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The Evaluation of the Effects of an Aqueous and Methanol Extracts of *Solanum incanum* on *Schistosoma mansoni* Infected Mice

John Mokua Mose[*1,3], Helen L. Kutima[2], Susy Muchika[2], Rebecca Waihenya[2], Dorcas S. Yole[3,4]

[1*]Department of Medical Laboratory Science, School of Medicine and Health Sciences, Kenya Methodist University (KeMU), Nairobi, Kenya.
[2]Department of Zoology, Faculty of Science, Jomo Kenyatta University of Agriculture and Technology (J.K.U.A.T), Nairobi, Kenya.
[3]Institute of Primate Research (I.P.R), National Museums of Kenya, P.O. Box 24481-00200 Nairobi, Kenya.
[4] Kenya Polytechnic University College, P.O Box 52428-00200 Nairobi, Kenya.

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***Corresponding author:**

E-mail: mokuaj@yahoo.com
Phone: +254-724-709 120

ABSTRACT

Interest in medicinal plants as a re-emerging health aid has been fuelled by increasing concern about the development of parasite resistance and the rising costs of prescription drugs in the maintenance of personal health and well-being, and the bioprospecting of new plant-derived drugs. Some plant extracts have been used worldwide in traditional medicine for the treatment of human helminthes but not all have been screened for activity against adult *Schistosoma sp*. The objective of this study was to evaluate the biological effects of crude extracts prepared from dried roots of *Solanum incanum* using experimental mice infected with *Schistosoma mansoni*, assessing the worm recovery and immunological responses after treatment. The mice were infected with a single dose of 250 *Schistosoma mansoni* cercariae and treated with the aqueous and methanol crude extracts at a specific time point. Evaluation on the number of worms recovered and the humoral and cellular immune responses was made. The results obtained showed a 16.7 % maturation of penetrant cercariae. The *Solanum incanum* aqueous group recorded the highest worm reduction of 46.3% compared to control infected animals with 53.7% worm recovery observed. Cytokine levels peaked during the acute infection and declined to detectable levels after treatment. There was a marked rise in SWAP specific Interleukin-5 and also a rise in 0-3hr and SWAP specific IgG regardless of the time point after treatment. IL-5 production was significantly greater in the infected control and the treatment groups ($p<0.05$). 0-3hr and SWAP induced gamma interferon production however did not increase after treatment.

INTRODUCTION

Schistosomiasis mansoni is considered as one of the most important diseases in the world, with more than 300 million people infected and over one billion at risk. The disease is widely prevalent in the most parts of Africa, South America and Asia. Indeed, the highest mortality in human *Schistosoma mansoni* occurs in the minority of people who develop hepatosplenic schistosomiasis characterized by periportal fibrosis, portosystemic shunts and hematemesis [1-3]. In Kenya, it has been estimated that over 3.5 million people are infected with *S. mansoni* in endemic areas in Taveta, Kitui, Machakos and Nyanza particularly along the shores of Lake Victoria. In Taveta district, the towns that are mostly affected are Jipe, Eldoro and Kivalwa while in Kitui district schistosomiasis is mainly found in Mwingi on the eastern fringes of the central plateau. Around Lake Victoria, endemic areas include the North Nyakach, Mfangano and Rusinga islands while in the northern part of Nyanza the towns most affected are Bunyala, Samia and Nduru. Other regions where *S. mansoni* infections are found in the upper valley of the Tana River in the vicinity of Mwea and in the Rift valley around Lake Naivasha. No transmission has yet been documented on the north of the equator although hospital reports have recorded cases at Wajir and Mandera [4].

The control of schistosomiasis requires large scale population based chemotherapy in addition to environmental and behavioural modification. Praziquantel, a pyrazinoiquinoline derivative, is the mainstay of treatment and a critical part of community-based schistosomiasis control programs but resistance to it may be emerging after nearly 20 years of intensive use and is also expensive [5-8]. Concern is therefore increasing in determination of effective and cheaper drug.

