EFFICACY OF SCHITOZIM - A HERBAL MEDICINE IN THE TREATMENT OF *Schistosoma mansoni* INFECTIONS IN EXPERIMENTALLY INFECTED BALB/C MICE

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2014

DECLARATION

I hereby declare that this thesis is my original work and has not been presented for the award of a degree in any other University.

AYONGA DARLENE NYABOKE **SIGNATURE………………..**

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Dedication

I dedicate this thesis to my dear parents, George Memba Ayonga and Gladys Bonareri Ayonga, who have been very supportive throughout my study.

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ABBREVIATIONS

ANOVA:	Analysis of Variance
BSA:	Bovine Serum Antigen
CDC:	Center for Disease Control
CNS:	Central Nervous System
ELISA:	Enzyme Linked Immunosorbent Assay
IC:	Infected control
IFN-γ:	Interferon
IgG:	Immunoglobulin G
IL-4:	Interleukin 4
IPR:	Institute of Primate Research
PBS:	Phosphate buffer saline
PZQ:	Praziquantel
RPA:	Release Protein Antigen
SEA:	Soluble egg antigen
SSP:	Soluble Schistosomule Protein
SWAP	Soluble worm antigen preparation
TID:	Tropical Infectious Diseases
WHO:	World Health Organisation

ABSTRACT

The aim of this study was to test and compare the effectiveness of Schitozim against Praziquantel (PZQ). Six treatment groups of mice were tested for the study: 50 mg, 150 mg and 300 mg Schitozim dosages, PZQ, Infected control(IC) and Naïve. Balb/c mice were infected with *S. mansoni,* treated at week 4 and perfused for worm recovery at week 6. Phytochemical screening of Schitozim revealed the presence of Tannins, Steroids, Flavonoids, Glycosides and Saponins. Worm maturation was 24.4%, and percentages of worm reduction were highest in PZQ (63.93%) and lowest in 150 mg Schotizim dosage (32.79%). The 0-3 hr specific IgG responses were not significantly different among PZQ, IC, 50 mg, 150 mg and 300 mg. SWAP specific IgG responses were not significantly different among PZQ, IC and 300 mg, whereas 50 mg and 150 mg were significantly different from IC. SEA specific IgG responses were not significantly different among PZQ, IC, 150 mg and 300 mg, whereas 50 mg was significantly different from PZQ. Naive group was significantly different from all the other treatment groups ($p < 0.05$). PZQ had the least cases of liver inflammation and granulomas: 50 mg dosage was most comparable to PZQ. Histopathology results on granulomas showed that PZQ had a low mean whereas IC had the highest: 50 mg was most comparable to PZQ. Cellular reactions in mesenteric lymph nodes were numerous in the 300 mg group and very few in PZQ. Generally, the efficacy of Schitozim was comparable to PZQ in worm reduction, elevation of humoral responses and pathology.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

1.1.1 Schistosomiasis

Schistosomiasis is a parasitic infection caused by blood dwelling fluke worms belonging to the family Schistosomatidae, genus *Schistosoma*. There are six species in the genus *Schistosoma* that are of major pathological importance to humans, *Schistosoma haematobium*, *S. mansoni, S. japonicum, S. mekongi, S. intecalatum, and S. guineensis* (Webster *et al*., 2006). Schistosomiasis is also known as snail fever or bilharzia. Its transmission has been documented in 78 countries worldwide; however, the most at-risk population groups live in 54 countries. After malaria and intestinal helminthiasis, it is the third most important tropical disease in the world (Schistosomiasis Fact Sheet, 2010).

The disease affects almost 240 million people worldwide, and more than 700 million people living in endemic areas (WHO, 2014). It mainly affects the poor due to lack of portable water and adequate sanitation (WHO 2012). Globally, 200,000 people die annually due to Schistosomiasis (Chitsulo *et al*., 2000) with Sub-Saharan Africa accounting for 85% of the total number of infected people and 95% of schistosomiasis cases being due to infections with *S. mansoni* and *S. haematobium* (Chitsulo *et al*., 2000). The prevalence and intensity of infection shows a peak at the age of 5 – 15 years and decreases in adults (Dalton and Pole, 1978) with the main risk groups being school-aged

children, fishermen, farmers, irrigation workers, and women using infested water for domestic purposes (WHO Expert Committee, 2002). This project will focus on *S. mansoni*.

1.1.2 Epidemiology of Schistosomiasis

Schistosomiasis is common in rural areas, though cases in urban areas have been seen to be increasing in many countries (Mott *et al*., 1990). Common sources of infection are natural streams, ponds, lakes, man-made reservoirs and irrigation systems. However, population growth and migration has greatly contributed to the spread of Schistosomiasis (Gryseels *et al*., 2006; McManus and Loukas, 2008). As the population size increases, so is the need for power and water which results in development schemes and environmental modifications that also lead to increased transmission (WHO Schistosomiasis, 2013). The distribution of Schistosomiasis is focal, depending on the variations of snail populations and human-water contact behavior (Gryseels and Nkulikyinka, 1988; Eldryd et.al., 2004).

S. mansoni is one of the most widespread of all the schistosomes, and is endemic in 54 countries especially in South America, Caribbean, Africa, and the Middle East where it causes intestinal Schistosomiasis. Worldwide, 83.31 million people are infected with *S. mansoni* (Crompton, 1999). In Kenya, *S. mansoni* is common in Machakos, Makueni, and Kitui districts. In the coastal region, *S. haematobium* is highly prevalent. There are mixed infections in Taveta region and near Lake Victoria, and *S. haematobium* is found mainly inland, while *S. mansoni* around the shore (Eldryd *et al*., 2004).

1.1.3 Symptoms of *Schistosoma mansoni* infection

Symptoms of infection are due to the body's reaction to the worms' eggs, and not to the worm itself. Early symptoms include fever, chills, muscle aches and coughs (CDC Fact Sheet, 2012). Other symptoms include diarrhea, abdominal pain, and blood in the stool. In advanced stages there is liver enlargement, frequently associated with an accumulation of fluid in the peritoneal cavity and hypertension in the abdominal blood vessels. This can also lead to an enlarged spleen (WHO Schistosomiasis, 2013). Repeated infection can cause damage to the liver, lungs, intestines and bladder (CDC Fact Sheet, 2012). In children, schistosomiasis causes anemia, stunted growth, and reduced ability to learn, although the effects are reversible with treatment (WHO Fact Sheet, 2012).

1.1.4 Life cycle of *S. mansoni*

The life cycle of *S. mansoni* is shown in Figure 1. The adult worms live in the portal veins that drain the large intestine. The male and female pair up and copulate, then move upstream into smaller veins, where the females deposit eggs, which are released into the lumen and excreted with faeces. About two-thirds of the eggs remain trapped in the host's body. The eggs are completely embryonated by the time they reach the outside, and hatch when exposed to the lower osmolarity of fresh water (Roberts and Janovy, 2006).

The miracidia have photoreceptors, and are positively phototropic which assist them in locating a snail host (intermediate host). The intermediate hosts of *S. mansoni* are snails of

the genus *Biomphalaria*. *Biomphalaria alexandrina* is the major transmitter in northern Africa, Saudi Arabia, and Yemen. *B. pfeifferi* and *B. rupellii* transmit *S. mansoni* in other parts of Africa, whereas *B. glabrata* transmit in the western hemisphere, (Roberts and Janovy, 2006). After penetration into the snail, a miracidium sheds its epithelium and develops into a mother sporocyst within the snail. By asexual multiplication, the mother sporocyst produces daughter sporocysts after 2 weeks, and the daughter sporocysts migrate to other organs of the snail. The mother sporocyst continues to produce daughter sporocysts for up to seven weeks (Okabe, 1964). There is no redial generation. The daughter sporocysts form furcocercous cercariae after another 2 weeks which have a bifurcated tail for swimming purposes. Cercariae emerge from the infected snail daily during the daylight hours and use water turbulence, negative phototaxix, and skin-derived chemicals to locate a human (definitive) host (Roberts and Janovy, 2006).

When in contact with the human skin, the cercariae attach and creep about, seeking a suitable place to penetrate. They are attracted to skin secretions, showing strong positive responses to the amino acid arginine. They then begin to produce arginine themselves from postacetabular glands, thus attracting other cercariae (Granzer and Haas, 1986). They penetrate the epidermis in less than 30 minutes, and disappear from the surface in 10 to 30 seconds. During penetration, the tail drops off and the schistosomules enter the peripheral circulation within 24 hours. They are swept off into the heart and migrate through the pulmonary capillaries to the left heart and systemic circulation. M

However, most schistosomules are eliminated as they migrate through the lung capillaries (Wilson, 1990). The schistosomules that enter the mesenteries, traverse the intestinal capillary beds, and reach the liver by the hepato-portal system continue to grow. After 3 weeks of development in the liver sinusoids, the male and female worm pair up and migrate to the gut wall where the female begins producing eggs (Nollen, 1997). Adult schistosome can live for up to 30 years (Jordan, 1985) and an infected person can harbour upto an average of hundreds of worms (Gryseels & De Vlas, 1996).

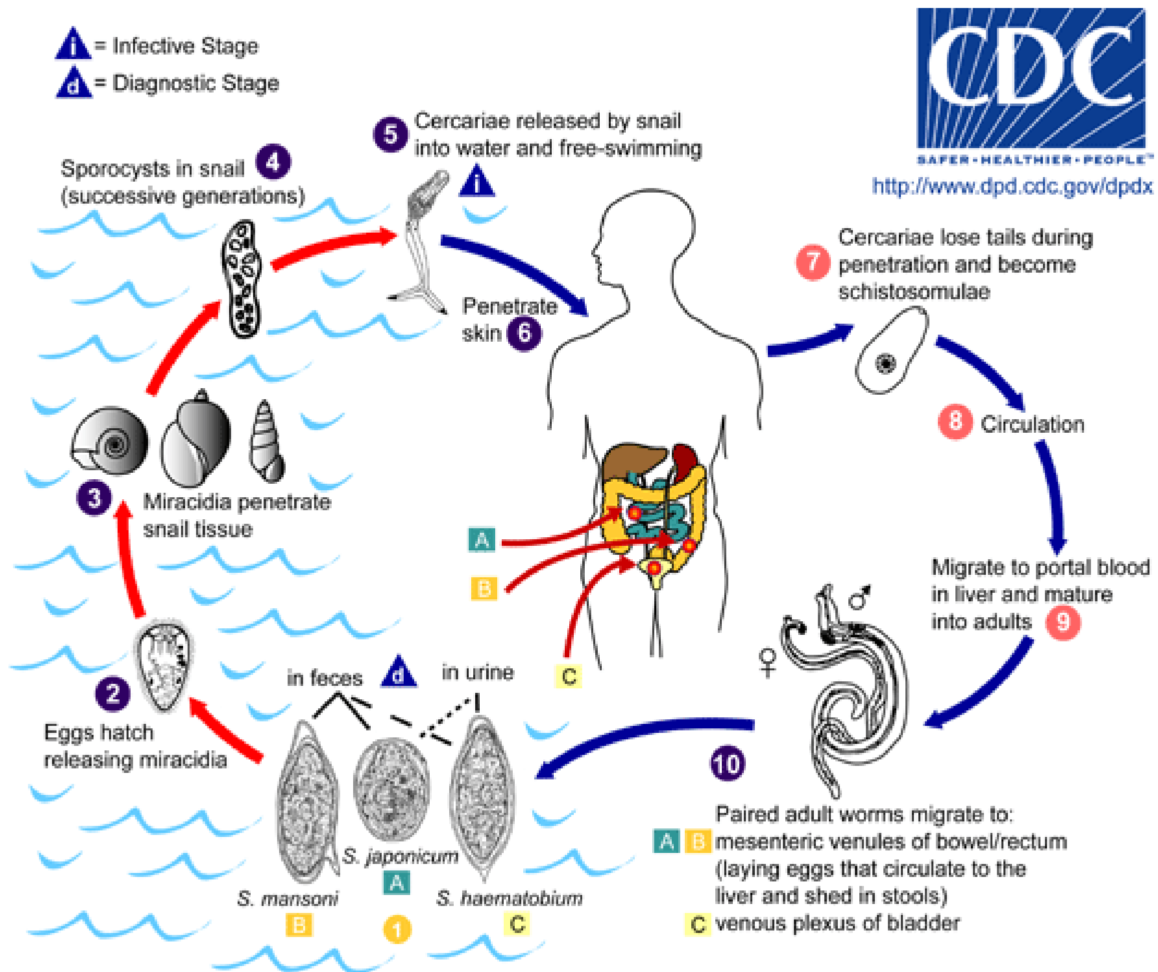


Figure 1: The life cycle of *Schistosoma mansoni* (CDC Fact Sheet, 2012)

