ANTISCHISTOSOMAL EFFECTS OF SELECTED PLANT EXTRACTS ON SWISS WHITE MICE INFECTED WITH *SCHISTOSOMA MANSONI*

By NAOMI NJOKI WAIGANJO (M.Sc)

A thesis submitted to the School of Biological Sciences, University of Nairobi in fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Applied Parasitology.

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DECLARATION

This thesis is my original work and has not been presented in any other institution for degree award or other qualification

Signature..... **Date**.5th November, 2012

Naomi Njoki Waiganjo, Reg. No. I80/80215/2010

This thesis has been submitted for examination with our approval as university supervisors

1. Signature..... **Date**..5th Nov., 2012

Prof. Horace Ochanda

School of Biological Sciences and Associate Dean,

Kenya Science Campus,

University of Nairobi

2. Signature..... **Date**.5 November 2012

Prof. Dorcas S. Yole

School of Pure Science and Technology

Kenya Polytechnic University College / Institute of Primate Research

DEDICATION

This work is dedicated to my family: my husband John Waiganjo, my daughters Gladys, Ruth and my son Peter for their encouragement, patience and support on a course to follow the desires of my heart. May God bless them richly.

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My profound thanks to my beloved family members; husband John Waiganjo, my daughters Gladys and Ruth and my son Peter for their prayers, financial and moral support.

To all those I have not mentioned by name but in one way or another contributed to this work am grateful. May God bless you all.

- Naomi Njoki Waiganjo

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LIST OF ABBREVIATIONS

ANOVA- Analysis of Variance

ADCC- antibody dependent cell mediated cytotoxicity

B - Bleeding

BSA – Bovine Serum Albumin

CD- Cluster Designation

C - Cell

Co - Company

CON A- Concavalin

DCs- Dendritic cells

n- Centimetres

SO_4 -Copper sulphate

M- Dichloromethane

– Distyrene Plasticizer Xylene

A- Enzyme -linked immunosorbent assay

lour

Horse Radish Peroxide

am

nfection

fected control

terferon

unoglobin Gamma

unoglobin Alpha

IgE- Immunoglobin Episilon

IgM- Immunoglobin Mu

IL - Interleukin

IPR- Institute of Primate Research

MAb- monoclonal antibodies

Mg -Milligrams

Ml- Mililitres

P - Perfusion

PBS- Phosphate buffered Saline

Pi - Post infection

PZQ- Praziquantel

RT- Room Temperature

S - Schistosoma

SDS- Sodium dodecyl sulphate

SWAP- Soluble Worm Antigen Prepation

T- Treatment

TH1 - Thelper - 1

TH2 - Thelper - 2

TLRs- Toll-like receptors

TID- Tropical and Infectious Disease Department

TMB - Tetramethylbenzidine

WHO - World Health Organisation

µl –Microlitre

ABSTRACT

Schistosoma mansoni is responsible for causing schistosomiasis in humans; a major public health problem worldwide. It is estimated that 200 million people, mostly children are infected with *schistosomes.* Schistosomiasis occurs in 76 tropical countries, 85% of the infections in Africa. In Kenya, it is estimated that 3 million people are infected. Schistosome eggs are responsible for most pathology. Even though conventional drugs are effective in the treatment of the disease, very little progress has been achieved on treatment of schistosomiasis in Kenya. Praziquantel (PZQ) is the most effective drug against all adult stages of human schistosomiasis. Being the only drug used for treatment, other drugs should be sought to avoid development of drug resistance. The use of plant extracts in treatment of diseases is universal. Many plants have been used locally for treatment of bilharziasis in Kenya. The aim of this study was to determine Phytochemistry and antischistosomal activity of five plant extracts, effective dosage and immunological responses of the efficacious extracts, and *in vitro* assessment of cercaricidal and wormicidal effects. Extracts from the following plants were used: *Sonchus luxurians*, *Ocimum americanum, Bridelia micrantha, Croton megalocarpus and Aloe secundiflora*. Plants were collected, identified, dried then extracted using hexane, Dichloromethane/methanol and water. Swiss white mice were infected, treated with plant extracts and worms recovered. Pathological, immunological, cercaricidal and wormicidal assays were carried out. The results obtained showed that *Ocimum americanum hexane* (OAH) and *Bridelia micrantha* (BMW) water extract had antischistosomal activity. This was indicated by low worm recovery, high worm reduction, and reduced gross pathology with histopathology showing no or few granulomas in the liver tissue, which was similar to PZQ. The effective dosage of efficacious plants was 300mg/ml which was used for OAH and BMW extracts. The two extracts had both cellular and humoral immunity as demonstrated by IFN-γ, IL-5 and IgG responses. OAH and BMW were significantly similar to PZQ; however BMW had higher IgG responses. BMW had higher IFN-γ responses for both spleen and lymph node cells. This implied that treatment groups were able to produce the TH-1 response which is important for cell mediated immunity. Although both extracts induced production of IL-5 for both lymph node and spleen cells, OAH generated more IL-5. IL-5 is responsible for humoral responses. The plant extracts with the highest cercaricidal effects were *Bridelia micrantha, Ocimum americanum and Sonchus luxurians* Dichloromethane and

Methanol extracts. The two efficacious extract, BMW and OAH exhibited wormicidal effect. In conclusion the two plants extract, *Ocimum americanum* hexane and *Bridelia micrantha* water were efficacious as demonstrated by high worm reduction, reduced gross and histopathology, humoral and cellular responses, similar to PZQ. In addition they were very effective in killing adult worms *in vitro*. Therefore the two plant extracts are possible candidates for drug development.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Background Information

Schistosomiasis, also known as bilharzia, is an infectious disease caused by trematode flatworms of the genus *Schistosoma.* It is endemic in 74 developing countries with more than 90% of infected people living in sub-Saharan Africa. It is estimated that 240 million people are infected worldwide and a close to 800 million are at risk of infection (WHO, 2011). Schistosomiasis ranks second only to malaria as the most common parasitic disease and is the most deadly neglected tropical disease killing an estimated 280,000 people each year in African region alone (CDC, 2010). Its distribution range has continued to expand due to increased establishment of water resources, development projects to boost food and cash crop production and hydroelectric power generation for industrialization (Hunters *et al.*, 1993). Four species, *Schistosoma mansoni, S. haematobium, S. japonicum* and *S. mekongi* are important agents of human schistosomiasis although other species, *S. matheei, S. intercalatum* and *S. bovis* parasitic in other mammalian hosts may produce infections in humans (Kibiki, 2004).

S. mansoni occurs in Africa, Madagascar, the Arabian Peninsula, South America and the Caribbean region (Rollinson and Southgate, 1987).

Schistosoma mansoni is the most prevalent and lives in the inferior mesenteric veins, causing intestinal schistosomiasis. S. *haematobium* lives in the venous plexus near the urinary bladder and urethra causing urinary schistosomiasis (WHO, 2009). S. *japonicum*

lives in the superior mesenteric veins of both the large and small intestine producing clinical and pathology similar to those for *schistosoma mansoni* (Savioli and Mott, 1989).