Many plant species have been used throughout the world in traditional medicine for the treatment of both veterinary and human helminthes [9-11], but some have not been screened for activity against adult *Schistosoma sp*. Myrrh, a gum extract from the stem of *Commiphora myrrha* (molmol-Somali) of the family *Burserceae* has been used to treat *Schistosomiasis. A*n extract of *Commiphora molmol* (myrrh) has been licensed and marketed for

clinical use against *Fasciola* and schistosome infections in Egypt. The extract has some antischistosomal properties that cause worm pairs to separate. The female worms then shifts to the liver, where they are destroyed [12].Tests carried on vernodalin, a highly toxic sesiquiterpene lactose compound, extracted from *Vernonia amygdalina* also showed significant activity against schistosomes as well as *Plasmodium* and *Leishmania* species. Crushed seeds of the plant *Nigella sativa* (Rununculaceae) were found to have antischistosomal activity against different stages (cercariae and juvenile) of *S. mansoni in vitro* [13].

The objective of this study was to evaluate the antischistosomal activities (in vivo) of Methanol and Aqueous extracts prepared from dried roots of *Solanum incanum*. The plant is extensively found in many regions in Kenya and it is frequently discussed as possible source of novel drugs and in recent years, it has been documented as a possible sources of novel antischistosomal agent. It is therefore necessary to carry out a study to determine it's immunological effects and determine if can be used alongside praziquantel and this formed the basis of this study.

MATERIALS AND METHODS

Animals, parasite and Plants extracts

The research was performed at the Institute of Primate Research (IPR) and at Jomo Kenyatta University of Agriculture and Technology (JKUAT). A strain of *Schistosoma mansoni* parasite originally obtained from humans and maintained in Olive baboon (*Papio anubis*) at IPR was used for the study. Swiss mice strain weighing about 30g and 7 weeks old were acquired from the Kenya Medical Research Institute (KEMRI) Nairobi and maintained at IPR.

Plant material

The roots of *Solanum incanum* were collected, stored in plastic bags and transported to the laboratory for processing. Taxonomic identification of the plant was done by Botany department of Jomo Kenyatta University of Agriculture and Technology and a voucher specimen was deposited in the department. Roots were dried at room temperature (25˚C) for one month and then crushed into small particles using Mekon Micromiller Single phase and passed through a 0.5mm mesh to standardize the particles. Two kilograms of the ground plant material was separately placed in different clean large bottles.

Preparation of the plant extract

Two extraction solvents were used in this process. Grounded fine powder was separately soaked in 98% methanol for 72hours and 36 hours for distilled water, followed by filtration using Whatman No.1filter paper consequently for three times. The three filtrates from aqueous extraction were lyophilized using a freeze drying machine (Model, FD-A, Japan) for a month after which the aqueous extract was obtained in powder form. The methanol filtrates were processed using a rotary vacuum evaporator (RE-100 Bibby, Japan) at 70˚C, and methanol was further removed by placing the samples on a water bath until there was no evaporation (methanol extract).

Infection, treatment, perfusion, and worm counts

White Swiss mice weighing 30g were used in the study. The animals had free access to a standard commercial diet and water ad libitum and were kept in cages (same sex) maintained at 25˚C with 12 hrs of light /12 hrs darkness photoperiod, ambient temperature (20˚C) and relative humidity (50-60%).

In this experiment, a group of mice were anaesthetized with Ketamine /Rompun mixture (ratio 2:1) and infected with *S. mansoni* at a rate of infection of 250 cercariae/mouse using the ring method described by Smithers and Terry. The lower abdomen of each mouse was carefully shaved and moistened with water before exposure. A metal ring of 1cm in diameter was firmly held to the abdomen with strips of tape. The cercarial suspension was gently stirred and the volume of 1ml containing the desired number of cercarial was pipetted into the ring and allowed 30 minutes to penetrate [14].

After 30 days of infection, the mice were divided into four subgroups of 18. In two groups, mice were separately (n=6) and individually orally treated with 300 mg/kg body weight in 200µl suspension, of either aqueous or methanol extracts of *Solanum incanum* two days apart. The other two groups served as controls; one infected and not treated and the other infected and dosed by 900mg/kg of praziquantel two days apart.