1.1.5 Control of Schistosomiasis

Control measures against *Schistosoma mansoni* are directed towards preventing occurrence of new infections by interrupting the life cycle of the parasite. This is achieved by:

1) Eliminating the intermediate host: This can be done by using molluscicides, altering the aquatic environment, and biological control (Schistosomiasis Research group, 2010).

2) Eliminating the parasite from the definitive host: This is achieved by giving regular treatments to infected persons. This is currently the main control method for schistosomiasis. The two main drugs used are Oxamniquine and Praziquantel. However, these drugs are expensive, and require repeated drug treatments at relatively short intervals due to re-infections (Schistosomiasis Research group, 2010).

3) Prevention of infection of the definitive host: This is done by reducing human-water contact with infected water (CDC Schistosomiasis, 2012). Schemes made to facilitate this however require a lot of funding.

4) Prevention of infecting intermediate host: This is simply preventing eggs from reaching water sources where snails breed (Schistosomiasis Research group, 2010).

5) Use of vaccine: This would be the ideal method of controlling schistosomiasis, however, none has been produced yet to date (Schistosomiasis Research group, 2010).

1.2 LITERATURE REVIEW

1.2.1 Pathology due to *S. mansoni* infection

The disease process is divided into 4 stages: 1) invasion of the skin by cercariae, and subsequent migration of schistosomula through the lungs to the liver, 2) maturation of adult worm and early egg laying, 3) established infection, with formation of granulomas around eggs deposited in tissues, 4) late infection with irreversible lesions caused by extensive fibrosis of egg granulomas (Eldryd *et al.*, 2004).

The first stage occurs within 12 hours of infection and is characterized with skin dermatitis at the site of cercaria invasion. The second stage is characterized by systemic hypersensitivity, also known as Katayama fever. It is caused by maturation and migration of schistosomes to their anatomical locations. It occurs at 5-7 weeks after infection, and involves fever, myalgias, chills, cough and peripheral eosinophilia (Kali, 2011).

The third stage is the chronic stage, which persists until the patient is treated (Kali, 2011.) Chronic intestinal schistosomiasis manifests a few years after infection. It causes cellular, granulomatous inflammation around eggs trapped in the tissues, with subsequent fibrosis. It affects the small and large intestines, with the large intestine showing the most severe lesions, whereas severe pathology in the small intestine is only rarely observed, even though large numbers of eggs may be deposited here. Chronic hepato-splenic schistosomiasis is characterized by cellular, granulomatous inflammation around eggs trapped in the liver, leading to fibrosis and hepato-splenic disease (Schistosomiasis Research group, 2010).

The flukes also release toxins that initiate a TH-2 mediated host response that causes peri-portal fibrosis. This is what causes portal hypertension and splenomegaly, but spares the hepatocytes. There is a potential progression to hepato-cellular carcinoma in patients infected with hepatitis B or C in the liver (Yosry, 2006). Invasion of the eggs in the colon can cause ulceration, stricture, iron-deficiency anemia and obstruction. Polyps may develop where there is granulomatous inflammation (Kali, 2011).

The pathology is due to immunological reactions to the eggs trapped in the host tissues. The eggs release antigens that stimulate a granulomatous reaction involving T cells, macrophages, and eosinophils, resulting in clinical disease. The inflammatory reaction is easily reversible. In latter stages, there is collagen deposition and fibrosis causing organ damage that is partly reversible. The eggs can end up in the eyes, skin, adrenal glands, brain, and muscles. There can be embolization of the eggs from the portal mesenteric system to the brain and the spinal cord via the para-vertebral venous plexus (Shadab, 2011). When the eggs are carried away by blood to the liver, they trigger cellular hypersensitivity which is supposed to prevent damage to hepatocytes (Black, 2005). Granulomas are formed in the process. This immune response in the intestines can obstruct the colon and cause blood loss and the individual develops a potbelly.

Eggs lodged in the liver cause high blood pressure in the liver, enlarged spleen, and ascites, i.e. a build-up of fluid in the abdomen. It also leads to potentially life-threatening dilations in the esophagus or gastrointestinal tract that can tear and bleed profusely

(esophagus varices). The CNS is rarely affected. Chronic Schistosomiasis affects an individual's ability to work, and can result in death (WHO Fact Sheet, 2012).

1.2.2 Chemotherapy of *S. mansoni* infections

1.2.2.1 The effects of chemotherapy on *S. mansoni* infections

Oxamniquine (Vansil) and Praziquantel are the only two drugs used to effectively treat schistosomiasis (Ferrari, Coelho et. al.; 2003). Oxamniquine (Vansil) is an antihelmintic agent that is effective against all stages of infection with *Schistosoma mansoni*, but not against other *Schistosoma spp*. (Miller-Keane, 2003). It is prescribed as a dosage of 15 mg/kg for 1 day and causes the worms to shift from the mesenteric veins to the liver where the male worms are retained. Here the cellular host responses finally eliminate the male worms; the female worms can move back to the mesentery, but do not produce eggs (Martidale, the extra Pharmacopoeia). Oxamniquine is associated with an irreversible inhibition of nucleic acid metabolism of the parasite. A schistosome sulfotransferase enzyme converts oxamniquine into an ester, which subsequently dissociates, and the resulting electrophilic reactant is capable of alkylation of schistosome DNA. The drug is well absorbed orally and its half life is 1-2.5 hours (Filho *et al*., 2006).

The side effects of this drug are not serious. A third of patients experience dizziness, with or without drowsiness, which occurs three hours after taking the dose and lasts for about six hours. Other common side effects are nausea, vomiting, diarrhea, and headache. There

are also allergic-type reactions like utricaria, pruritic skin rashes, and fever. Patients who have convulsive disorders report having epileptiform convulsions. Hallucinations and excitement rarely occur. Patients also have a reddish discoloration of urine, probably due to a metabolite of oxamniquine (Roberts and Janovy, 2006).

Praziquantel (PZQ) is said to work by increasing permeability of the membranes of schistosome cells towards calcium ions. This causes contractions of the parasites, leading to paralysis in this contracted state. Therefore, the parasites dislodge from their active site, and are released into the systemic circulation or are destroyed by phagocytosis. It also alters the morphology of the parasite, increasing the exposure of schistosomal antigens on the parasite surface. The parasites are thus easily destroyed by the immune system. When treated with PZQ, the ratio of IL-4: IFN-γ increases in children. There is also a significant increase in cell proliferation after treatment (Mduluza *et al*., 2009). Praziquantel, however, cannot work against juveniles. The general proliferations prior to treatment are low towards the worm antigen, but high towards the egg antigen. Immune responses towards the worm are elevated after treatment, while that towards the egg antigen remains high (Roberts and Janovy, 2006).

The current chemotherapy in use is PZQ which is a wide-spectrum schistosomicide. PZQ has no prophylactic properties and is ineffective against schistosomula, therefore it has to be given repeatedly on a regular basis in order to control the infection. This, however, may result in decrease in efficiency and risk onset of resistance. Therefore, the need for better alternatives is great.

1.2.3 Medicinal plants

Plants have been in use traditionally to heal different diseases, especially across Africa and Asia (Ndamba *et al*., 1994). They were prepared by traditional healers with empirical knowledge and skill. Any medication used as an anthelmintic should have a broad spectrum of action, be high in efficacy, be non-toxic to the host, and cost effective. Most synthetic drugs,however, do not meet these criteria; they have side effects that vary with each medication. A summary of some selected plants with antihelminthic properties is given in table 1.

Table 1: Plants with antihelminthic properties

Plant species	Plant part	Extract	Activity against	Reference
Ocimum sanctum		Oils	*Caenorhabditis elegans*	(Handa and Kapoor, 1988).
Piliostigma thonningii	stem and bark	ethanol extract	*Ascaridia galli*	(Asuzu *et al*., 1994).
Melia azedarach		Ethanolic extracts	*Taenia solium*,	(Szewezuk *et al*., 2003)
Punica granatum	root and stem bark	alcoholic extract, Pelletierine	*Haemonchus contortus*	Prakash *et al*., 1980
Moghania vestita/ Flemingia vestita	fleshy tubers		*Ascaris suum*,	Tandon *et al*., 1997
Mimusops elengi	bark	Saponins and tanins	cause paralysis and death of worms	Kirtikar and Basu, 1935; Thompson and Geary, 1995
Calotropis procera			*Ostertagia, Nematodirus, Dictyocaulis, Taenia, Ascaris,* and *Fasciola*	Shvikar and Kumar, 2003
Xylopia aethiopica	seeds	Tannis, flavanoids or terpenoids	gastrointestinal helminth parasites; reduces worm count	Lahlou, 2002
Butea monosperma		Palasonin	*Ascaris lumbricoides* and *Fasciola hepatica*	Kumar *et al*., 1995
Gynandropsis gynandra)	leaves and stem	Methanol extracts	helminthes	Ajaiyeoba *et al*., 2001
Evolvulus alsinoides		Ethanolic extracts	causes paralysis, followed by death of worms	Dash *et al*., 2002
Carica papaya		Benzyl isothiocyanite	targets the metabolic pathways, carbohydrate pathways, and neuromuscular coordination	Sharma, 1987; Kumar *et al*., 1991
Nigella sativa		Oil	tapeworms, hookworms and nodular worms	Roy and Tandon, 1997
Commiphora mukul	oleo-gum resin		tapeworms and hookworms	Roy and Tandon, 1997
Cannabis sativa	leaves		*Fasciolopsis buski*	Roy and Tandon, 1997
Trifolium repens	Aerial shoot	Aerial shoot extracts	*Hymenolepis diminuta*	Tangpu *et al*., 2004

Flavonoids, glycoside, saponins, steroids and tannins were present in Schitozim aqueous extract. Flavonoids, sesquitepenes, saponins, tannins, anthraquinones, steroids, glyocises and generally phenolics have been reported to be potent plant secondary metabolites with broad spectrum of bioactivities. The presence and quantity of these phytochemical constituents in a given extract determines the extent of extracts' bioactivity. In addition, the presence of more than one class of secondary metabolites in a given plant extract determines the nature and extent of the extract's biological activity (Wang *et al*., 2010).