Schistosomiasis has earned a Category II disease ranking next to malaria in importance as a target tropical disease by the World Health Organization Special Programme for Research and Training in Tropical Diseases. Deaths are associated with the severe consequences of infection, including bladder cancer or renal failure as in *Schistosoma haematobium* and liver fibrosis and portal hypertension in *S. mansoni*. The rapture of the oesopharyngeal varices may lead to fatal haemorrhage (Jondan *et al.*, 1993).

Intestinal schistosomiasis is prevalent in areas where freshwater snails belonging to the family Planorbidae breed in water contaminated by faeces of infected persons. Activities linked to development of resources such as irrigation schemes and hydroelectric power project have spread the disease to previously no endemic areas (Chitsula *et al.*, 2000). In sub-Saharan Africa where schistosomiasis constitutes an important public health problem, the disease-specific mortality is reported that 70 million individuals out of 682 million had experienced haematuria and 32 million dysuria associated with *S. haematobium* infection (King and Dangerfield-cha, 2008).

It was estimated that 18 million suffered bladder wall pathology and 10 million hydronephrosis. Infection with *S. mansoni* was estimated to cause diarrhoea in 0.78 million individuals, blood in stool in 4.4 million and hepatomegaly in 8.5 million. Using the very limited data available, mortality rates due to non-functioning kidney (from *S.*

haematobium) and haematemesis (from *S. mansoni*) have been estimated at 150 000 and 130 000 per year, respectively (King and Dangerfield-cha, 2008).

Although these are global estimates of the schistosomiasis disease burden, the public health impact of schistosomiasis in the field has been poorly evaluated and is still subject to controversy. Apart from a few situations where schistosomiasis is or was recognized as an obvious public health problem, as in Brazil, China, Egypt, the Philippines, northern Senegal and Uganda, the disease is often not a priority for health authorities. Moreover, the lack of a simple clinical case definition does not enable rapid identification of the disease by health personnel (WHO, 2010; Kabatereine *et al.*, 2004).

1.1.2 *Schistosomes* and Schistosomiasis

Schistosomes (also known as blood flukes) are digenetic trematodes. The schistosomes belong to phylum *Platyhelminths* (flatworms); class *trematoda,* family *Schistosomatidae*, sub family *Schistosomatinae* and they fall under genus *Schistosoma* (Rollinson and Simpson, 1998).

These are parasites in the blood-vascular system of vertebrates, endothermic birds and mammals. A characteristic feature of members in this family is that mature female worms are more slender than the male worms and are normally carried by the males in a ventral groove known as the gynaecophoric canal, which is formed by ventrally flexed lateral outgrowths of the male body (Arbaji *et al.*, 1998).

Schistosomes utilize aquatic snails as intermediate hosts for the completion of their life cycles. Five genera are currently recognized in this group viz. *Schistosoma*, *Schistosomatium*, *Bivetellobilharzia*, *Heterobilharzia* and *Orientobilharzia* (Chitsula *et al.*, 2000). The genus *Schistosoma* is important from the medical and economic viewpoint, as it comprises species that parasitize and cause disease in humans and domestic animals (Engles *et al.*, 2002).

Human schistosomiasis afflicts at least 200 million people, mostly children, in 76 tropical countries with another close to 800 million people being at risk of contracting the disease (WHO, 2011), and the number of people estimated to be infected or at risk continues to increase as population growth increases. It is also estimated that 85% of the total number of infected people worldwide are in Africa (Lichtenberg, 1987).

1.1.3 Geographical distribution of Schistosomiasis

S. mansoni is found in Africa, the Caribbean, and South America and *S. mekongi* is found in Laos and Cambodia. Swimmer's Itch, a non-disease form of schistosomiasis in humans, is marked by a short-lived, itchy rash (Fenwick *et al.*, 2003). It is caused by certain other species of schistosomes that are found in both fresh and saltwater areas worldwide. In the United States, Swimmer's Itch is common along the Atlantic, Gulf, and Pacific coasts and in the lakes of northern Michigan, Wisconsin and Minnesota (Haymann, 2004). Urinary schistosomiasis, caused primarily by *Schistosoma haematobium*, is found throughout Africa and the Middle East. *S. intercalatum* is confined to the countries within the rainforest belt of Africa; *S. japonicum* is restricted to

the Pacific region including China, East Asia, the Philippines and Malaysia (WHO, 2004).

In Kenya the two important species are S. *mansoni* and S. *haematobium*. S. *mansoni* is mainly found in Machakos and Kitui districts, Murang'a, Kiambu, Mwea, Kirinyaga, Embu, along the shores of L. Victoria, L.Kanyaboli and L. Jipe, Rusinga Island, Nandi hills, Kericho, Kajiado and Mwea (WHO, 1985). S. haematobium is found in Kwale, Lamu, Malindi, Kilifi, Mombasa, and areas of Tana River, shore of L. Victoria, Kajulu, Nyakach, Siaya, Kisii, Nandi hills, Kericho, Machakos and Kitui districts. It is found in scattered foci in Muranga, Kiambu and Western Kenya (WHO, 1996).The distribution of schistosomiasis around the world is shown in Figure 1.1.

