae than the other extracts. The mortality rate of *Carica papaya* against cercaria was 92%, 40% and 70% for methanol, ethanol and butanol, respectively. In the absence of the plant extracts, cercariae showed normal viability without any morphological changes (tail loss). The LC₅₀ of methanol, ethanol and butanol extracts of *Carica papaya* against cercariae were 2, 20 and 4 mg/L, while the respective LC₉₀ values were 13.5, 80.5, 18.5 mg/L respectively, after 1 hour from exposure to different plant extracts.

3.4 Antibodies and Flowcytometry

Fig. 4 and Table 1 showed that, the proportion of CD4, CD25, FOXP3 Treg in infected group were decrease significantly ($p < 0.001$) in compared to that in healthy control group. However, there was a significant ($p < 0.001$) increase in levels of TGF- β in comparison to healthy control group. Treatment of *S. mansoni* infected mice with MeOH, EtOH and BuOH *Carica papaya* affects the incidence of peripheral blood Tregs and levels of cytokine TGF- β . A comparison of levels of CD4, CD25, and FOXP3 Treg from infected group and MeOH, EtOH and BuOH treated groups were done using flowcytometry. We found that there was a significant decrease in levels of CD4, CD25, FOXP3 Treg and increase in TGF- β than infected group after treatment with MeOH, Et OH and Bu OH.

4. DISCUSSION

Schistosomiasis is a neglected tropical disease caused by blood-dwelling digenetic trematodes of the genus *Schistosoma*. It remains a major problem in more than 70 countries, and the disease is the second most important human parasitic disease after malaria, with more than 200 million people infected and approximately 800 million people living at risk of infection [1,19,20]. There is no vaccine for schistosomiasis and chemotherapy depend mainly on praziquantel, causing a development of drug resistance; therefore, there is a strong need to develop a new drug [21]. The importance of natural products in modern medicine has been described in a number of earlier reviews and reports [22,23,24]. In the present study, snails exposed to three extracts from *Carica papaya* were adversely affected, the snails were weak and could neither eat nor retract into their shells and finally died. Mortality increased with the increase in concentration of the extracts. The LC₅₀ for methanol, ethanol and butanol extracts of *Carica papaya* were 180, 499.3 and 509.1 mg/L, respectively. The results indicated that methanol extract was more effective than ethanol and butanol extracts of *Carica papaya* against molluscicidal activity.

Controlling of *Biomphalaria alexandrina* snails by plant molluscicides is the cornerstone in treating schistosomiasis in Egypt, it is important to investigate the possible toxicity of this plant molluscicides extract to aquatic organisms, especially invertebrates. Hence, the use of biological method, affect on

snails and not harm to the ecosystem therefore can be used as potent molluscicides to control schistosomiasis. Recent researches focus on finding an alternative natural source instead of the chemical molluscicides [25].

Table 1. Effect of *Carica papaya* extract on levels of peripheral tregs cells

Groups	Mean ± SEM			
	CD4	CD25	FOXP3	TGF-β
Healthy control	66.82±3.71	47.43±1.94	54.88±2.14	14.53±0.98
Infected group	12.34±0.61	7.29±0.17	1.82±0.40**	47.75±2.23
MeOH	44.76±1.75***	21.19±1.19**	37.60±1.13***	26.62±0.66**
Et OH	28.82±2.47**	11.31±0.49	29.15±2.49***	29.14±0.50*
BuOH	23.83±0.65*	8.66±0.40	12.32±0.92**	37.88±0.46