Glycosides and Saponins are able to bind physically to cell walls thereby preventing the adhesion of pathogens to human cell walls, and hence display antimicrobial activity (Nostro *et al*., 2000). Glycosides have been long used as cardio tonic, in nephrological diseases, and have also been shown to be useful in managing infections. Tannins are useful in the treatment of inflamed or ulcerated tissues, and for treating intestinal disorders such as diarrhoea and dysentery (Dharmananda 2003). Flavonoids have shown to exhibit wide range of biological activities like antimicrobial, anti-inflammatory, anti-allergic, anti-analgesic cytostatic, and anti-oxidant properties (Shobha and Arunima, 2012). Biological activity is attributed to the presence of various secondary metabolites in plants (Mazid *et al*., 2011). These secondary metabolites detected in the Schitozim aqueous extract, therefore, have activities against various ailments.

Granulomas are tiny pinhead sized foci on the surface of the liver that indicate cellular infiltration consisting mainly of eosinophils, macrophages, fibroblasts, and lymphocytes surrounding a schistosome tissue trapped egg. Though pathogenic, granulomas serve as an essential host protective function (Hagan *et al*., 1991); Severe granulomas can be attributed

to a large number of egg laying female worms in the mesenteries, releasing many eggs whose miracidia produce secretions that led to both humoral and cellular immune responses, leading to formation of many granulomas (Theodore and Braden, 2000). Inflammation is a reaction of a tissue to infection due to cellular infiltration and it is characterized by redness, swelling, pain and heat. Adhesions are clear membranes that form in response to infection, and attach different tissues or parts of the same tissue (Muchirah *et al*., 2012). Adhesions in the liver are an indication that there were inflammatory responses as a result of cell mediated immunity (Theodore and Braden, 2000).

The granulomatous hypersensitivity reaction around deposited eggs is mediated by T-cell responses to the secreted soluble egg antigens (SEAs) (Borros D. L., 1989). In the past, great effort has been made to determine the egg antigen(s) that elicited T-cell responses and induced granuloma formation (Lukacs and Borros, 1992).It was found that some of these egg antigens are both granulomogenic and cross-reactive with schistosomular antigens (Lukacs and Boros, 1991).

When an infection occurs, the first line of defense responds, the innate immune response. It remains active for the first several days. The second line of defense, adaptive immunity, becomes active when the disease persists, and is specific to the pathogens. The lymph nodes have T-cells which proliferate and induce B-cells to produce antibodies specific to the pathogens' antigens, which results in a swollen lymph node.

1.2.3.1 Plants with anti-schistosomal properties

Seven plants most commonly used against schistosomiasis infection in Zimbabwe are: *Abrus precatorius* (Leguminosae), *Ozoroa insignis* (Anacardiaceae), *Dicoma anomala* (Cornpositae), *Ximenia caffra* (Oleaceae), *Lamnea edulis* (Anacardiaceae), *Elephantorhiza goetzei* (Leguminosae), and *Pterocarpus angolensis* (Leguminosae). The effectiveness of the extracts from *Pterocarpus angolensis* (Leguminosae) are almost comparable to PZQ (Ndamba *et al*., 1994). Stem and root extracts from *Abrus precatorius* (Fabaceae) and stem bark from *Elephantorhiza goetzei* (Mimosaceae) produce the best results against *S. mansoni* larvae (Mølgaard *et al*., 2001).

Artemether and artesunate, artemisinin derivatives, have anti-schistosomal properties (Utzinger *et al*., 2001; Xiao *et al*., 2002). Artemether has proved to be effective against immature schistosomes, but is less effective against adult worms (Utzinger *et al*., 2001). It interacts with heme, which cleaves the endoperoxide bridge of the drug to produce free carbon radicals that alkylate parasite proteins (Golenser *et al*., 2006).

Extract of rhizomes from the plant *Zingiber officinale* Roscoe (Zingiberaceae), when tested *in vitro*, cause morphological alteration on the tegument. Male worms are more susceptible than female worms. It affects the females by reducing egg production. It has not been tested on schistosomula. Extract from plant *Curcuma longa* L. (Zingiberaceae) has schistosomicidal activity and reduces egg production (Magalhães *et al*., 2009). Extract from garlic *Allium sativum* L. (Liliaceae) is active against *S. mansoni* in mice (50mg/kg), but not effective in high doses (100mg/kg). It affects development and maturity in eggs,

and protects hepatic tissues against oxidative damage. The main constituent in garlic, allicin, causes alterations on the male tegument in high doses (10-20µg/ml). It has, however, not been tested on schistosomula (El Shenawy *et al.*, 2008; Lima *et al.*, 2011; Riad *et al.*, 2007).

The extract from the plant *Clerodendrum umbellatum* Poir (Verbenaceae) is effective in the *S. mansoni* model (Jatsa *et al.*, 2009). The extracts from *Zanthoxylum naranjillo* Griseb (Rutaceae), phloroglucinol compounds from plants of the *Dryopteris* genus (Dryopteridaceae), essential oils from *Baccharis dracunculifolia* DC. (Asteraceae) and *Ageratum conyzoides* L. (Asteraceae), piplartine, an amide isolated from *Piper tuberculatum* Jacq. (Piperaceae), Dermaseptin 01, and Epiisopiloturin, an alkaloid isolated from plant *Pilocarpus microphyllus* Stapf ex Holm (Rutaceae), all have antischistosomal properties, when tested in vitro: they act against the adult worms, causing alterations on the tegument of the worms. They have not been tested on schistosomula, and are not toxic in mammalian cells (Braguine *et al.*, 2009; Magalhaes *et al.*, 2010; Parreira *et al.*, 2010; de Melo *et al.*, 2011; Moraes *et al.*, 2011; Leite *et al.*, 2011;). Oil from the plant *Nigella sativa* L. (Ranunculaceae) is effective against Schistosoma miracidia, cercariae, and adult worms. It also inhibits egg-laying. It has not been tested on schistosomula (Mahmoud *et al.*, 2002; Mohamed *et al.*, 2005). *Jatropha curcas* has little antischistosomal properties, producing a worm reduction of 8.33%, when compared to PZQ which produced 97% worm reduction (Adamu *et al.*, 2006).

Various secondary metabolites have been isolated from the family *Piperaceae*, and have biologically active metabolites e.g. pyrones, terpenes, lactones, chromenes, lignoids, amides, and alkaloids (Kato and Furlan, 2007; Parmar et.al., 1997). Piperlartine, an amide found in several *Piper* species has demonstrated in vitro schistosomicidal activity. At low concentrations of 95 µm, the amide kills *S. mansoni* adult worms (male and female coupled) and the sub-lethal concentrations of 6.3 µm caused a 75% reduction in egg production. Piperlatine was not cytotoxic against mamalian cells when given at concentrations up to three times higher than the dosage needed for schistosomicidal effect (31.5 µm) (Moraes et.al., 2011).

1.2.3.2 Schitozim

Schitozim is a herbal medicine (not registered) that is sold over the counter in selected towns where there is schistosomiasis infection, i.e Coast, Kisumu and ukambani. It is used as treatment against *S. mansoni* and *S. haematobium* infections, and is given together with vitamin supplements. It is made of several plant extracts that have been packed into tablets. It is cheaper than Praziquantel, and can be found in local drug stores.

1.3 Problem statement

Schistosomiasis is one of the neglected tropical diseases, mainly because it is a disease of the poor. It is found in areas with poor sanitation, and where the source of water is polluted with waste products. This poses a challenge in developing countries due to lack of funding to deal with the problem. The communities affected are often too poor to afford PZQ, and lack the basic knowledge about the disease and its propagation around the region. PZQ is an effective drug against all schistosome parasites; however, over-reliance will no doubt increase the chances of parasites developing resistance against it.

1.4 Justification

Herbal medicines are non-toxic, locally available, and cheap (Jayashree and Maneemegalai, 2008). Therefore, Schitozim will provide an alternative to PZQ, which is currently the only drug of choice, and is under immense drug pressure and possible drug resistance. Furthermore, it is not readily available to the poor due to its high cost. Herbal medicines have been noted to be effective against their specified diseases, have little if any side effects, and have no cases of resistance. They are also easier to find since they are acquired locally (Karachi, 2006). Schitozim is an anti-schistosomal drug sold over the counter, and its low cost makes it affordable. However, in vivo testing is suggested to determine how effective it is and, thus validate the drug.

1.5 Objectives

1.5.1 General objective

To determine the effectiveness and humoral response of Schitozim, a locally available herbal medicine, against *S. mansoni* infection.

1.5.2 Specific objectives

1) To determine antischistosomal properties of Schitozim in mice experimentally infected with *S. mansoni*.
2) To determine whether Schitozim can elicit humoral responses in *S. mansoni* infected mice.
3) To assess the effects of treatment with Schitozim on the pathology caused by *S. mansoni* infection in mice.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study site

The study was carried out at the Institute of Primate Research (IPR), Nairobi. The experimental mice were obtained and maintained at the Rodent facility at Animal Science Department. The snails were obtained from irrigation canals found at Mwea, Eastern Province. They were collected and transported to IPR, and were reared and maintained at the snail laboratory throughout the experiment. Snail infection, mice infection, perfusion, gross pathology, and IgG ELISA assays were done in the Schistosomiasis laboratory at the Tropical and Infectious Disease Department (TID). Preparation and reading of histopathology slides were carried out at the Diagnostic Laboratory (IPR).

2.2 Phytochemical analysis of Schitozim

Chemical tests were carried out on the aqueous extract of the drug for the qualitative determination of phytochemical constituents as described by Harborne, 1998 and Siddiqui and Ali,1997. The various extracts were tested for terpenoids, steroids, flavonoids, saponins, tannins, glycosides and alkaloids.

2.2.1 Test for flavonoids

One gram of the drug was mixed with 10 ml of distilled water to form an aqueous extract. Two methods were used to test for flavonoids; firstly, dilute ammonia (5 ml) was added to a portion of the aqueous filtrate of the drug sample. Concentrated sulphuric acid (1 ml) was then added. A yellow coloration indicated the presence of flavonoids. Secondly, a portion of the filtrate was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was thereafter filtered and 4 ml of the filtrate shaken with 1 ml of dilute ammonia solution. A yellow coloration indicated the presence of flavonoids.

2.2.2 Test for saponins

Schitozim (0.5 g) was mixed with 20 ml of distilled water and then agitated in a graduated cylinder for 15 minutes. The formation of 1 cm layer of foam showed the presence of saponins. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

2.2.3 Test for tannins

In a test tube, 0.5 g of Schitozim powder was boiled in 10 ml of water and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

2.2.4 Test for alkaloids

Schitozim (0.5 g) was added to 5 ml of 1% HCl in a steam bath. The solution was then filtered and 1 ml of the filtrate was treated with two drops of Mayer's reagent. Turbidity of the extract filtrate on addition of Mayer's reagent was regarded as evidence for the presence of alkaloids in the extract. Secondly, 5 ml of the extract was added to 2 ml of HCl. To this acidic medium, 1 ml of Dragendroff's reagent was added. An orange or red precipitate produced immediately indicated the presence of alkaloids.