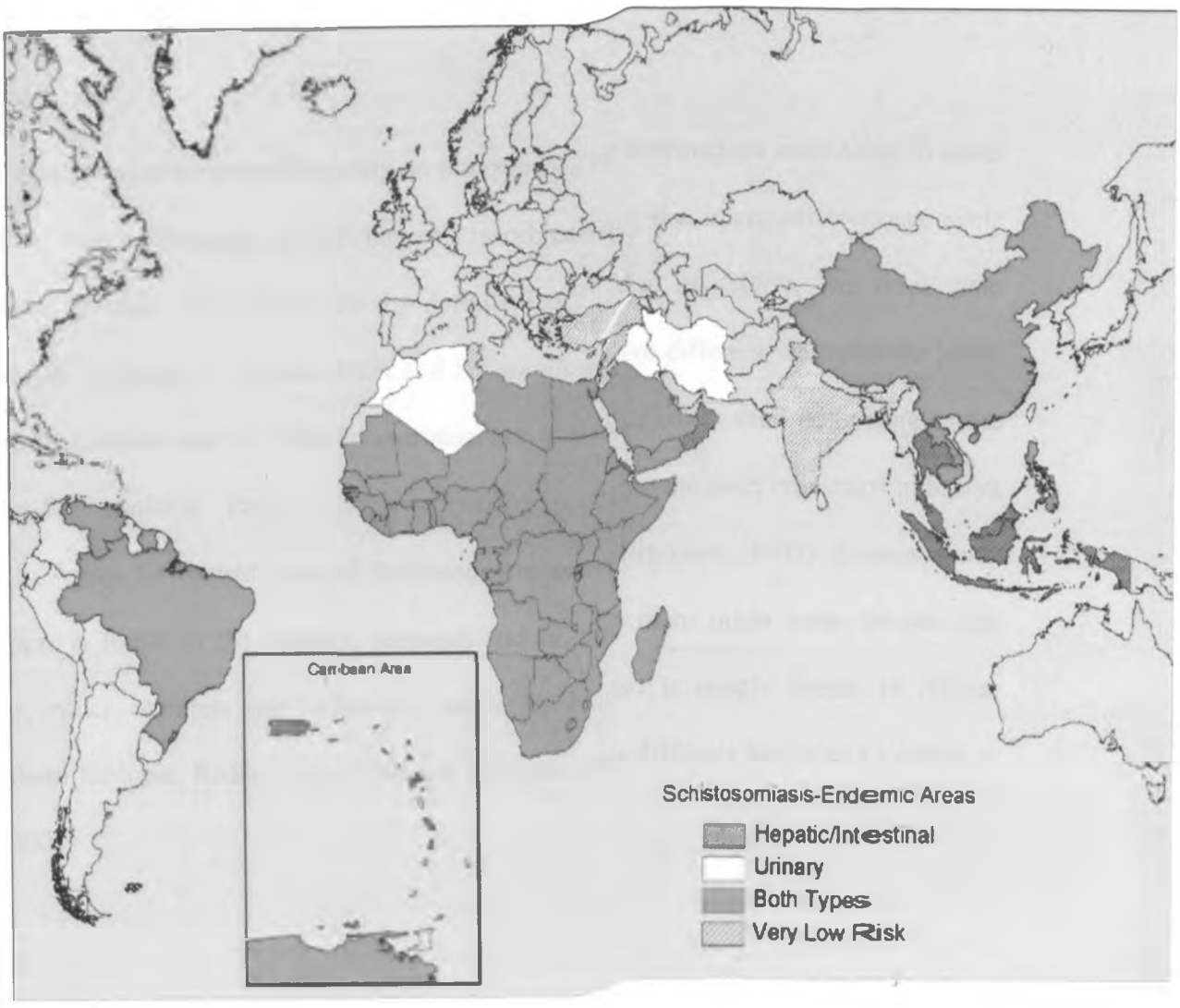

Figure 1.1: Worldwide distribution of Schistosomiasis (WHO, 2001)

1.1.4 Hosts

The transmission depends absolutely on the presence of intermediate snail hosts in water bodies. Snails belonging to the family Planorbidae are the intermediate hosts while humans, primate and rodents are the definitive (Colley *et al.*, 2001). The three main flukes, S. *mansoni*, S. *haematobium* and S. *japonium* have different intermediate hosts. The intermediate host of *Schistosoma mansoni* is a fresh water snail belonging to the genus *Biomphalaria*. The *Biomphalaria pfeifferi* species are the most important in Kenya and in Africa for transmission of *Schistosoma mansoni* (Sturrock, 1993). *Biomphalaria pfeifferi* is found in the streams, seepages and in many man- made water bodies like dams, water channels and swimming pools. *B. Pfeifferi* is mostly found in Africa, Northern Ethiopia, Sudan, Lake Chad and Senegal within different localities (Leshem *et al.*, 2008).

The snail hosts of S. *haematobium* belong to the genus *Bulinus* while S. *japonicum* uses snails in the genus *Onchomelania* (Cheesbrough, 1987). In Kenya S. *mansoni* is transmitted by *Biomphalaria pfeifferi* and *B. sudanica* while *S. haematobium* is transmitted by *Bulinus globosus* and *B. nasutus*. In *S. japonicum* the intermediate hosts are *Oncomelania nosophora, O. hupensis* and *O. quadrasi* mainly found in China, Phillipines and Celebes (Basch, 1991).

Transfer of infection does not require direct contact between man and the snail. Man gets infected by coming into contact with water infested with cercariae released into water by snails (Sturrock, 1993; Abel and Dessein, 1998).

1.1.5 *Schistosoma mansoni* stages and morphology

The egg of *Schistosoma mansoni* is characterized by lateral spine and it measures 115 to 175μm by 4 to 70μm. The eggs hatch into miracidia (Cheever, 1997). The miracidia is ciliated. The body of miracidia is covered by flat epidermal plates, beneath which a well developed sub- epidermal musculature (Smyth and Halton, 1983). The miracidi*a* infects snail through the foot of the snail (Kojima, 1998). The miracidia transforms in to primary and secondary sporocyst within the snail host. The snails shed cercariae after 4 to 5weeks and has bifurcated tail. It measures 400 to 600μm (Jang-Lee et al., 2007; Ross *et al.*, 2002).

The male *S. mansoni* is approximately 1 mm long (0.6 to 1.4 mm) and is 0.11 mm wide white and has a funnel-shaped oral sucker at its anterior end (Criscione *et al* 2009). The female has a cylindrical body, longer and thinner than the male. The female adult worm is slender and dark in appearance. The female lives in the gynaecophoric canal of male where they mate (Wilson, 1987; Loverde and Chem, 1991).

1.1.6. Life Cycle of *Schistosoma mansoni*

Schistosomes, like other digenean trematodes have a complex life cycle involving parasitic forms in a snail intermediate host, free-living larval forms in water and parasitic stages in a definitive host (Rollinson and Southgate, 1987). The adult male and female worms live paired in the blood-vascular system of humans or other susceptible mammalian hosts, where they mate and reproduce (Boros, 1970). A detailed review of

the development, reproduction, and host-parasite relations in schistosomes is described by Basch (1991).

The female worms produce eggs in the blood stream. The eggs work their way into the host tissue and eventually into the lumen of the intestine, from where they are passed out into the environment via faeces (Stadecker and Colley, 1992). The schistosome eggs are fully developed and contain ciliated larval forms (miracidia) when they leave the body of the definitive host (Sturrock, 1993). On coming into contact with fresh water, the eggs hatch and the miracidia, which are free-swimming forms, are released into the water, and immediately embark on the search for a suitable snail host. If a suitable snail host is found, the miracidium enter, transform into mother sporocyst then into daughter sporocyst in which cercariae (another free-swimming larval form that is infective to the definitive host) develops (Jordan & Sturruck, 1993).

Each miracidium is either male or female, and the resulting cercariae or an adult worm is of a single sex. A single miracidium, however, gives rise to many cercariae (Damian, 1976). When the cercariae are released into water they search for a suitable mammalian host, and enter by penetrating unbroken skin and transform into schistosomula. The schistosomula incorporate host proteins, and blood group antigens, in their integuments. Each schistosomule spends a few days in the skin and then enters the circulation starting at the dermal lymphatics and venules. Here they feed on blood, regurgitating the haem as hemozoin. (Olivera *et al.*, 2010). The schistosomule migrates to the lungs (5–7 days post-penetration) and then moves via circulation through the left side of the heart to the

hepatoportal circulation (>15 days) where it feeds on blood and matures. If it meets a partner of the opposite sex, it develops into a sexually mature adult and the pair migrates to the mesenteric veins. Such pairings are monogamous (Beltran & Boissier, 2008).

Male schistosomes undergo normal maturation and morphological development in the presence or absence of a female, although there are behavioral, physiological and antigenic differences between males from single-sex, and those from bisexual. On the other hand, female schistosomes do not mature without a male. Females' schistosomes from single-sex infections are underdeveloped and exhibit an immature reproductive system. Although the maturation of the female worm seems to be dependent on the presence of the mature male, the stimuli for female growth and for reproductive development seem to be independent from each other (Beltran & Boissier, 2008).