Humoral responses

Blood was collected by heart puncture at week 4 and week 6 for Infected control and week 6 for all the other groups prior to perfusion. Centrifugation was done at 7000xG for 20 minutes and the serum stored at -20°C for IgG ELISA.

Worm recovery

At week 6 post-infection and two weeks post-treatment, six mice from each group were perfused and the worm recovery was determined according to the method described by Yole *et al* [15] Worm maturation was calculated using the following formula:

$$\text{Worm maturation} = \frac{\text{Number of worms recovered from infected control}}{\text{Initial number of infecting parasites}} \times 100$$

$$\text{Worm recovery} = \frac{\text{Number of worms recovered from treatments}}{\text{Number of worms recovered in Infected - untreated control}} \times 100$$

Cellular responses

Cytokine production can be studied by following their profiles in infected hosts. In order to study this, mice were sacrificed at week six following infection; spleen and lymph nodes (auxillary and the inguinal) were removed aseptically and separately placed in 5cm Petri-dish containing incomplete medium (RPMI 1640, 0.1% Gentamycin, $5x10^{-5}$ Beta mercaptoethanol). They were later separately placed on sterilized wire gauze, in a sterile petri dish containing incomplete media in a sterile culture hood, squashed and the cell dispersed. The cells were dispensed in 15ml tubes and incomplete RPMI 1640 added up to 10ml mark before centrifugation at 450g for 10min at RT, supernatant discarded and pellet resuspended. Washing was repeated two times and after the final wash, the supernatant was removed using a pasteur pipette and 4mls of Complete medium (Incomplete medium fortified with 10% Foetal calf serum) added to the pellet to make cell

suspension. Lymphocyte viability was determined by the trypan blue exclusion test and counting done using a haemocytometer. Cell suspension was made up to 3 x 10^6 cells per ml in complete medium.

Lymphocyte culture stimulation

Flat-bottomed 48-well microtiter plates were used for culture and 6 x 10^5 cells were dispensed in each well. Negative control had only medium and cells while positive control had 1µg/ml of Concanavalin A; and test wells, 10µg/ml per well of SWAP/0-3hr release protein. Duplicate wells were set for each regimen and the total volume of culture medium per well was 400µl. The plates were incubated at 37ºC, 5% CO_2 for 48hrs for Con A and 72hrs for the other set-ups. Supernatants were collected from the wells after the specified periods (BIO-RAD). The lymphocyte cultures were collected and stored at -70^{0}c. IFN-γ and IL-5 levels were determined by cytokine-capture ELISA.

Analysis of Results

Analysis of worm recovery was presented as Mean ±S.E.M. Immunological data obtained was analyzed using SPSS-14 statistical software; Chi squared test was used to determine association and P-values P<0.05 were considered statistically significant.

RESULTS

Parasitological outcomes: Worm recovery and maturation

Mean total worm recovery from various groups is detailed in Table No.1. Consistent results were achieved for some groups with some exceptions. The infected control group had a mean worm recovery ± SEM of 41.8 ± 4.54 which represented 16.7% maturation of penetrant cercariae. Comparatively, more worms were recovered in the group *Solanum incanum* methanol than *Solanum incanum* aqueous with no significant difference (p>0.05). The mean worm recovery for the infected control was significantly higher compared to praziquantel (p<0.05).

Table No. 1: Effect of *Solanum incanum* on Worm recovery.