2.2.5 Test for steroids and triterpenoids

Ten milliliters (10 ml) of aqueous extract (1 g Schitozim and 10 ml water) was placed in a small beaker (50 ml) and evaporated to dryness. The residue was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. The solution was transferred into a dry test tube and concentrated solution of sulphuric acid (2 ml) added slowly. Brownish red, pink or violet rings at the zone of contact with the supernatant and blue or green color or a mixture of these two shades denoted the presence of terpenoids and steroids respectively.

2.2.6 Test for glycosides

Plant powder was put into two separate beakers in equal amounts (1 g). to one beaker, 5 ml of dilute sulphuric acid was added while 5 ml of water was added to the other beaker. The two beakers were heated for 3–5 min and the contents filtered into labeled test tubes. The filtrates were made alkaline with 5% sodium hydroxide and heated with Fehling's solution

for 3 min. The presence of a reddish precipitate in the acid filtrate and the absence of such a precipitate in the aqueous filtrate was regarded as positive for glycosides.

2.3 Collection of snails

Biomphalaria pfeifferi snails, the intermediate hosts of *S. mansoni,* were collected from water canals at Mwea irrigation scheme using scoopers. They were picked from under floating water plants on the river and placed in a basin. They were then placed on a layer of cotton wool inside a well aerated plastic container. Another layer of wet cotton wool was added when the first one was full, creating a layer-upon-layer until the container was full. A final layer of cotton wool was placed on top of the container and the snails transported to IPR Snail laboratory.

2.4 Laboratory maintenance of snails

Plastic tanks were washed in 3% hydrochloric acid, to eliminate possible contaminants, and rinsed thoroughly with chlorine free (snail water). Sand and gravel from the area where the snails were collected was sterilized by heating at 150° C for 11 hours and cooled before being placed in a layer inside the tanks. Snail water was added into the tanks until three quarter full and the snails then washed and placed into the tanks. Daphnia was added to the tanks for aeration. The temperature of the Snail lab was controlled at 28° C, with a

period of 12 hours of light and 12 hours of darkness (Yole, 1993). They were fed mainly on dried lettuce and screened for cercariae for 5 weeks.

2.5 Experimental mice

Balb/c mice of the same cohort were purchased from IPR, and maintained at the experimental unit in the Rodent House, Animal Science Department, IPR. They were fed on nutrient pellets, supplemented with kales, carrots and water *ad libitum*.

2.6 Collection of *S. mansoni* eggs and hatching miracidia

The eggs were obtained from faecal samples of chronically infected baboons (experimentally infected with *S. mansoni*) maintained at the Animal Science Department. Twenty-four hour stool was collected, placed in sealable containers and transferred to the Parasitology laboratory. The stool was thoroughly mixed with saline in a 1 liter plastic container and the suspension passed through 2 sieve meshes (sizes 600 and 250 µm) and the filtrate collected in a metal tray. The filtrate was poured into a urine jar and left to stand for 45 minutes in a dark cupboard. The supernatant was drained, and fresh saline added to the deposit, re-suspended and left to stand for another 30 minutes in the dark. This was repeated twice, and the deposit was then layered onto a petri dish containing water using a pipette. The petri dish was placed under an artificial light (20–25° C) for 30 minutes to allow the eggs to hatch into miracidia (Yole, 1993).

2.7 Infection of snails with *S. mansoni* miracidia

The petri dish containing hatched miracidia was placed onto a dissecting microscope. A glass pipette was used to pick 5-7 miracidia and transfer them into 24 wells of a culture plate. Each snail was placed inside a well and the plate covered to prevent the snails from crawling out. They were left to stand for 30 minutes. The snails were then transferred into the tanks and left under a 12 hours light/12 hours dark cycle for 3 weeks. On the 4th week, they were placed in the dark. The tanks were fully covered with dark clothes to prevent trickle shedding of cercariae by the infected snails.

2.8 Shedding of cercariae from infected snails

On the 5th week post-infection, the snails were removed from the dark and placed in 10 ml beakers with snail water. The beakers were then exposed to light (100 watts lamp) shaded with glass to release cercariae. The cercariae suspension was pooled in a 100 ml beaker and mixed well. Three aliquots of 50 µl suspension were drawn into a petri dish, 3 drops of iodine was added, and the cercariae were viewed under a dissecting microscope and enumerated. An average volume containing 250 cercariae was determined (Yole, 1993).

2.9 Infection of mice with *S. mansoni* cercariae

The mice were exposed to cercariae using the ring method (Smithers and Terry, 1965). They were first anaesthetized by injecting 0.02 ml of Ketamine/Rompun mixture (ratio of

7:3) intraperitoneally. The sedated mice were shaved on the stomach area and the area was made wet using a wet cotton wool. This was to allow easy penetration of the cercariae. A metal ring of 1 cm in diameter was placed on the shaved area and a suspension with 250 whole cercariae was dispensed using a micropipette into the metal ring, then left in position for at least 30 minutes to allow the cercariae to penetrate. The mice were maintained under the natural light/ dark cycle of 12/12 hours, at ambient temperature of about 20° C and relative humidity of 50 – 60% at the Rodent House and were fed on commercial pellets and water *ad labium*.

2.10 Experimental design

Sixty-four mice were obtained for the study from the Animal Science Center. There were 6 experimental groups, 5 of them having 12 mice each, and the naïve group had 4 mice. Twelve mice were randomly assigned to each group except naïve group. They were marked using picric acid to differentiate them. On the first day, the first 5 groups of mice were infected with *S. mansoni* cercariae. On the 4th week, the three experimental groups and PZQ group were treated. On the 6th week, blood samples were obtained from each mouse and the mice were also perfused for worm recovery. The experimental design is depicted on Table 1.

Table 2: Experimental design schedule

GROUP	TREATMENT	WEEK 0	WEEK 4	WEEK 6
1	PZQ	I	T	S, C, P
2	Schitozim 50 mg	I	T	S, C, P
3	Schitozim 150 mg	I	T	S, C, P
4	Schitozim 300 mg	I	T	S, C, P
5	IC	I	-	S, C, P
6	NC	-	-	S, C

KEY:

PZQ: Praziquantel
I: Infections
Schitozim: Herbal medicine

T: Treatment
IC: Infected control
S: Sampling for serum

NC: Naïve control
P: Perfusion
C: Sampling for cells

2.11 Treatment of mice with Praziquantel and Schitozim

A dozing needle was used to administer all treatments. There were three dosages of the herbal medicine (Schitozim): 50 mg, 150 mg, and 300 mg suspended in 200 µl of distilled water for each mouse. It was then thoroughly mixed on a plastic plate using an applicator stick, sucked into the dozing needle and administered to the mouse. These regimes were given on alternate days, for five days in total. The positive control group was treated with PZQ; two oral doses of 900 mg/Kg in 200 µl suspension on two alternate days (Muchirah *et al*., 2012).

2.12 Collection of blood from mice

The mice were first anaesthetized using Ketamine/Rompun mixture as described in section 2.9. A small incision was made at the center of the abdominal skin, and the skin was torn around the "waist" of the mouse. The skin was peeled upwards and downwards to expose the thoracic region. An incision was made on the abdominal wall, which was then cut horizontally from the thorax to the groin and vertically on either side, taking caution not to puncture the lungs, heart or the two main blood vessels on the sternum. The ribs were trimmed on either side of the sternum to expose the heart. A 1 ml syringe was inserted on the right ventricle of the heart, and blood was sucked by small jerks to create a vacuum and prevent the heart from collapsing. The whole volume of blood collected (approximately 1 ml) was dispensed into a 1.5 Eppendorf tube and left to stand at room temperature to clot, and then stored in the refrigerator (4°C) overnight.

2.13 Perfusion of mice and adult worm recovery

After bleeding, the mouse was placed on the left palm of the hand, with the back of the mouse lying on the palm. The hepatic portal vein was located and incised. The cut area was quickly flushed with perfusion fluid to remove any worms. A 21G perfusion needle containing perfusion fluid (0.85% Sodium Chloride and 1.5% Sodium Citrate) was inserted on the left ventricle of the heart. The mouse was turned up-side-down with the intestines hanging above a glass petri dish, then the perfusion pump was turned on and perfusion was carried out until the liver, and mesenteries were clear. The intestines were explored for any trapped worms using a sharp pair of forceps. The mouse was placed on a petri dish, intestines down, and soaked in perfusion fluid so that any worms that were still trapped in the vessels would creep out. The perfusate collected was transferred to a urine jar and allowed to settle then topped with Phosphate Buffered Saline (PBS). The blood cells in the perfusate were lysed using Haemolyser DL-1 (Erma Inc. Japan). After the worms had settled, the supernatant was sucked out, and PBS was added and the worms, then re-suspended and allowed to settle again. Washing was repeated three times using PBS after which the worms were counted and the mean and standard error (±S.E.) was worked out for each group.

2.14 Preparation of serum

The clotted blood from the refrigerator was centrifuged at 7000 rpm for 10 minutes. The clear supernatant, serum, was transferred to a sterile Nunc tube and properly labeled, then stored at 20°C until use.

2.15 Preparation of 0-3 hour release protein (0-3 Hr RAP)

S. mansoni cercariae were obtained by shedding infected snails with a patent infection of five weeks. Heads and tails of *S. mansoni* cercariae were separated and isolated on a discontinuous percoll gradient (Ramalho-Pinto *et al*., 1974), and washed twice in RPMI 1640 media (supplemented with 10% foetal calf serum (BDSL, Kilmarnock UK), 0.1% gentamycin AND 5 x 10^{-5}) *β*- mercaptoethanol (Ladzins *et al*., 1982). The schistosomule heads were transferred to bijou tubes and incubated at 37ºC, 5% CO_2 . After 3 hour of incubation, the suspension was centrifuged (10 minutes at 450 g, at 37º C) to obtain a supernatant containing the proteins released by penetrating shistosomula between 0-3 hours of penetration. Protein concentration was assayed as described below.

The concentration of the protein was assayed using the Bradford method. The optical densities of the standards serial diluted BSA were automatically plotted against their concentrations. The optical densities of different dilutions of the SSP were obtained at the same wavelength as BSA dilutions. The concentration of SSP dilutions was then calculated using the equation of the curve given by the spectrometer (Cell Double Beam

Spectrophotometer, CE 6600R). The protein was aliquoted and sterilized by exposure to UV light (10 minutes, 5 cm from a 30 watt ultraviolet OSRAM bulb) before use in *in-vitro* assays. Aliquots were stored at -20ºC and the protein concentration adjusted to 1 mg/ml before use.