The male and female adults' pair off with the thin female entering and remaining in the gynaecophoric canal of the male worm. The pair now migrates together along the endothelium, against portal blood flow to the mesenteric veins (*S. mansoni)* where they begin to produce eggs (WHO, 2001). A summary of the life cycle is shown in Figure 1.5

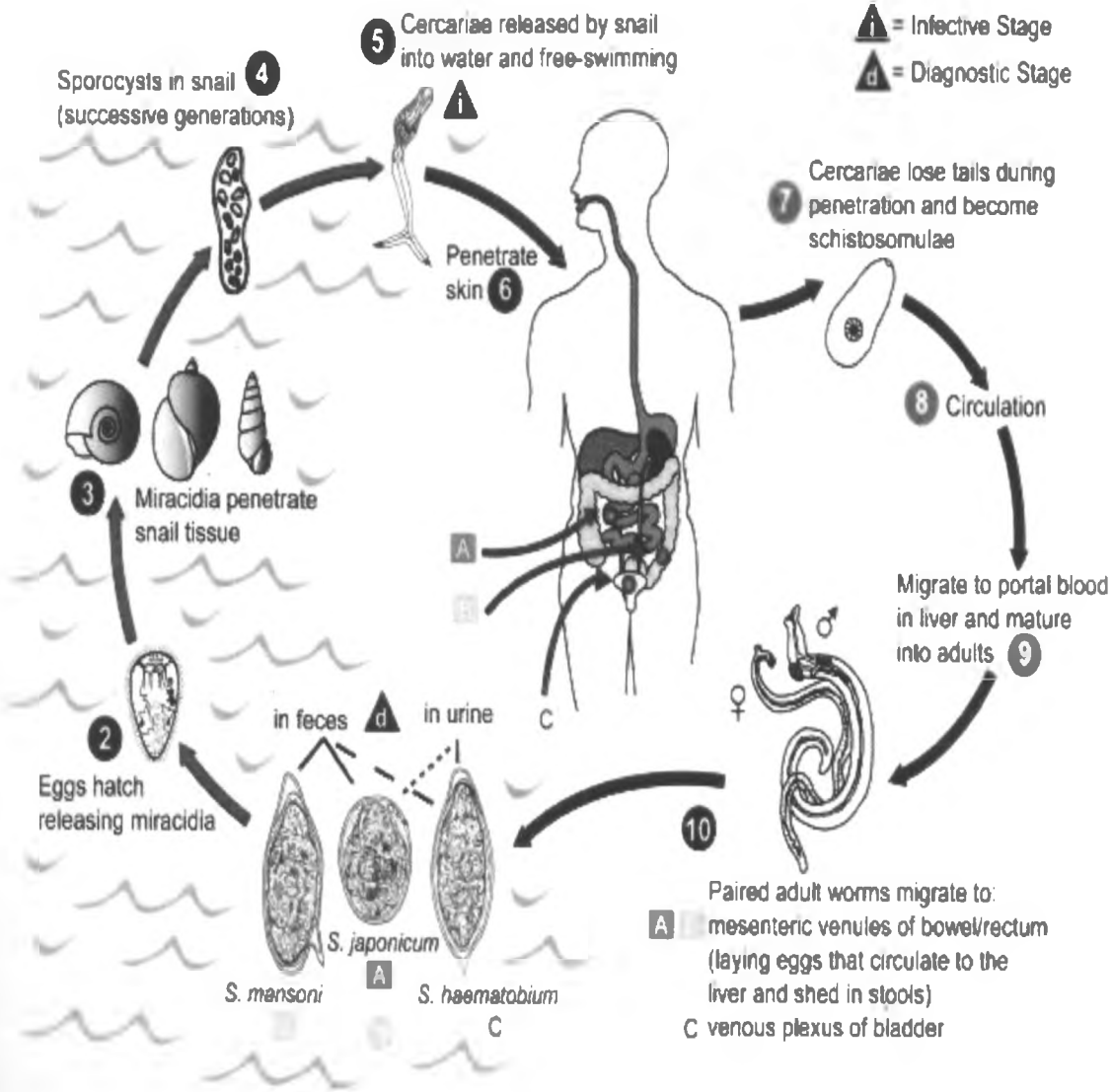

Figure 1.1: Life cycle of mammalian Schistosomes (WHO /TDR, 2002)

1.1.7 Symptoms of *Schistosoma mansoni* infection

1.1.7.1 Swimmer's itch

Many individuals do not experience symptoms. If symptoms do appear, it usually takes four to six weeks from the time of infection. The first symptom of the disease may be a general ill feeling. Within twelve hours of infection, an individual may complain of a tingling sensation or light rash commonly referred to as "swimmer's itch", due to irritation at the point of entrance. The rash that may develop can mimic scabies and other types of rashes (Stadecker *et al.,* 1992).

1.1.7.2 Katayama fever

Another primary condition, called Katayama fever or acute toxaemic schistosomiasis is the most important sequel of the migration of schistosomulae and adult worms as well as the initial deposition of eggs, and it can be very difficult to recognize. The condition, which resembles serum sickness, is related to the deposition of the large amounts of antibody-antigen complexes as a result of excess circulating antigens. The clinical signs are non-pathognomonic but include persistent pyrexia, abdominal pain, diarrhea and myalgia, lethargy, the eruption of pale temporary bumps associated with severe itching (urticaria) rash, liver and spleen enlargement, bronchospasm and characteristic peripheral eosinophilia (Ross *et al.*, 2002).

7.3 Intestinal schistosomiasis

ntestinal schistosomiasis, eggs become lodged in the intestinal wall and cause an
ıune system reaction called a granulomatous reaction. This immune response can lead
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Diagnosis and treatment of *Schistosoma mansoni* infection

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1.1. 7.3 Intestinal schistosomiasis

In intestinal schistosomiasis, eggs become lodged in the intestinal wall and cause an immune system reaction called a granulomatous reaction. This immune response can lead to obstruction of the colon and blood loss. The infected individual may have what appears to be a potbelly (Leshem *et al.*, 2008) Eggs may also become lodged in the liver, leading to high blood pressure through the liver, enlarged spleen, the buildup of fluid in the abdomen, and potentially life-threatening dilations or swollen areas in the esophagus or gastrointestinal tract that can tear and bleed profusely (esophageal varices). Rarely, the central nervous system is affected. Individuals with chronic schistosomiasis may not complain of typical symptoms (King *et al.*, 2003).

1.1.8 Diagnosis and treatment of *Schistosoma mansoni* infection

Diagnosis of infection is confirmed by the identification of eggs in stools. Eggs of *Schistosoma mansoni* are approximately 140 by 60 μm in size and have a lateral spine. The diagnosis is improved by the utilization of the Kato-Katz technique (quantitative stool examination technique). Other methods which can be used are enzyme linked immunosorbent assay (ELISA), circumoval precipitation test (COPT) and alkaline phosphatase immunoassay (APIA).