Group	Dose (mg/kg)	Mean number of worms recoverd per group (Mean±SE)			% worm recovery
		Total males	Total females	Total worms Mean±S.E	
Praziquantel	900x2	9.8 ± 0.65	4.2 ± 0.40	14.0 ± 0.856	33.5
S. incanum Methanol (SIM)	300x2	16.8 ± 2.96	11.5 ± 2.66	28.3 ± 5.282*	67.7
S. incanum Aqueous (SIA)	300x2	12.4 ± 1.21	10.0 ± 1.67	22.4 ± 1.806*	53.7
Infected control	0	24 ± 3.67	17.8 ± 1.11	41.8 ± 4.535	-

Values are expressed as the mean ± SEM of six observations
*P<0.05 Statistical comparisons are made between: Control vs SIA and SIM groups
Dose=mg (plant extract/PZQ)/kg X number of doses

Schistosome specific IgG ELISA

Correlations were observed between 0-3Hr and SWAP antigen specific IgG responses and resistance to infection before and after treatment in both the treatment and the control groups by ELISA. The levels of 0-3hr and SWAP specific IgG, in serum were examined throughout the course of infection (Figure No.1). Humoral immune responses demonstrated expected increases in IgG in response to 0-3Hr and SWAP antigen as the infections progressed. Comparably, the IgG antibody responses in the group *Solanum incanum* methanol, was lower to *Solanum incanum* aqueous with no significant difference (p>0.05).

The SWAP IgG responses were higher than 0-3hr. Generally, the results showed an increasing trend of IgG responses in infected control from week 4 to 6 and they were significantly higher than praziquantel. Generally, solanum treated groups had similar responses to praziquantel with no significant difference (p>0.05).

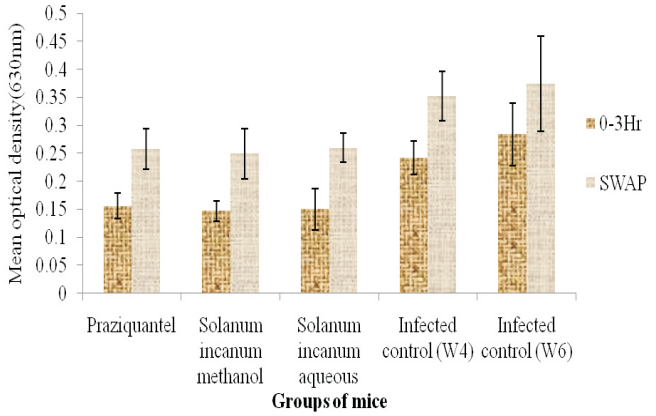

Figure No. 1: Levels of 0-3hr and SWAP specific IgG in serum after *S.mansoni* infection. Each time point represents the mean± SEM of six observations

Cellular responses

0-3Hr and SWAP antigen specific IFN-γ responses from the lymph node and the spleen cells

0-3hr and SWAP-specific IL-5 and IFN-γ production by cultured splenic and lymph node lymphocytes were quantified by ELISA. The 0-3hr and SWAP antigen specific IFN-γ responses in the lymph node and in the spleen are shown in Figure No. 2 [A] and [B] respectively. For the lymph node, the results obtained generally showed that the group, *Solanum incanum* methanol had similar response profile for both SWAP and 0-3hr antigens. It was also observed that both the infected control and praziquantel had low responses to 0-3hr and higher responses to SWAP. The IFN-γ response for infected control was significantly higher than PZQ

($p<0.05$) for both antigens. On the other hand, the *Solanum incanum* aqueous group had higher responses to 0-3hr antigen (but similar to other groups) but lower responses to SWAP

In the spleen cells for both antigens, infected control had significantly higher IFN-γ responses than PZQ and treatments ($p<0.05$). Comparably, *Solanum incanum* aqueous had lower IFN-γ response for both antigens. Responses of the group praziquantel were similar to those in the group *Solanum incanum* aqueous. Generally, IFN-γ responses stimulated by the SWAP antigen were higher compared to those stimulated by the 0-3hr antigen.

Interleukin 5 (IL-5) responses

The IL-5 production in spleen cells stimulated by both 0-3hr and SWAP antigen in the infected control was significantly higher compared to praziquantel group ($p<0.05$) (Figure No.3). When compared to PZQ, the IL-5 production in *Solanum incanum* methanol was significantly higher compared to PZQ ($p<0.05$) while *Solanum incanum* aqueous showed similar response with no significant difference ($p>0.05$). In the spleen cells, the responses indicated that all scenarios induced IL-5 production. However, there was higher IL-5 production in PZQ as compared to other groups with no significant difference ($p>0.05$). The other groups generally showed similar responses with no particular trend.