2.16 Preparation of *S. mansoni* soluble worm antigen preparation (SWAP)

S. mansoni worms were obtained by perfusion of BALB/c and Swiss mice with a five week worm infection. The worms were washed in PBS, and then sonicated (23 khz, 16 μm amplitude, for 10 minutes) and the homogenate was centrifuged for 1 h at 10,000 g, 4΄ΙC to obtain the soluble protein. The protein estimation, aliquoting and sterilization were done as for SSP and the protein concentration adjusted to 1 mg/ml in complete medium before use.

2.17 Preparation of *S. mansoni* Soluble Egg Antigen (SEA)

Eggs were collected from liver tissues of infected baboon and purified. They were then suspended in 4°C PBS at a concentration of 100,000 eggs/ml. The eggs were then homogenized on ice using the pre-chilled homogenizer, using a tight pestle. During the process, which took 10-15 minutes (depending on the number of plunging cycles and the force used), a small sample was withdrawn and examined using a dissecting microscope. A crude determination was made of the percentage of eggs that had been broken apart. Both

empty egg shells and intact eggs were easily distinguished. When approximately 95% (or more) of the eggs had been disrupted, the crude mixture was centrifuged at 4°C at 200 xg for 20 minutes. The supernatant was collected and centrifuged for 90 minutes at 100,000g at 4°C. Protein assay and concentration were carried out as for SSP. The supernatant was sterilized by passing it through a 0.2 µm filter and stored at -20° C.

2.18 ELISA for Schistosome Specific IgG

The antigens used in the experiment were Soluble Worm Antigen Preparation (SWAP), Soluble Egg Antigen (SEA), and 0-3 hour Release Antigen Preparation (0-3 Hr RAP). Each well of Nunc-Immuno^{TM} plates (MaxiSorp^{TM} Surface) ELISA plates were coated with 50 µl of 10 µg/ml of the respective antigen (diluted in carbonate/bicarbonate buffer, pH 9.6), one plate per antigen, and incubated overnight at 4°C. The antigen was dispensed on a blotting paper, and blocking of non-specific binding sites with 100 µl of 3% BSA in PBS/Tween 20 (0.05%) was done. The plate was then incubated overnight at 4°C, and washed 6 times with PBS/Tween 20 (0.05%). Test serum samples were diluted in 0.5% BSA/PBS/Tween at a dilution of 1:200. The blank (control) was 0.5% BSA/PBS/tween. Fifty microlitres of the diluted serum samples and blanks were added in duplicates to the plate and incubated for 2 hours at 37°C. The plate was then washed 6 times as above. Fifty microlitres of 1:2000 peroxidase conjugated goat anti-mouse IgG horse radish peroxidase (HRP) was added into the wells and incubated for 1 hour at 37°C. The unbound conjugate was washed off , and 50 µl TMB microwell peroxide substrate (Kirkguard and Perring

laboratories, USA) was added. The plates were incubated at 37°C in the dark for 30 minutes and optical density readings obtained at 630 nm using an ELISA microplate reader (Molecular Devices, Palo Alto, England).

2.19 Pathology

2.19.1 Gross pathology

The mice were opened up and observed before perfusion and the size of the liver, as well as presence and number of granulomas recorded. Based on the number of granulomas observed, the granulomas were categorized as either few (1 – 3), moderate (4 – 10) or severe (> 10 granulomas). Inflammation of the liver was categorized as: slightly inflamed, and inflamed, whereas adhesions were recorded as either present or absent.

2.19.2 Histopathology

Liver and mesenteric lymph nodes were fixed in 10% formalin and left to stand for 2 weeks. The tissues were cut widthwise into small pieces of 1-2 mm using a scalpel blade and placed into a tissue processing capsule (cassette). The tissues were dehydrated in ethanol, cleared (removing alcohol from the tissues) in toluene, infiltrated in hot paraffin and embedded on tissue-embedding paraffin wax. The tissue blocks were trimmed using a Rotary microtome, then stored at 4°C before sectioning at 6 microns (Leitz, Germany). The sectioned tissues were placed onto the water bath using a camel hair brush, then

picked onto a new slide and left to air dry before drying in an oven at 50-60°C to melt the excess wax for 1 hour. The slides were left to cool and then stained using Hematoxylin and Eosin stain and then left in xylene overnight. Cover slips were mounted using Distyrene Plasticizer Xylene (DPX), left to dry for about 1 h, and observed under light microscope.

For the liver samples, the sizes of the granulomas containing an ovum only were measured. Horizontal and vertical diameters of granulomas with a centrally placed egg were measured using an ocular micrometer, and the average taken (Farah *et al*., 2000).

In the lymph nodes, the severity of cellular reaction was determined. It was measured at a scale of 1–6 where 1 stood for few cellular reaction, and 6 for severe cellular reaction.

2.20 Data Management and Analysis

Analysis of data was performed using the Student t test for the comparison of two paired data. This was done using Excel. ANOVA was used for the comparison of in comparing data involving more than two groups. Chi square was used to analyse nominal data, and Kruskal-Wallis for ranked data. Significant levels were defined as P value of < 0.05. Pathology was noted by visual observation of tissues.

CHAPTER THREE

RESULTS

3.1. Qualitative analysis of the crude extracts

Phytochemical analysis of the aqueous extract of Schitozim revealed the presence of tannins, steroids, flavonoids, glycosides and saponins, but alkaloids and terpenoids were not detected in the aqueous extract (Table 2).

Table 3: Secondary phytochemicals in the aqueous extracts of Schitozim

Extract	Alkaloids	Tannins	Saponins	Glycosides	Terpenoids	Steroids	Flavonoids
Aqueous	ND	+	+	+	ND	+	+

KEY: Phytochemical detected (+);

Phytochemical not detected (ND)

3.2. Worm Maturation

Worm maturation was calculated as described by the method of Yole *et al*., (1996):

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

Worm maturation was 24.4% which is about a quarter of the total parasite used in infection.

3.3. Worm Recovery

The mean numbers of worms recovered from IC (61 ± 15), PZQ (22 ± 3), 50 mg (33 ± 4), 150 mg (41 ± 9), and 300 mg (24 ± 2) are shown in Table 3. IC was significantly different from PZQ (0.014) and 300 mg (0.021), but not significantly different from 50 mg (0.099) and 150 mg (0.319) at P value of < 0.05. There were no significant differences between PZQ and 50 mg, PZQ and 150 mg, and PZQ and 300 mg.

Percentage worm recovery was calculated as follows:

$$\% \text{ Worm recovery} = \frac{\text{Mean of total worms in experimental group}}{\text{Mean of total worms in infected control}} \times 100\%$$

The percentages of worm recoveries were: PZQ - 36.07%, 50 mg - 54.1%, 150 mg - 67.21%, and 300 mg - 39.34%.

Percentage worm reduction was calculated as follows:

$$\% \text{ Worm reduction} = \frac{\text{Mean of total worms in IC - Mean of total worms in experimental group}}{\text{Mean of total worms in IC}} \times 100\%$$

The percentages of worm reduction in PZQ (63.93%), 50 mg (45.9%), 150 mg (32.79%), and 300 mg (60.66%) are shown in Table 3. There were significant differences between IC and 300 mg (0.021), but no significant differences between IC and 50 mg (0.099), and IC

and 150 mg (0.319). There were also no significant differences between PZQ and 300 mg (0.999), PZQ and 50 mg (0.782), and PZQ and 150 mg (0.362).

Treatment Groups	D1Schitozim (50mg/kg)	D2 Schitozim (150mg/kg)	D3 Schitozim (300mg/kg)	PZQ Control	Infected control
(mean ± SE) of Total worm	33 ± 4	41 ± 9	24 ± 2	22 ± 3	61 ± 15
% Worm Recovery	54.1	67.21	39.34	36.07	
% Worm Reduction	45.9	32.79	60.66	63.93	

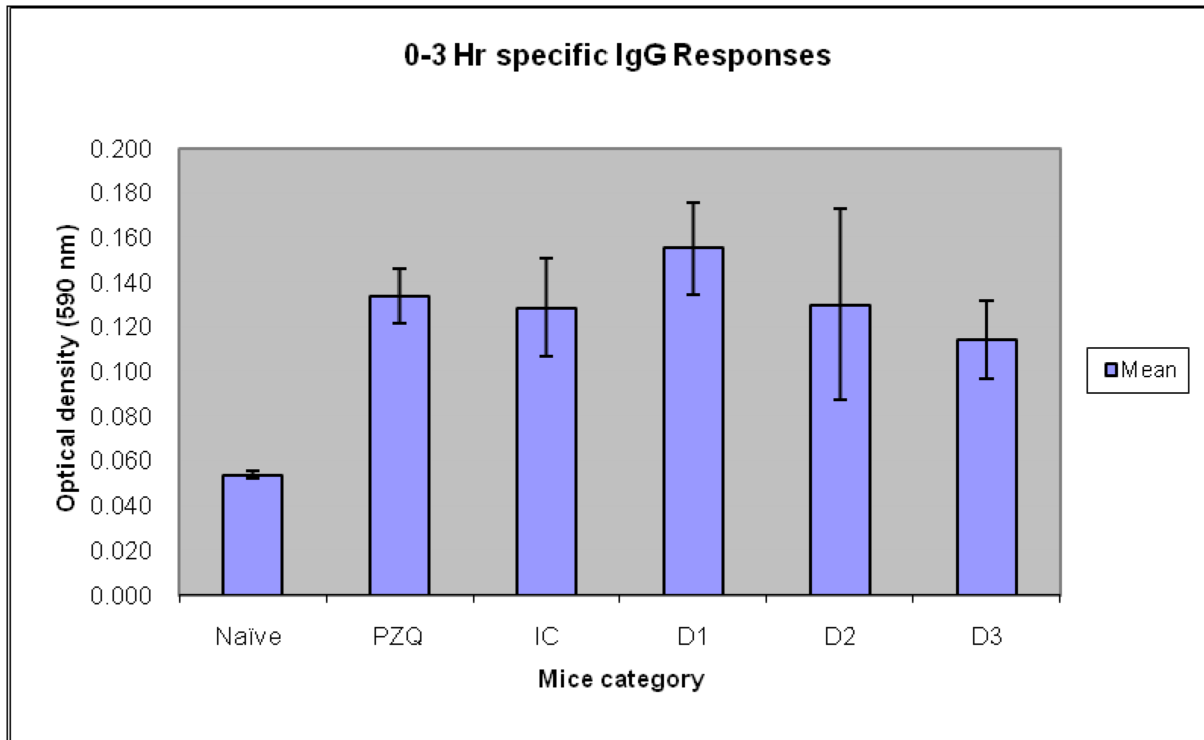
Table 4: Mean (± SE) Number of worms recovered from experimental groups.

3.4 Antibody response to schistosome antigen

3.4.1 Specific IgG response to 0-3 hr Release Protein Antigen

The IgG levels in response to 0-3 Hr RPA are shown in Figure 2. Naïve (0.054 ± 0) had the lowest immunological response, followed by 300 mg (0.115 ± 0.02), IC (0.129 ± 0.02), 150 mg (0.13 ± 0.04), PZQ (134 ± 0.01), and the highest IgG response was 50 mg (0.155 ± 0.02), $p < 0.05$.

Naïve was significantly different from all the groups ($p < 0.05$). There were no significant differences between IC and PZQ, IC and the Schitozim groups, PZQ and the Schitozim groups, and among the Schitozim groups, (Appendix 1).