Currently there are two drugs available, Praziquantel and Oxamniquine, for the treatment of *Schistosoma mansoni* (Davis, 1979). They are considered equivalent in relation to efficacy and safety. Due to its lower cost per treatment, Praziquantel is generally considered the first option for treatment (Hotez, 2009). The recommended dose is:

Praziquantel, 60mg/kg of body weight for children up to 15 years old, and 50mg/kg of body weight for adults; Oxamniquine, 15mg/kg for adults, and 20mg/kg for children up to 15 years old (King,1989).

Both drugs are administered as a single dose treatment. The treatment objective is to cure the disease and to prevent the evolution of infected patients to the chronic form of the disease. All confirmed cases should be treated (Ali, 2006).

1.2 LITERATURE REVIEW

1.2.1. Pathogenesis of *Schistosoma mansoni*

In infection by *S. mansoni*, the major pathologic changes are not caused by the adult worm itself but by eggs which do not reach the intestinal lumen, but instead, become trapped in other body tissues. At these sites, areas of local inflammation are produced, cumulating in the formation of granulomas around eggs (Giboda and Smith, 1994). The eggs of Schistosomes are lethal to a human's body system. When the body detects these eggs, an inflammatory reaction is triggered (Burke *et al.*, 2009).

The encapsulated eggs cause liver damage. Eggs undetected by the body's defense system also harm the body. The embryos in the un-capsulated eggs release a toxin that damages the liver (Reynolds *et al.*, 2002). The formation of granuloma around schistosome eggs in the liver and the intestine is the major cause of pathology in schistosome infections. Granuloma and the subsequent fibrosis in the liver appear to be primarily responsible for mortality and morbidity by this highly endemic parasitic disease (El-Banhawey *et al.*, 2007). Ultrasound is used for detection of complications of infection in animals (Akpinar and Metin, 1999; Oliveira *et al.*, 2004).

Visible to the human eye are the enlargement of the liver and spleen. Once symptoms have been noticed treatment can occur. *In vivo* microscopy revealed in addition to these lesions, dilatation and sacculation of sinusoids (Aly and Hamed, 2006). These lesions were associated with varying degrees of reduction of blood flow due to the parasite (El-

Banhawy *et al.*, 2007; Aly and Hamed, 2006). Ascites of the liver and esophageal varices may proceed .Spleen is enlarged with fibrosis and contains Gamma- Gandy bodies. Among severe cases hepatomegaly and enlargement of the spleen are found in high number of cases (Talaat and Miller, 1998; Smyth, 1983).

Other clinical manifestations of chronic schistosomiasis include core pulmonale due to egg deposition in the lungs and subsequent development of pulmonary hypertension. Pulmonary complications of *S. mansoni* infection manifest with bronchopulmonary symptoms (Akpinar and Metin, 1999) Total protein is reduced in bilharzail infection. This could be attributed to cellular damage caused by parasite toxins. The main fraction of total protein content is albumins and the reduction in total protein may be due to reduction in albumin fraction level that in turn may be result from decreased anabolism or increased catabolism; hence, malnutrition and/or malabsorption (El-Fakahani *et al.*, 1993; Rizk *et al.*, 2000; El-Ansary *et al.*, 2007).

The significant decrease in total protein is mainly due to increase in messenger RNA degradation which is the possible cause for the hypoalbuminemia of murine schistosomiasis (Metwally *et al.*, 1990). Perioval granulomas in the liver lead to fibrosis. The *Schistosoma mansoni* eggs embolizing in the hepatic microvasculature precipitate a perioval granulomatous inflammatory reaction, which may lead to hepatic fibrosis, portal hypertension, gastrointestinal hemorrhage, and death (Bica *et al.*, 2000)

1.2.2. Immune-related pathologies during *Schistosoma mansoni* infection

Schistosomiasis causes a range of morbidities, the development of which seems to be influenced to a large extent by the nature of the induced immune response and its effects on granuloma formation and associated pathologies on target organs (Brunnet *et al.,* 1998). Field studies in endemic areas, combined with animal experiments, have led to the view that host genetics, infection intensity, in utero sensitization to schistosome antigen and co- infection status, all influence the development of the immune response and, so, disease severity. Two main clinical conditions are recognized in *S. mansoni*-infected individuals, acute schistosomiasis and chronic schistosomiasis (Pearce and MacDonald, 2002; Omer-Ali *et al.*, 1989).

1.2.3. control of Sschistosomiasis

The two main reasons for the spread of schistosomiasis are contamination of the environment by people because of lack of education and insufficient attention to hygiene. It is also associated with poverty and poor living conditions, inadequate sanitation and water supply as well as unplanned water resource development (Austin, 1979). Various strategies are recommended for schistosomiasis control, and these include chemotherapy, snail control, health education, community participation, and provision of clean water and improved sanitation (WHO, 1993). Chemotherapy, using the antischistosomal drug Praziquantel, however, is considered the most cost effective approach for control of human schistosomiasis (WHO, 1990).

1.2.3.1 Environmental control

Schistosomiasis can only be transmitted by water contacts mostly for domestic and recreational purposes and also occupational .Therefore, providing safe water supply, sanitary facilities, washing facilities, cattle watering facilities and bathing, not only reduce the risk of infection with schistosomiasis, but also reduce the source of infection with other parasite and bacterial infections (Doenhoff et *al.*, 2000; Engles, 2002).

1.2.3.2 Control by using molluscicides

The control of schistosomiasis is through disrupting its life cycle. Snails, which are schistosomiasis intermediate host, once destroyed, will prevent miracidia from changing into cercariae (Yuan, 1989). The use of molluscicides for the control of the snail is important since it prevents the reinfection of people after treatment. Metallic salts, such as copper sulfate, were among the first agents used and were most effective when applied to standing bodies of water (Andrew *et al.*, 1983). Newer molluscicides, such as nicotinanilide, organotin, dibromo-nitraozo-benzene, sodium pentachlorophenate, tritylmorpholine, sodium dichloro-bromopheno, niclosamide and acetamide analogs replaced copper sulfate, as these were deemed safer to the environment (Zhang *et al.*, 1996). Niclosamide is still in use and effective molluscide today. However it affects non-target organisms is costly and toxic to fish (Weiss, 1985).

1.2.3.3 Biological control

It was shown that *Microsporidium* sp. can interfere with the development of the sporocyst stage of *S. mansoni (*Duncan, 1987*)*. More of these kinds of associations most likely exist in nature and discovering them may result in the development of a useful adjunct to current control strategies (Dunne *et al.*, 1995; Zhang, 1990). For control of schistosomiasis, one strategy is based on the premise that snails resistant to parasitic infection could be used as biological competitors to replace existing susceptible snails in endemic areas (Yuan, 1989). This approach, however, requires a more thorough understanding of the genetics of the complex interrelationship between parasites and snails (El-Ansary, 2001).