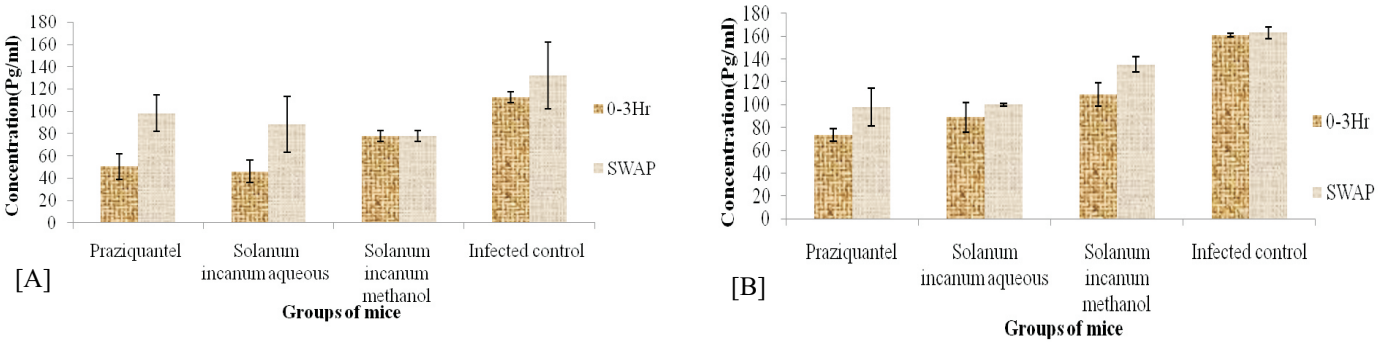

Figure No. 2: 0-3hr and SWAP specific IFN-γ cytokine levels in the **[A]** Lymph node and **[B]** Spleen cells. Lymph node cells and splenocytes were cultured at 6 x 10^5/ml in the presence of 10μg/ml per well of 0-3hr or SWAP and supernatants were harvested at 72hours. Cytokine secretion was compared to that at the corresponding time points after infection

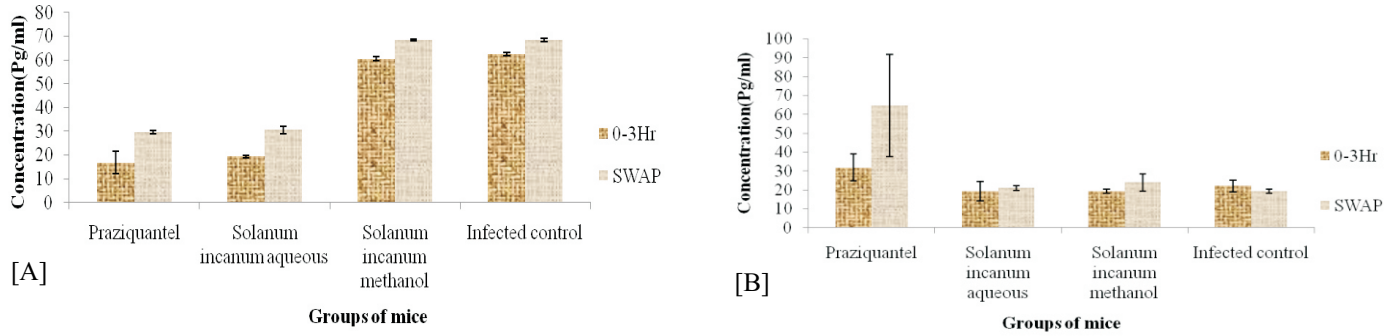

Figure No. 3: 0-3hr and SWAP specific IL-5 cytokine levels in the **[A]** Lymph node and **[B]** Spleen cells. Lymph node cells and splenocytes were cultured at 6 x 10^5/ml in the presence of 10μg/ml per well of 0-3hr or SWAP and supernatants were harvested at 72hours. Cytokine secretion was compared to that at the corresponding time points after infection

DISCUSSION

The results showed that *Solanum incanum* portrayed comparable effects to praziquantel. These results showed that primary infection followed by treatment with the extracts produced significant reduction in worm burden. The host immune response to *S. mansoni* infection has been shown to be a T-cell dependent process. Classically, the host initially responds with a Th1 type response which has been shown to be directed against early stages of the parasite and to be important for the induction of the cell mediated protective immunity to *S. Mansoni*.