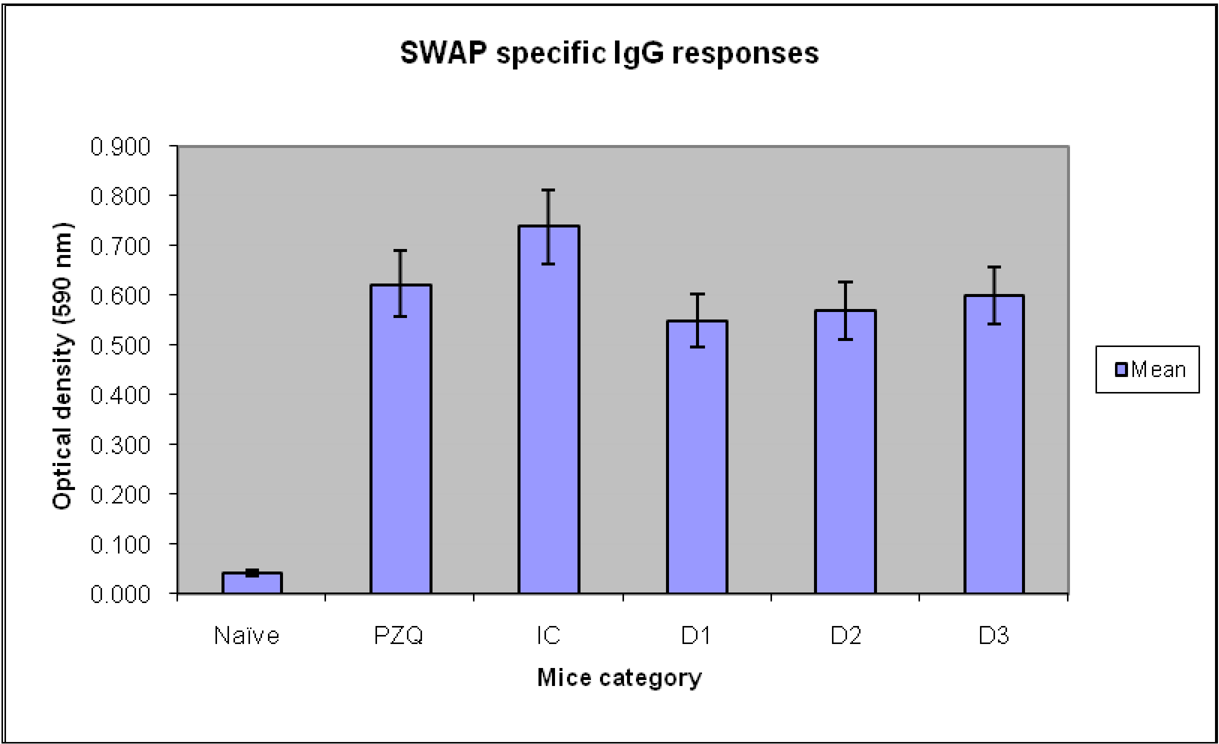
Mean (± SE)

Figure 2: 0 – 3 hour Antigen specific IgG Response

3.4.2 Specific IgG response to SWAP Antigen

The levels of IgG in response to SWAP antigen are shown in Figure 3. The trend was as follows: Naive (0.041 ± 0) had the lowest followed by 50 mg (0.549 ± 0.053), then 150 mg (0.569 ± 0.058), 300 mg (0.599 ± 0.057), and PZQ (0.623 ± 0.067), while the highest IgG response was observed in IC (0.739 ± 0.074).

Naïve was significantly different from all the other ($p < 0.05$). There were no significant differences between IC and PZQ, and IC and 300 mg, but there were significant differences between IC and 50 mg, and IC and 150 mg. PZQ was also compared to the Schitozim groups, and showed no significant difference with any of them. There were no significant differences observed between 300 mg and 150 mg, 300 mg and 50 mg, and 150 mg and 50 mg, (Appendix 2).



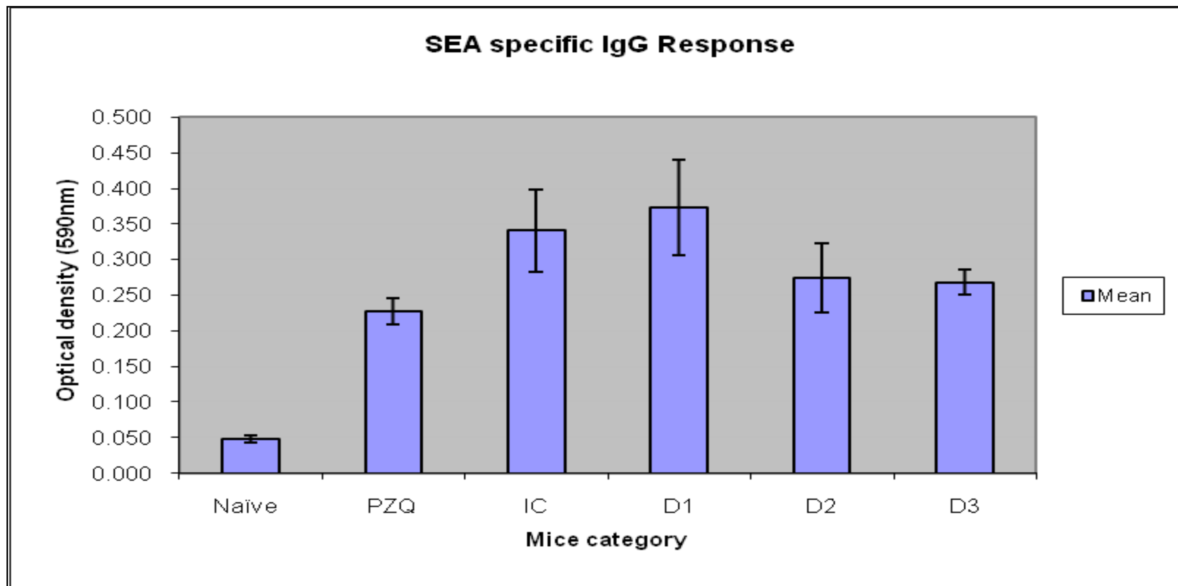
Mean (± SE)

Figure 3: Specific IgG responses to SWAP antigen

3.4.3 Specific IgG responses to SEA

The IgG levels for SEA are shown in Figure 4. Naive (0.048 ± 0) had the lowest levels, followed by PZQ (0.227 ± 0.018), then 300 mg (0.268 ± 0.018), 150 mg (0.274 ± 0.049), and IC (0.34 ± 0.058) while the highest IgG levels were observed in 50 mg (0.373 ± 0.067).

There were significant differences between Naïve and all the groups ($p < 0.05$). There were no significant differences when IC was compared to PZQ, 50 mg, 150 mg and 300 mg, and no significant difference when the Schitozim groups were compared among themselves. PZQ was significantly different from 50 mg, but not significantly different from 150 mg and 300 mg, (Appendix 3).



Mean (± SE)

Figure 4: Specific IgG responses to SEA

Of the three IgG responses in all the treatments, the SWAP specific IgG response was highest (response ranged from 0.7388 to 0.0415) followed by SEA specific IgG response whose response ranged from 0.366 to 0.0485 while the least IgG response was observed in the 0-3 hrs release protein antigen (response ranged from 0.1556 to 0.0545). In the three schistosome-specific responses, the least IgG responses were observed in the Naïve groups. This is shown in Figure 5 below.

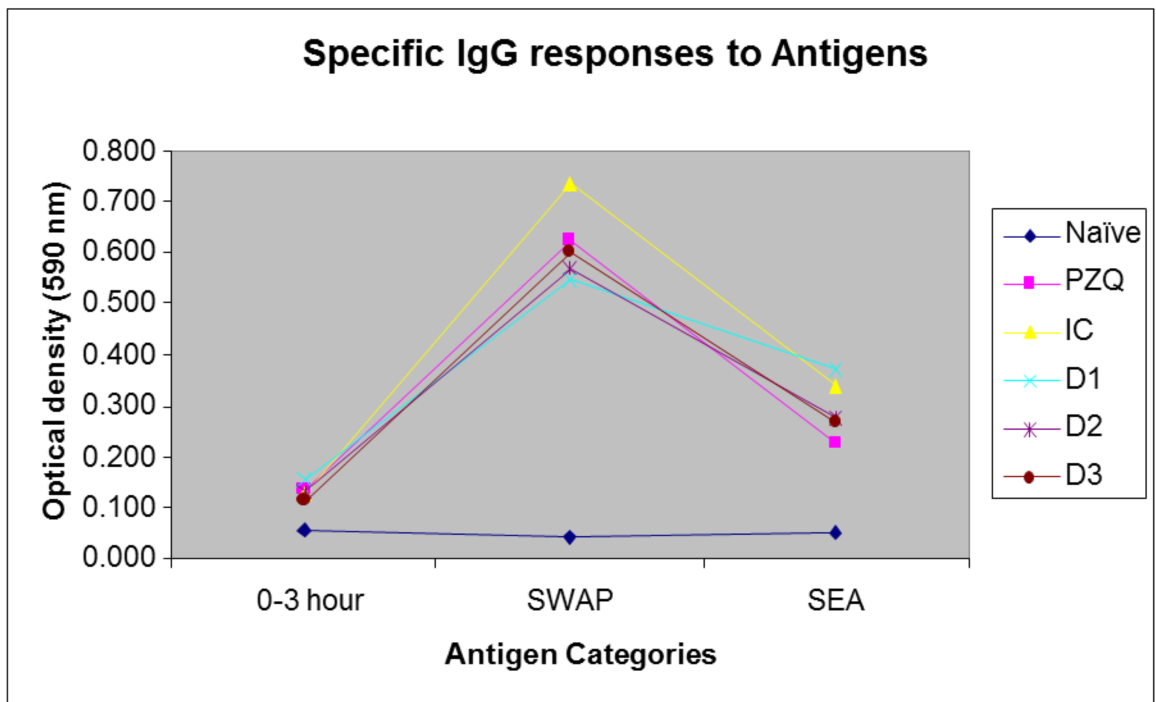


Figure 5: Specific IgG responses to various antigens.

3.5 Pathology results

3.5.1 Liver gross pathology

Gross pathology observations were done on week 6 post infection by physical observation of the liver to detect presence of granulomas, inflammation and adhesions. The results are shown in Table 4. Mice treated with PZQ and 50 mg had the least adhesions having only one mouse each with adhesion; 150 mg had two mice; 300 mg had three and IC had four mice with adhesions. There was slight inflammation of the liver observed in one mouse in the PZQ group, two mice in 50 mg, two in 300 mg, and two in IC. Very inflamed livers were observed in one mouse in 50 mg, two mice in 300 mg, four mice in 150 mg, and four mice in IC. PZQ had two mice with few granulomas; 50 mg had two mice with few granulomas, and three mice with moderate granulomas; 150 mg had one mouse with few granulomas, two mice with moderate granulomas, and two mice with severe granulomas; 300 mg had three mice with few granulomas, two mice with moderate granulomas, and 1 mouse with severe granulomas; and finally IC had two mice with few granulomas, two mice with moderate granulomas, and two mice with severe granulomas.