A number of predator/competitor snail species are receiving more and more attention as potential control agents, as well in well-controlled situations, such as small, artificial ponds. In Grenada, Martinique, Guadelupe, Puerto Rico and St. Lucia it was shown that *Ampullariidae* (*Pomacea glauca* and *Marisa cornuarietis*) and *Thiaridae* (*Tarebia granifera* and *Melanoides tuberculata*) snails out-competed *Biomphalaria* species for space and resources (Duncan and sturrock, 1987; Zhang, 1996). Competitor snail species were also used successfully as a follow up measure after molluscicide use in some rivers of central Venezuela (Katz, 1998).

1.2.3.4 Health education

Many of the countries lack the funds, or do not realize the inherent need to implement health education programs that inform the populace of ways to protect themselves from

schistosomiasis (Zhen, 1993). Currently, in countries that have been able to implement such programs, they tend to emphasize the control of human behavior as they relate to the spread of schistosomiasis. Earlier versions of these programs stressed the need for snail control and the attendant reduction of transmission. Educated, informed people are able to adopt control strategies at the personal level, even if it requires reducing their contact with contaminated water sources, or making water safe to drink.

1.2.3.5 Chemotherapy of schistosomiasis

This is the most effective method for reducing the infection rates of schistosomiasis. Access to very effective drug in the last decades has resulted in reduction in prevalence and morbidity of the disease in many areas of the world and reducing the public health importance of Schistosomiasis in some countries (Doenhoff, 1998; Austin *et al.*, 1979). Although, several drugs were used for treatment of schistosomiasis in the past, now only a few drugs are used. Introduction of praziquantel has transformed the treatment of schistosomiasis (Colley *et al.*, 2001; Doenhoff *et al.*, 2008). This drug is effective, generally in a single dose, against all species of the parasite. Other drugs include metrifonate, which is active against *S. haematobium* and oxamniquine, which is effective against *S. mansoni* (Botros *et al.*, 1989; Stelma, 1997).

ziquatel is an acylated quinoline-pyrazine (piperazine). Chemically piperazine is 2-lohexylcarbonyl-1,2,3,6,7,11b-hydropypyrazino{2,1-a}isoquinolin-4-one ndley,1987; Beck *et al., 2001*). Only the enantiomor is active. It-is present as a

white, hygroscopic, bitter tasting, crystalline powder, either odorless or having a faint odor. It is very slightly soluble in water and freely soluble in alcohol (Ali, 2006).

Praziquantel has activity against a wide spectrum of trematodes, including all species of pathogenic schistosome. In humans Praziquantel with a single dose of 40 mg kg^{-1} is effective in the treatment of all forms of schistosomiasis in both adults and children. The cure rate is usually 60-90% with egg reductions of 90-95% in those not cured (Andrews, 1981), but is generally ineffective against other organisms such as nematodes. The exact mechanism of action of praziquantel against cestodes has not been fully determined (Doenhoff, 2000).

At low concentrations *in vitro*, the drug appears to impair the function of the worms' suckers and stimulates their movement. At higher concentrations *in vitro*, praziquantel increases the contraction (irreversibly at very high concentrations) of the worm's strobilla (chain of proglottids) and causes irreversible focal vacuolization with subsequent cestodal disintegration at specific sites of the cestodal integument (Ali, 2006).

In schistosomes and other trematodes, praziquantel directly kills the parasite, possibly by increasing calcium ion (Ca^{2+}) influx into the worm. There is experimental evidence that praziquantel increases the permeability of the membranes of parasite cells for Ca^{2+} Fenwick *et al.*, 2003; Fallon *et al.*, 1995). The drug thereby induces contraction of the arasites resulting in paralysis in the contracted state. The dying parasites are dislodged om their site of action in the host organism and may enter systemic circulation or may

be destroyed by host immune reaction (ADCC). Additional action of PZQ are focal disintegrations and disturbances of ovipositor (laying of eggs) as are seen in other types of sensitive parasites (Metwally, 1990).

Praziquantel may adversely affect the parasite's glutathione and intracellular calcium concentrations, with secondary effects on the metabolism and antigenicity (Utzinger *et al.*, 2003 and Mandour *et al.*, 1990). The side effects occur in a relatively large proportion of patients (30- 60%) but they are transient and disappear within 24 hours after treatment (Cioli and Mattocia, 2003). In a study in Cote D'ivoire (Raso *et al.*, 2004) most frequent side effect were abdominal pain, dizziness and diarrhoea, urticaria and rectal bleeding in patients with heavy worm loads (Beck *et al.*, 2001).

Praziquantel is the drug of choice but there are reports of a possibility of developing drug resistance (Cioli, 2002). Resistance to praziquantel in the treatment of schistosomiasis has been reported from Egypt where the drug has been used aggressively for more than 10 years (Doenhoff *et al.*, 2000; El-Banhawey *et al.*, 2007; El-Ansary *et al.*, 2007). There are some reports of the possibility of resistance of some strains of schistosomiasis to praziquantel (Fallon *et al.*, 1995; Mandour *et al.*, 1990). However, some believe this resistance does not exist (Ismail *et al.*, 1999).

Treatment of infected cases provides the most effective short-term results in control of schistosomiasis (Castro *et al.*, 2002). It also reduces morbidity and rate of the transmission. Treatment of cases as a control method require proper planning such as

defining objectives of treatment, using the most reliable case finding method, selection of appropriate drug, section of the population to be treated, correct dosage schedule and having adequate information on the drug and its side-effects (Cioli *et al.*, 1995).

1.2.4: Plant extracts used in treatment of *Schistosoma* and other hhelminthes

Egyptian healers have used myrrh, the dried extracts from the plant Mommiphora mol mol since pharaonic ages. Modern Egypt has a special formulation prepared by the PHARCO pharmaceutical company (Bruneton, 1999 and Maghraby *et al.*, 2010 a) and have been subjected to many clinical studies for safety as well as efficacy in the treatment of *Schistosomiasis, fasciolopsis* and other parasitic worm infestations (Sheir *et al.*, 2001 and Maghraby *et al.*, 2010 b). Indigenous knowledge on herbal medicine can be used as a possible alternative to synthetics antihelmintics. To exploit this indigenous knowledge, both basic and applied research and evaluation of herbs have been carried by different people against different parasites and infectious agents (Bruneton, 1999 and Kokwaro, 1993). Some of medicinal plants are *Cassia auriculata* and *Erythrina variega*te which are useful in treatment of leprosy, ulcer and have antihelmintic activity (Patil, 2003; Weiss, 1995).

1.2.4.1. Plants used for antischistosomal activity

1.2.4.1.1 *Artemisia annua*

Artemisinin, a sesquiterpene lactone with a peroxide group, is the active principle derived from the leaves of *Artemisia annua* and is best known for its antimalarial properties (N'Goran, 2003; Abdel *et al.*, 2000). Several semisynthetic derivatives with even higher

antimalarial activities were developed, namely, arteether, artemether, artesunate, and dihydroartemisinin (Frederich *et al.*, 2002). The initial work was done in the early 1970s, and over the last decade, derivatives of artemisinin have gained tremendously in importance for the treatment and control of malaria. It is anticipated that their popularity will further increase, particularly also in combination with other antimalarial drugs with unrelated mechanisms of action (Nosten and Brasseur, 2002; Xian *et al.*, 2002; Genovese *et al.*, 2000).