The results obtained showed the infected control group with significantly higher IFN-γ responses than all the treatments groups and praziquantel in lymph node cells stimulated with SWAP and spleen cells stimulated with both 0-3Hr and SWAP antigens. This is expected as IFN-γ is required in the initiation of granulomatous infection (cellular infiltration.

An important function of the TH2 response during infection is to produce cytokines that can prevent or dampen the production or effector functions of potentially dangerous inflammatory mediator. The production of eggs brings about the most dominant responses that occur at the acute stage of the disease. This response is mediated by $CD4^+$ cells that are highly Th2-polarized with the production of large amounts of IL-4, IL-5 and IL-13 among other cytokines [8,10,16-17].

In this study, the two extracts induced production of IL-5. The praziquantel and *Solanum incanum* aqueous groups showed similar lower IL-5 responses and also similar IgG levels and also *Solanum incanum* aqueous had the closest worm reduction to

Praziquantel. The higher levels of IL-5 production by infected control and *Solanum incanum* methanol could be as a result to non-specific schistosome antigens, which are not responsible for protection to schistosomes. These groups had high worm recovery as compared to praziquantel and *Solanum incanum* aqueous. This would translate to high levels of non-specific IgG, as suggested by high levels of IgG recorded in these groups. This state of high production of IgG is referred to hypergammaglobulinaemia.

For both 0-3Hr and SWAP antigens, the IgG responses in infected control increased from week 4 to week 6. This increase is expected because worms are more mature at week 6 compared to week 4 hence more antigens were being released stimulating increased antibody response at week 6. Similar results have also been reported by Yole *et al*[15]. There had also been no treatment in this group and there are no adult worms killed by extracts and hence there was continued release of antigens from ova and further stimulation of B- lymphocytes. In addition, the infected control group showed higher responses than both the treatment groups and praziquantel. This corresponds to the number of worms, and in turn the antigens being released into circulation.

The *Solanum incanum* aqueous group shoewd similar IgG response to praziquantel for both antigens. It also recorded the closest worm reduction among the treatment groups as compared to praziquantel. High IgG levels signify a high protective immunity. This implies that *Solanum incanum* aqueous treatment sustained a higher activity of B cells of the immune system and has a higher protective immunity. This was emphasized by the student t-test which showed that the two groups (praziquantel and *Solanum incanum* aqueous) were not significantly different ($p>0.05$).

These results also revealed that serum stimulated by 0-3hr antigen exhibited low IgG response as compared to SWAP. This is usually expected since the assay was done at the time when the parasites had developed into mature worms. What the 0-3hr antigen detected was shared antigens between the different stages of schistosomes.

The IgG production to SWAP antigens was high because of the consistency in antigens exposed on the surface due to maturity of the worm into adult worm. This variation in the level of IgG to Schistosome specific antigens shows that there is greater protective immunity to adult worm than to immature ones as high IgG level indicated increased immune protection. These results agree with the SWAP antigen and IL-5 responses where infected control showed higher response than PZQ. The cytokine IL-5 is responsible for antibody production. This therefore means that, an increase in IL-5 also directly increases the production of antibodies.

CONCLUSION

From the results, it can be concluded that both the methanol and aqueous extracts processed from the dried roots of *Solanum incanum* and at a concentration of 10mg/ml have a protective action against the effects of *Schistosoma mansoni*. In particular, the extracts have shown significant reduction in worm recovery i.e it is a potential antischistosomal agent, it has also significant immunological effects

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