***P < 0.001, **P < 0.01, *P < 0.05 relative to infected control

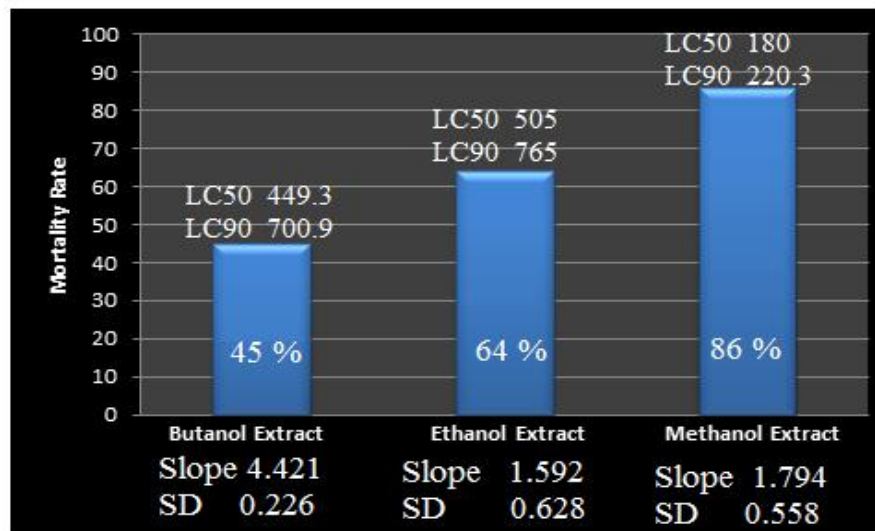


Fig. 1. Anti- molluscidal effect of *Carica papaya* extract after 24 hours from exposure

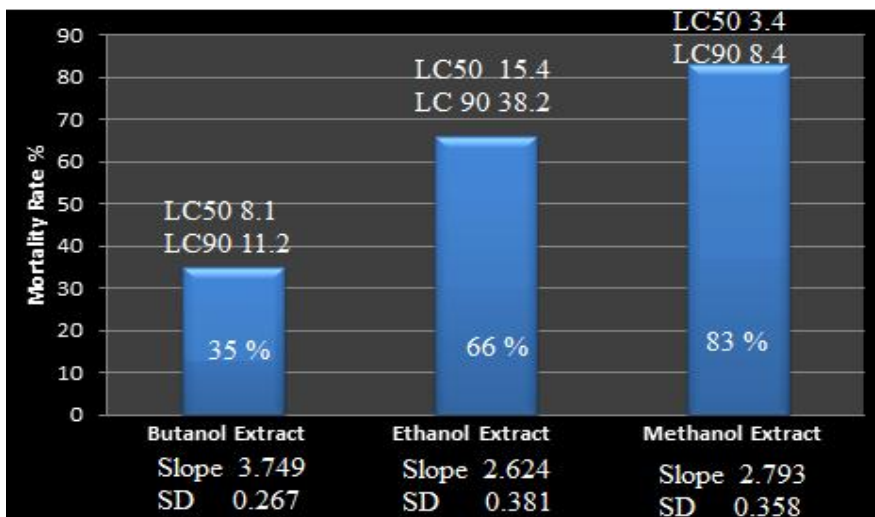


Fig. 2. Anti-miracidal effect of *Carica papaya* extract after 1/2 hour from exposure