The antischistosomal activities of artemisinin, artemether, and artesunate were discovered in the early 1980s, with the initial experiments focusing on *S. japonicum*. More recent studies confirmed that arteether and dihydroartemisinin also display antischistosomal properties (Abdel and El-Badawy, 2000; Xian *et al.*, 2001). Laboratory experiments conducted so far in different animal models found that artemether is active against the three major human schistosome parasites (Utzinger *et al.*, 2001).

1.2.4.1.2. *Glinus oppositifolius* [AIZOACEAE]

Glinus oppositifolius (Aizoaceae) has also been described under the names *Mollugo spergula* or *Mollugo oppositifolia*. The plant is a slender spreading or ascending annual herb and the stems can get 40 cm high. The leaves are opposed two by two, but they seem verticillate by three to five according to the development of axial leaves. The plant grows on damp sandy sites, occurring across West Africa from Senegal to South Nigeria, and is widely distributed in the tropics and sub-tropics generally. In Mali the plant can be found in the Gourma, Dire, and Hauossa areas and in the region near Lac Horo. In Mali, an

aerial part of *G. oppositifolius* is used for treating abdominal pain and jaundice. Fresh leaves are reported to be used against dizziness and to stimulate the appetite (Herborne and Baxter, 1993).

The plant has also been reported used against joint pains, inflammation, diarrhoea, intestinal parasite, fever, malaria, furuncles, skin disorders and wounds. *G. oppositifolius* has previously been shown to possess antimalarial activity by its triterpenoid saponins. A DCM-extract was reported to have fungicidal activity by inhibition against *Candida albicans* and larvicidal activity against *Anopheles gambiae* and *Culex quinquefasciatus*. A molluscicide effect has been obtained by MeOH and DCM extracts on three types of snails, *Biomphalaria glabrata*, *Biomphalaria pfeifferi* and *Bulinus truncates*. (Inngjerdingen *et al.*, 2008).

1.2.4.1.3 *Parkia biglobosa* [MIMOSACEAE]

Local name: Nere. *Parkia biglobosa* is a tree being up to 20 m high, bole stout, not buttressed, low-branching, bearing a large wide-spreading crown, deciduous, flowering while leafless; flowers in pendulous capitula bearing also pendulous, large fruit pods; of the Soudanian/Guinean savannah and transition woodland, from Senegal and on into southern Sudan. The bark-infusion is taken in Kordofanan in Ivory Coast, Burkina Faso and South-East Nigeria as a tonic and anti-diarrhoetic. The bark is commonly sold in herbalist shops in the western part of the Region for the analgesic action it confers in mouthwashes and steam-inhalations for toothache. A red colour is imparted to the mouth

while the saponins in the bark contribute asepsis (Togola *et al.*, 2008; Taylor *et al.*, 2001).

In Casmance, Senegal, the bark has wide usage: alone for female sterility, baths and by draughts, and similarly administrated in prescription with other drug-plants for skin-infections and leprosy, and for blennorrhoea. Fula and Tukulor people of Senegal drink a decoction against *Schistosoma* infection. In Ivory Coast and Burkina Faso the pounded bark with lemon juice added is applied to sores and ulcers; a decoction is considered anti-rachitic, tonic and febrifugal; and it is one of 32 other plants in a complex prescription used in the Kaya region for leprosy (Togola *et al* ., 2008).

1.2.5. Problem statement

Over 240 million people are infected with schistosomiasis and 800 million people are at risk of infection (WHO, 2011). Schistosomiasis continues to be a major public health problem in most African countries, parts of Asia, Caribbean, and South America. Moreover, it is endemic in 74 tropical developing countries. Schistosomiasis is the most prevalent water borne disease in rural areas with up to 500,000 attributable deaths (WHO, 2004).

Conventional drugs, though effective in the treatment of the disease, have achieved very little progress on treatment of schistosomiasis in Kenya due to varying drug responses. Praziquantel is the most effective drug against all adult stages of human schistosomiasis (Cioli, 2003) though not effective in juvenile stages. It is the drug of choice for morbidity

control of Schistosomiasis (Andrews, 1981). Its minimal side effects and high degree of efficacy against both trematodes and cestodes have made it the drug of choice for many human and veterinary parasitic infections. However, praziquantel has been in use for more than 20 years and concern is increasing that resistance has emerged, or will soon emerge, in human parasites (Utzinger *et al.*, 2003).

Loss of praziquantel efficacy would set back helminth control efforts (Cioli, 2000). The demand for praziquantel has been growing over the years, but production has hardly increased. The demand is mainly due to growing numbers of endemic countries implementing large-scale control programmes (WHO, 2009). It is therefore not a satisfying situation to have only one drug in use due to drug resistance (Raso *et al.*, 2004). Ideally other drugs should be available so that the strategy of avoiding development of drug resistance could be followed. Therefore there is need for new drugs which are cheap, readily available and easy to use.

1.2.6. Justification of the study

Praziquantel is not affordable and readily available to the local people. There is a need for an alternative drug for treating *Schistosoma mansoni* infection. Many plant species have been used throughout the world in traditional medicine for the treatment of both veterinary and human helminthes (Ndamba *et al.*, 1994), but few have been screened for activity against adult *Schistosoma* sp. Herbalist use the five plants to treat schistosomiasis to their clients in Athi river and Machakos town. It is therefore important to find out if the five plants, *Sonchus luxurians*, *Ocimum americanum*, *Bridelia micrantha*, *Croton*

megalocarpus and Aloe secundiflora have antischistosomal activity and can be used alongside Praziquantel.

1.2.7. Research questions

1. Which is the most effective plant extract in terms of worm and pathology reduction of *Schistosoma mansoni* infections among *Sonchus luxurians, Ocimum americanum, Bridelia micrantha, Croton megalocarpus and Aloe secundiflora?*
2. Which is the most effective dosage of the efficacious plants?
3. Do the efficacious plants also induce immune response in the infected mouse model?
4. Do the efficacious plants kill cercariae and worms *in vitro*?

1.2.8. Null Hypothesis

The plant extracts obtained from *Sonchus luxurians, Ocimum americanum, Bridelia micrantha, Croton megalocarpus* and *Aloe secundiflora* are not effective against *S. mansoni.*

1.2.9 Objectives

1. 2.9.1 General objective

To determine anti-schistosomal activities of five plants extract regimes in Swiss mice experimentally infected with *S. mansoni.*

1.2.9.2 Specific objectives

1. To determine the phytochemical constituents of the selected plant extracts.
2. To determine anti-schistosomal activities of the five plant extracts based on worm counts and pathology.
3. To determine the effective dosage of the efficacious extract(s)
4. To determine if the efficacious extract(s) induce cellular and humoral immunological responses.
5. To determine if the efficacious plant extract(s) have an effect on cercariae and worms *in vitro.*

CHAPTER 2: MATERIALS AND METHODS

2.1 Study site

The study was carried at the Institute of Primate Research (IPR) at the Tropical and Infectious Disease Department (TID). IPR is a biomedical research centre using both non-human primate models and rodents with the aim of improving human health. The institute has well developed elaborate research programs that use rodent and primate models. The Swiss mice were obtained from The Kenya Medical Research Institute, Nairobi. The maintenance of experimental mice, snails, infection treatment, gross pathology, perfusion, IgG and cytokine ELISA assay were carried out at IPR. Drying, crushing, soaking and filtering and extraction of plants using different solvents were carried out at Phytochemistry Laboratory at Museums of Kenya. Freeze drying of plant extract and histopathology was carried out at University of Nairobi, Chiromo campus in Chemistry and School of Biological Sciences respectively.