Chi square analysis on adhesions showed there was signicant difference between PZQ and 300mg dosage, whereas IC was significantly different from 50 mg dosage. Analysis on the inflammation and granulomas showed that both PZQ and IC were significantly different from the Schitozim groups, and from each other.

Treatment Group		Liver		
		Adhesions	Inflammation	Granuloma
PZQ	M			
	M			
	M		S	+
	F			
	F			
	F	*		+
IC	M	*	E	++
	M	*	E	+++
	M	*	S	+
	F	*	E	+++
	F		E	++
	F		S	+
50 mg	M		S	++
	M			+
	M	*	S	++
	F		E	++
	F			
	F			+
150 mg	M		E	++
	M		E	++
	M			+
	F			
	F	*	E	+++
	F	*	E	+++
300 mg	M	*		+
	M	*	E	++
	M	*	E	+++
	F		S	+
	F		S	+
	F			++

Table 5: Gross pathology of the liver

KEY: M – male **F** – female ***** - presence of adhesions

E – very inflamed **S** – slightly inflamed + - few granulomas

++ - moderate granulomas +++ - severe granulomas

3.5.2 Histopathology of the liver

The vertical and horizontal length of granulomas with a centralized egg or egg without a granulomatous reaction was recorded and the mean measurements calculated. PZQ had one granuloma with a mean size of 2.5, 50 mg had17 granulomas (4.67 ± 0.57), 150 mg had 24 granulomas (5.4 ± 0.45), 300 mg had 22 granulomas (4.74 ± 0.26), and IC had 22 granulomas (5.8 ± 0.27). PZQ was significantly different from all the other groups ($p < 0.001$). There was significant difference between IC and 50 mg, and IC and 300 mg, but there was no significant difference between IC and 150 mg ($p > 0.05$). There was no significant difference between 50 mg and 150 mg, 50 mg and 300 mg, and 150 mg and 300 mg ($p < 0.05$), (Appendix 4).

Figure 6 shows a normal liver without any granulomatous reaction. Figure 7 shows a liver which has an egg that has elicited granulomatous reaction, and a modulating granuloma in which the egg has been destroyed.

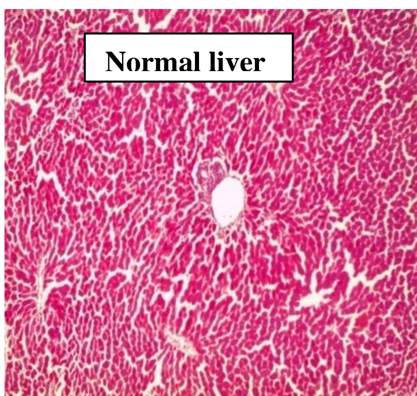


Fig. 6: Normal liver

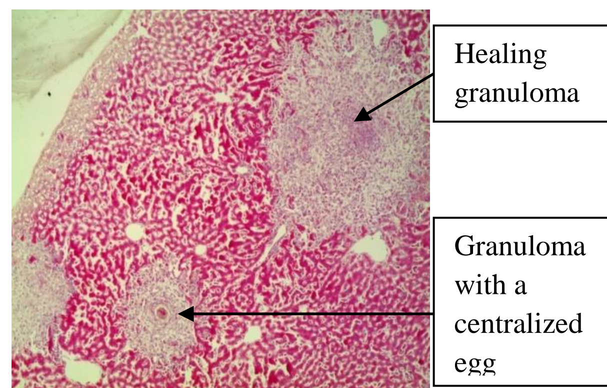


Fig. 7: Liver with granulomas

Key: Fig. - Figure

3.5.3 Histopathology of the mesenteric lymph nodes

Cellular reaction in the mesenteric lymph nodes was categorized depending on the number of reactive germinal centers: few cell reaction (1 - 3 proliferated germinal centers), moderate cell reaction (4 - 7 proliferated germinal centers) and severe cell reaction (more than 7 proliferated germinal centers). This is shown in Table 6. Mesenteric lymph nodes with the most active germinal centers were those of 300 mg, whereas those with the least active germinal centers were those of PZQ. When comparing which had the most active germinal centers to which had the least active, the trend was as follows: 300 mg – 1 few, 1 moderate, and 4 severe; 150 mg – 1 few, 4 moderate, 1 severe; 50 mg – 2 few, 3 moderate, and 1 severe; IC – 3 few and 3 moderate; and finally PZQ – 5 few and 1 severe. PZQ was significantly different from the Schitozim dosages, thus, none was comparable to PZQ.

Figure 8 shows an illustration of a normal lymph node. Figure 9 shows a lymph node with few cellular reactions. The germinal centers are clear and countable. Figure 10 shows a lymph node with moderate cellular reaction. The germinal centers are not clear, but the reactions are concentrated on a portion of the lymph node, creating a solid pattern. Figure 11 shows a lymph node with severe cellular reaction. The reactions are observed on the entire lymph node.

Treatment groups	**Sex of mouse**	**Mesenteric Lymph nodes**	
		Intensity of reaction of Germinal centers	**Category of reaction**
PZQ	Ma	**1**	**F**
	Ma	**1**	**F**
	Ma	**5**	**S**
	Fe	**1**	**F**
	Fe	**2**	**F**
	Fe	**2**	**F**
IC	Ma	**4**	**M**
	Ma	**4**	**M**
	Ma	**2**	**F**
	Fe	**3**	**M**
	Fe	**2**	**F**
	Fe	**2**	**F**
D1	Ma	**2**	**F**
	Ma	**3**	**M**
	Ma	**3**	**M**
	Fe	**2**	**F**
	Fe	**5**	**S**
	Fe	**3**	**M**
D2	Ma	**4**	**M**
	Ma	**3**	**M**
	Ma	**2**	**F**
	Fe	**5**	**S**
	Fe	**4**	**M**
	Fe	**4**	**M**
D3	Ma	**1**	**F**
	Ma	**5**	**S**
	Ma	**3**	**M**
	Fe	**6**	**S**
	Fe	**5**	**S**
	Fe	**5**	**S**

Table 6: Histopathology table

Key: Ma – male; **Fe** – female;

Category: 1 – 2 few cellular reaction (**F**); 3**-4** moderate cellular reaction (**M**);

5-6 severe cellular reaction (**S**)

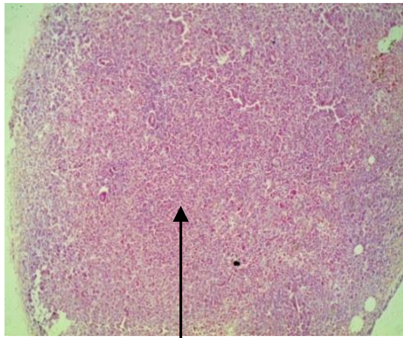


Fig. 8: Normal lymph node

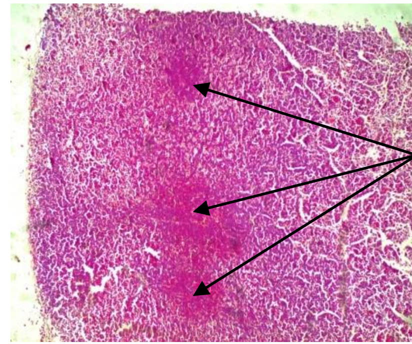


Fig. 9: L. N. with few cellular rxn

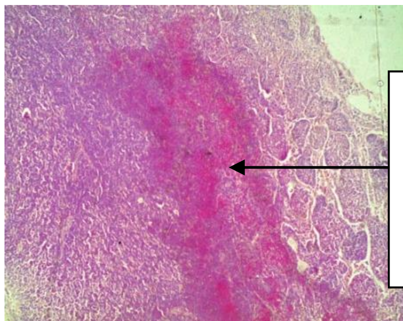


Fig. 10: L.N. with moderate cellular rxn

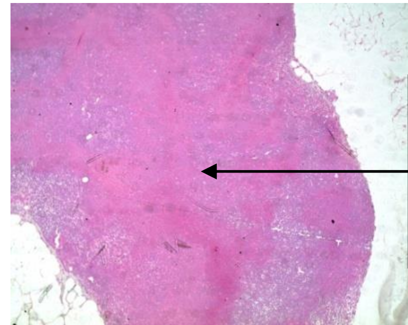


Fig. 11: L.N. with severe cellular rxn

Key: L. N. – lymph node **Rxn** – reaction **Fig.** - Figure

CHAPTER 4

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 DISCUSSIONS

Schitozim is sold in local chemists in the coast region of Kenya for treatment of schistosomiasis. This study was focussed on evaluating the effectiveness of Schitozim against *Schistosoma mansoni* infection in Balb/c mice. Phytochemical screening was done to test for the presence of active secondary metabolites, percentage worm reduction was calculated, ELISA assays were done to test for specific humoral responses, and gross and histopathology on the liver and lymph nodes were done to determine the effectiveness of Schitozim on pathology caused by *Schistosoma mansoni* infection.

Aqueous extracts of Schitozim used in the experiment were screened for the presence or absence of various classes of secondary metabolites.

Worm maturation was seen to be 24.4% , a quarter of the total parasite used in infection. In a mouse model system, only about 20% of initial cercarialinoculum makes it to the adult stage (WHO, 2002). The greatest loss of larval stages occurs during the migration through the lungs with relatively smaller losses during migration through the skin (WHO, 2002). Thus, the results obtained were within the range of percentage worm maturation recorded by WHO. A high number of worms was recorded in the IC group because most of the worms had matured and migrated to the mesenteries and were recovered during perfusion without any drug induced destruction. When drugs are normally administered, increase in

dosage generally decreased the number of worms recovered (Muchirah *et al*., 2012). This is the trend observed in the three Schitozim treatment groups.

The significant low worm recovery and more worm reduction in the PZQ group were due to the action of the drug that destroyed most of the worms. Though the mechanism of action of Schitozim is not yet known, it is evident that it acts on the parasite by directly killing it. According to Muchirah *et al*. 2012, IC recorded high number of worms because most worms matured and migrated to the mesenteries and were recovered during perfusion without any drug induced destruction.

Naïve had the lowest responses in all the three IgG responses. The background immunological responses could have been due to environmental antigens similar to schistosomes. The high IgG responses of IC can be explained by the fact that this group has a large number of worms releasing large number of antigens into the mouse circulation. This leads to the humoral response observed. Despite PZQ having fewer worms (less that 1/3 of IC), yet for all the antigens, its IgG response is similar to IC. This was probably due to PZQ inducing an immune response which resulted in the high IgG responses. This implies that PZQ was killing the worms directly, and also via immune system involvement. Other authors have recorded similar findings (Andrew, 1985; Fallon and Doenhoff, 1995: Woolhouse and Hagan, 1999; Mutapi, 2001; Muchirah *et al*., 2012). The three doses of Schitozim had worm counts as low as PZQ. It is probable that they were

also inducing an immune response that led to high IgG responses. Therefore, Schitozim probably was destroying the worms directly and also via immune system involvement.