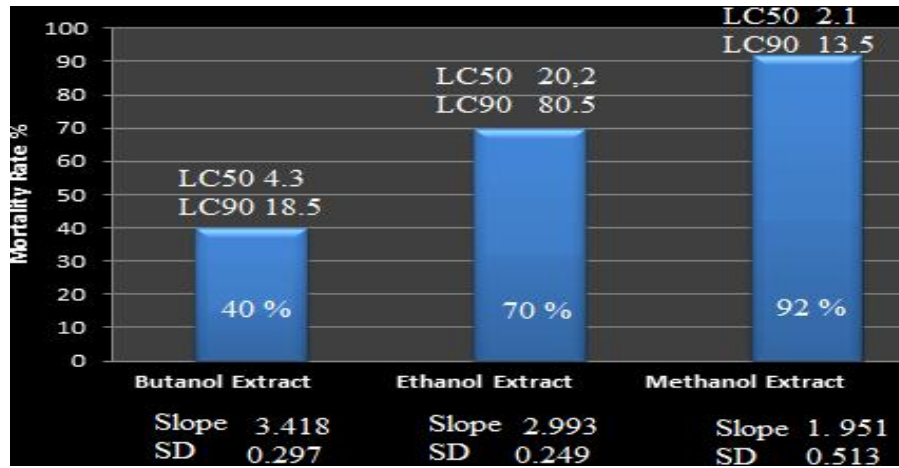
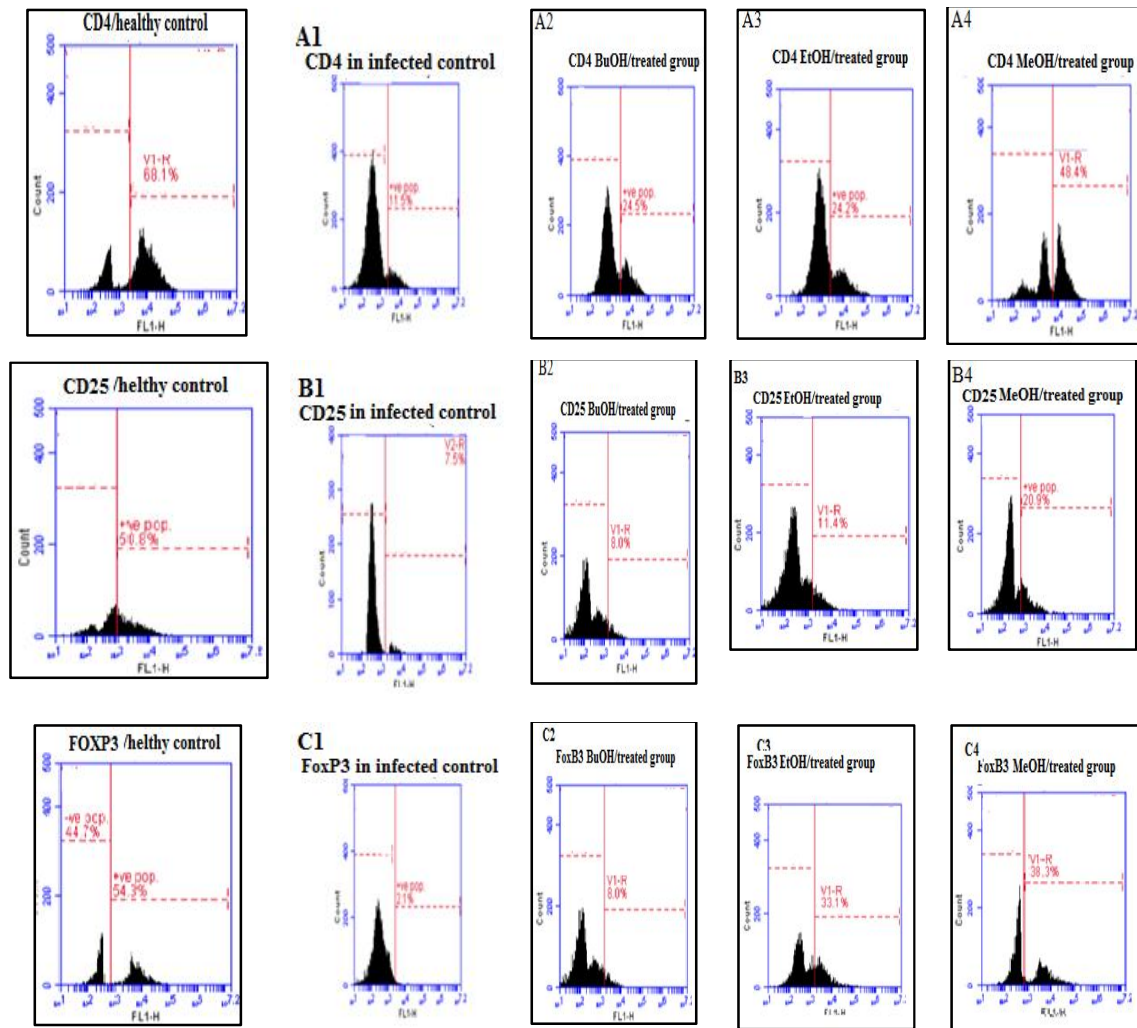


Fig. 3. Anti-cercaricidal effect of *Carcia papaya* extract after 1 hour from exposure



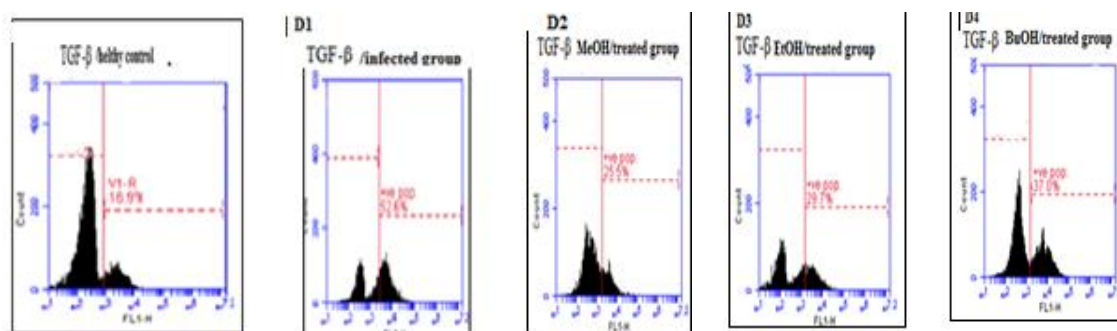


Fig. 4. Flow cytometric analysis showing levels of CD4, CD25, FOXP3 Treg and TGF- β cytokine after treatment with MeOH, Et OH and BuOH from *Carica papaya* extract