The antibody levels of specific IgG responses to 0-3 Hr RPA were the lowest since the schistosomula stage is vulnerable to host immunity (Terry, 1994; El-Ridi *et al*., 2001). Furthermore, at week 7 there were a low number of specific antibodies reserved against the antigens, and the interaction of the host's immune system against the schistosomules was quite brief; it lasted for about 24 hours at this stage. The antibody levels of specific IgG responses to SWAP antigens were highest because the experiment was terminated at 7 weeks post-infection, which by then the parasites had fully developed into adults. Thus the immune system had produced large amounts of specific antibodies to fight against the adult worms. The levels of IgG responses to SEA was higher in 0-3 Hr RPA, but lower than SWAP because at the time the experiment was terminated, the egg production by the adult worms had just commenced. Therefore, although there were immunological reactions against the eggs, these had not yet reached optimal level.

In this study, PZQ was the best dosage for reducing egg associated pathology, followed by 50 mg, then 300 mg. This shows that the two treatments were able to destroy *Schistosoma* egg associated pathology. However, 150 mg was not as effective.

In an experiment conducted by Muchirah *et al*., (2012), PZQ (900 mg dose) had no adhesions, the livers were slightly inflamed, and there were no granulomas. IC had adhesions, and the livers were severely inflamed. Few and moderate granulomas were recorded. Therefore the results obtained on PZQ and IC in this experiment was comparable

to Muchirah's experiment. Histopathology results recorded normal liver tissues which indicated that the treatment was able to destroy Schistosoma egg associated pathology.

4.2 CONCLUSION

Flavonoids, glycoside, saponins, steroids and tannins are secondary metabolites present in Schitozim aqueous extract. They have been proven to be constituents with biological activity that are effective against a number of infections. The results of the study showed that PZQ had the greatest effect on worm reduction, worm recovery, IgG specific immunological response, pathology on liver tissue and mesenteric lymph nodes compared to other groups. IC had the highest worm recovery, IgG specific immunological response, and the worst pathology on liver tissue. However it had less active germinal centers in the mesenteric lymph nodes when compared to the three Schitozim groups. Of the three Schitozim groups, 300 mg/ml had the best worm reduction of the three treatment groups. Although the IgG responses were similar in the three treatment groups, 300 mg/ml was the most similar to PZQ, and therefore, had the best results. In pathology, 50 mg/ml had the best results in the gross pathology and histopathology of the liver. In the histopathology of the lymph nodes, 300 mg/ml had the most active germinal centers, whereas 50 mg/ml had the least active germinal centers. Therefore the most effective dosage and most comparable to PZQ is that of 50 mg/ml. From the overall results given, I can therefore conclude that Schitozim has similar anti-schistosomal qualities as Praziquantel in the management of Schistosomiasis. The study suggests the use Schitozim in the management of

Schistosomiasis is justifiable. Furthermore, as it is locally available and affordable it makes it a great alternative treatment against Schistosomiasis. However, it is recommended that further tests be carried out to establish the MIC and better ascertain Schitozim's drug properties.

4.3 RECOMMENDATIONS

1. Further research is needed to characterize Schitozim and determine their mode of action.

2. Studies similar to this are needed which will involve the use of lower dosage levels to determine the most effective dose, its toxicity level, and the possible side effects of the drug when administered at different dosages.

3. Further research should also be done on *S. haematobium* using the same drug.

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APPENDICES

Appendix 1: LSD analysis of 0-3 hrs release protein IgG response

Absorbance

LSD

(I) Treatments	(J) Treatments	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PZQ	50 mg	-.02133	.03257	.517	-.0878	.0452
	150 mg	.00383	.03257	.907	-.0627	.0703
	300 mg	.01958	.03257	.552	-.0469	.0861
	Naive	.07975*	.03257	.020	.0132	.1463
	IC	.00550	.03257	.867	-.0610	.0720
50 mg	PZQ	.02133	.03257	.517	-.0452	.0878
	150 mg	.02517	.03257	.446	-.0413	.0917
	300 mg	.04092	.03257	.219	-.0256	.1074
	Naive	.10108*	.03257	.004	.0346	.1676
	IC	.02683	.03257	.416	-.0397	.0933
150 mg	PZQ	-.00383	.03257	.907	-.0703	.0627
	50 mg	-.02517	.03257	.446	-.0917	.0413
	300 mg	.01575	.03257	.632	-.0508	.0823
	Naive	.07592*	.03257	.027	.0094	.1424
	IC	.00167	.03257	.960	-.0648	.0682
300 mg	PZQ	-.01958	.03257	.552	-.0861	.0469
	50 mg	-.04092	.03257	.219	-.1074	.0256
	150 mg	-.01575	.03257	.632	-.0823	.0508
	Naive	.06017	.03257	.075	-.0063	.1267

	IC	-.01408	.03257	.669	-.0806	.0524
Naive	PZQ	-.07975*	.03257	.020	-.1463	-.0132
	50 mg	-.10108*	.03257	.004	-.1676	-.0346
	150 mg	-.07592*	.03257	.027	-.1424	-.0094
	300 mg	-.06017	.03257	.075	-.1267	.0063
	IC	-.07425*	.03257	.030	-.1408	-.0077
IC	PZQ	-.00550	.03257	.867	-.0720	.0610
	50 mg	-.02683	.03257	.416	-.0933	.0397
	150 mg	-.00167	.03257	.960	-.0682	.0648
	300 mg	.01408	.03257	.669	-.0524	.0806
	Naive	.07425*	.03257	.030	.0077	.1408

*. The mean difference is significant at the 0.05 level.

Appendix 2: LSD analysis of SWAP IgG response

Absorbance

LSD

(I) Treatments	(J) Treatments	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PZQ	50 mg	.07392	.08052	.366	-.0905	.2384
	150 mg	.05358	.08052	.511	-.1109	.2180
	300 mg	.02350	.08052	.772	-.1410	.1880
	Naive	.58125*	.08052	.000	.4168	.7457
	IC	-.11608	.08052	.160	-.2805	.0484
50 mg	PZQ	-.07392	.08052	.366	-.2384	.0905

	150 mg	-.02033	.08052	.802	-.1848	.1441
	300 mg	-.05042	.08052	.536	-.2149	.1140
	Naive	.50733*	.08052	.000	.3429	.6718
	IC	-.19000*	.08052	.025	-.3545	-.0255
150 mg	PZQ	-.05358	.08052	.511	-.2180	.1109
	50 mg	.02033	.08052	.802	-.1441	.1848
	300 mg	-.03008	.08052	.711	-.1945	.1344
	Naive	.52767*	.08052	.000	.3632	.6921
	IC	-.16967*	.08052	.044	-.3341	-.0052
300 mg	PZQ	-.02350	.08052	.772	-.1880	.1410
	50 mg	.05042	.08052	.536	-.1140	.2149
	150 mg	.03008	.08052	.711	-.1344	.1945
	Naive	.55775*	.08052	.000	.3933	.7222
	IC	-.13958	.08052	.093	-.3040	.0249
Naive	PZQ	-.58125*	.08052	.000	-.7457	-.4168
	50 mg	-.50733*	.08052	.000	-.6718	-.3429
	150 mg	-.52767*	.08052	.000	-.6921	-.3632
	300 mg	-.55775*	.08052	.000	-.7222	-.3933
	IC	-.69733*	.08052	.000	-.8618	-.5329
IC	PZQ	.11608	.08052	.160	-.0484	.2805
	50 mg	.19000*	.08052	.025	.0255	.3545
	150 mg	.16967*	.08052	.044	.0052	.3341
	300 mg	.13958	.08052	.093	-.0249	.3040
	Naive	.69733*	.08052	.000	.5329	.8618

*. The mean difference is significant at the 0.05 level.

Appendix 3: LSD analysis of SEA specific IgG response

Absorbance

LSD

(I) Treatments	(J) Treatments	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PZQ	50 mg	-.13892*	.06097	.030	-.2634	-.0144
	150 mg	-.04733	.06097	.444	-.1718	.0772
	300 mg	-.04150	.06097	.501	-.1660	.0830
	Naive	.17858*	.06097	.006	.0541	.3031
	IC	-.11333	.06097	.073	-.2378	.0112
50 mg	PZQ	.13892*	.06097	.030	.0144	.2634
	150 mg	.09158	.06097	.144	-.0329	.2161
	300 mg	.09742	.06097	.121	-.0271	.2219
	Naive	.31750*	.06097	.000	.1930	.4420
	IC	.02558	.06097	.678	-.0989	.1501
150 mg	PZQ	.04733	.06097	.444	-.0772	.1718
	50 mg	-.09158	.06097	.144	-.2161	.0329
	300 mg	.00583	.06097	.924	-.1187	.1303
	Naive	.22592*	.06097	.001	.1014	.3504
	IC	-.06600	.06097	.288	-.1905	.0585
300 mg	PZQ	.04150	.06097	.501	-.0830	.1660
	50 mg	-.09742	.06097	.121	-.2219	.0271
	150 mg	-.00583	.06097	.924	-.1303	.1187
	Naive	.22008*	.06097	.001	.0956	.3446
	IC	-.07183	.06097	.248	-.1963	.0527

Naive	PZQ	-.17858*	.06097	.006	-.3031	-.0541
	50 mg	-.31750*	.06097	.000	-.4420	-.1930
	150 mg	-.22592*	.06097	.001	-.3504	-.1014
	300 mg	-.22008*	.06097	.001	-.3446	-.0956
	IC	-.29192*	.06097	.000	-.4164	-.1674
IC	PZQ	.11333	.06097	.073	-.0112	.2378
	50 mg	-.02558	.06097	.678	-.1501	.0989
	150 mg	.06600	.06097	.288	-.0585	.1905
	300 mg	.07183	.06097	.248	-.0527	.1963
	Naive	.29192*	.06097	.000	.1674	.4164

*. The mean difference is significant at the 0.05 level.

Appendix 4: LSD analysis of granulomas measured in Liver Histopathology

Post hoc ANOVA using LSD

Multiple Comparisons

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
IC	50 mg	1.1900*	.51663	.026	.1495	2.2305
	150 mg	.4600	.51663	.378	-.5805	1.5005
	300 mg	1.1200*	.51663	.035	.0795	2.1605
	PZQ	3.3600*	.51663	.000	2.3195	4.4005
50 mg	150 mg	-.7300	.51663	.165	-1.7705	.3105
	300 mg	-.0700	.51663	.893	-1.1105	.9705

150 mg	50 mg	.7300	.51663	.165	-.3105	1.7705
	300 mg	.6600	.51663	.208	-.3805	1.7005
300 mg	50 mg	.0700	.51663	.893	-.9705	1.1105
	150 mg	-.6600	.51663	.208	-1.7005	.3805
PZQ	50 mg	-2.1700*	.51663	.000	-3.2105	-1.1295
	150 mg	-2.9000*	.51663	.000	-3.9405	-1.8595
	300 mg	-2.2400*	.51663	.000	-3.2805	-1.1995
	IC	-3.3600*	.51663	.000	-4.4005	-2.3195

*. The mean difference is significant at the 0.05 level.

Appendix 5: Chi square analysis of nominal data.

$\chi^2_{0.05.1} = 3.841$

		50 mg	150 mg	300 mg
Adhesions	PZQ	0.00	1.2	4.8
	IC	6.75	3.0	0.75
Inflammation	PZQ	4.8	10.8	10.8
	IC	10.5	4.67	4.67