A bioassay of whole plants or parts in which snails are killed within 24 hours at a dosage below 200 mg/L indicates that the molluscicide is released quickly and the material may be a good candidate for LC₅₀ determination [26,27]. These efficacy on *B. alexandrina* snails may be according to the phytochemical composition of *Charica papaya* plant including polysaccharides, minerals, vitamins, proteins, enzymes, alkaloids, glycosides, fats and oils, lectins, saponins, flavonoids, sterols, etc. [28]. The antimicrobial, antifungal and antihelminthic activities of the seed and latex of the plant have been extensively reported [29,30]. With regard to the effect of extracts against cercariae, the exposure of *S. mansoni* cercariae to the methanol extract of *Carica papaya* showed an increase in the mortality rate of cercariae when compared to other extracts. This activity was both time and dose dependent. These results agreed with Tekwu [31] who showed that the extracts from *R. vomitoria* was active at concentrations from 31.25 μ g/mL to higher concentrations 1000 μ g/mL, and this activity was also both time and dose dependent. Similarly [32,33] reported the cercariocidal activity of *Glinuslotoides* fruits and *Nigellasativa* crushed seed, respectively, as both time and concentration dependent. In the present study, *S. mansoni* infection was associated with increase in levels of Tregs CD4, CD25, and FOXP3 in comparison to healthy control group. This finding is consistent with reports showed increased numbers of FOXP3 Tregs in peripheral blood from children 8–13 years old with active schistosomiasis [34]. In accordance with many reports [5,7,34,35,36] that indicated that schistosome infection stimulates the production of Tregs that play a key role in controlling Th1 and Th2 responses. TGF- β is known for its immunosuppressive properties; can control the differentiation, proliferation, and

activation of immune and non immune cells in addition to its suppressive function of CD4⁺CD25⁺ FOXP3 Tregs [37]. Our data showed a significant increase in levels of TGF- β in *S. mansoni* infected mice in comparison to healthy control group. These results were in match with Farah [38] indicating that TGF- β is produced in increased quantity during *S. mansoni* infection of humans, non-human primates, and mice. In addition, Zhu [39] found higher levels of serum TGF- β in experimental group than control groups at 8, 10, 12 and 16 weeks after *S. japonica* infection. Similarly, El-Sayed [40] indicated that infection of mice with *S. mansoni* caused pronounced elevations in serum TGF- β levels compared with the control uninfected untreated group. We evaluated the effect of treatment with *Carica papaya* MeOH, EtOH, or BuOH extracts on Treg cells. The results revealed a significant decrease in levels of CD4, CD25, FOXP3Treg, however, *Carica papaya* MeOH extract was more effective. Several studies showed a reduction in the number of CD4, CD25 and FOXP3 Treg after treatment with praziquantel [7,36] which is in line with reports showing that drug induced clearance of *Schistosoma* parasites reduces Treg numbers defined. Treatment of *Schistosoma* infected mice with a single dose of them ethanol and aqueous seeds extract of *Carica papaya* showed anti-schistosomal activity [41]. In addition, a study on treatment with *Carica papaya* methanol extract showed effectiveness against schistosomiasis as it led to less recovery, indicating anti schistosomal properties of *Carica papaya* extracts against *Schistosoma mansoni* [42].

5. CONCLUSION

Finally, controlling *B. alexandrina* snails by medicinal plant is a promising way as it is an ecofriendly strategy in rural areas of developing

countries, where schistosomiasis is endemic. This study also showed that *S. mansoni* infection is related with changes of the activity of CD4⁺, CD25⁺ and FOXP3 Treg cells in addition to levels of TGF- β cytokine.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Handling and treatment of mice were conducted according to internationally valid guidelines and ethical conditions adopted by Theodore Bilharz Research Institute.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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