2.2. Plants used and their collection

Bridelia micrantha and *Croton megalocarpus* plants were collected from Kiambu and the bark part was used. The whole plant of *Ocimum americanum* and *Aloe secundiflora* were collected from Machakos area where the plants grow abundantly. The whole plant of *Sonchus luxurians* was collected from Nyandarua area. All the plants were collected in plastic bags and transported to the laboratory for preparation.

2.3 Drying of plants

The plants were dried as shown in the Figure 2.1 at room temperature for two months monitoring the material carefully to ensure that they do not turn moldy. The material was turned daily to ensure uniform drying. They were crushed into small particle using Mekon Micro miller single phase and passed through a 0.5 mm mesh to standardize the particle (Figure 2.2). The powder was then stored in glass bottles and kept at room temperature.

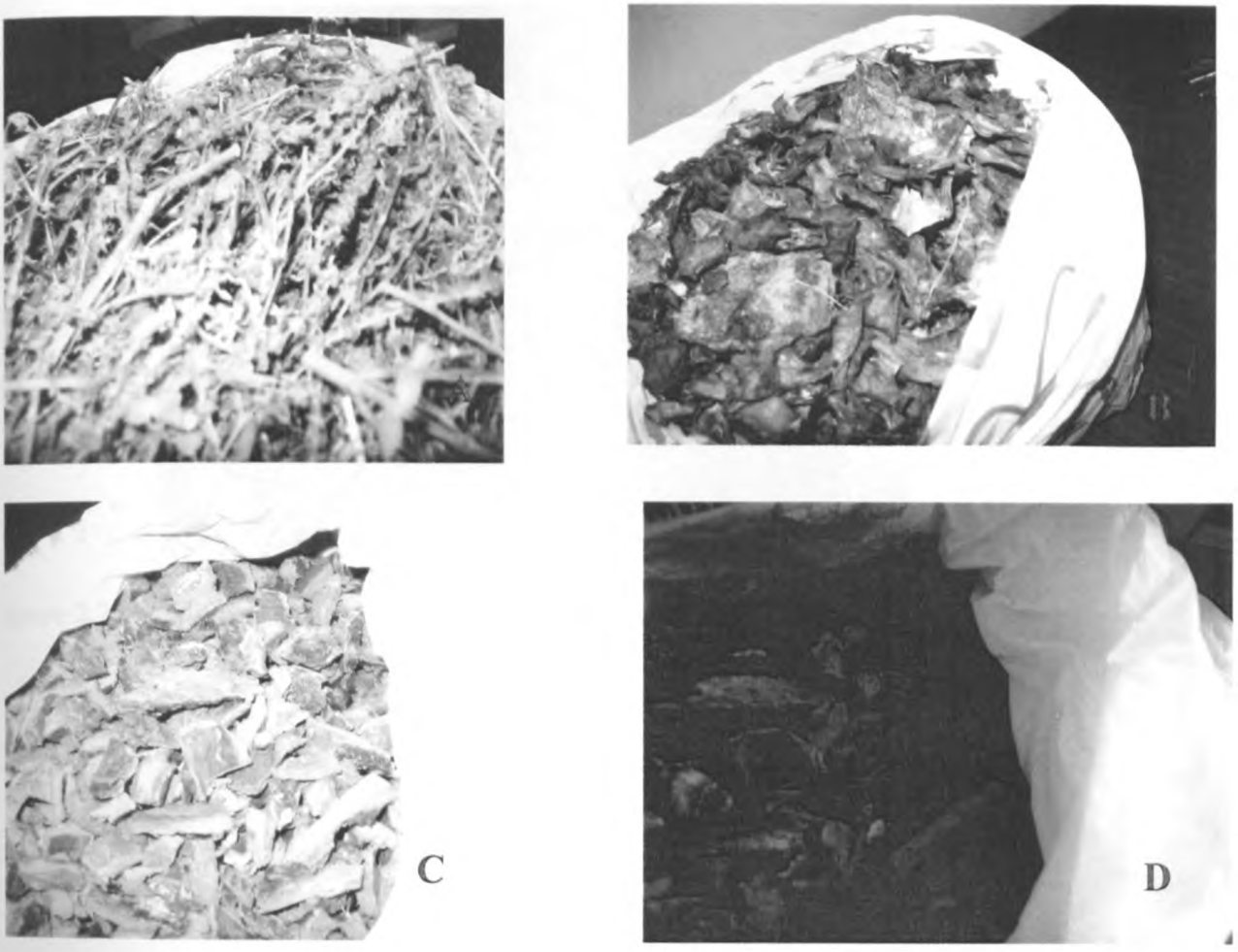


Figure 2.1: **Dried whole plant of *Ocimum americanum* (A), *Aloe secundiflora* (B), *Bridelia micrantha* (C) and *Croton megalocarpus* (D)**

Figure 2.2: ***Sonchus luxurians*** **whole plant ground ready for extraction**

2.4 Extraction of ground Plant materials

The mode of extraction naturally depends on the texture and water content of the plant materials being extracted or the type of substances to be isolated. The extraction for organic constituents from dried plant tissues was carried out in a Rotary evaporator. A range of solvents starting with which elute non-polar materials using n-hexane, to more polar components by using alcohols e.g. methanol was used.

The extract obtained was clarified by filtration through celite or a water pump and then concentrated *in vacuo*. This was carried out on a rotary evaporator as shown in Figure 2.3 which concentrates bulky solutions down to small volumes without bumping, at temperature 70°C.

2.4.1(a) Hexane Extraction

The powdered materials (2kg) from roots, stems, leaves and were first subjected to extraction by soaking in hexane 3- 8 litres depending with plant for 72 hours. The process was repeated three times using the same volume of hexane for each plant part in order to make sure that all non-polar materials are eluted. The contents were filtered using what man filter paper (No. 1 medium crystalline). The filtrates were concentrated using Rotary evaporator (RE-100 Bib by, made in Japan) at 70°C.

The filtrates from hexane were subjected to drying in fume cupboards at 25°C for 1-2 weeks to remove most of the solvents from the extracts to constitute to Extract I.

2.4.2 (b) Dichloromethane / Methanol Extraction

The residues from n-hexane filtration were soaked for 72 hours in Dichloromethane (DCM) / Methanol 1:1 to elute the medium polar materials. The process was repeated 3 times as described for n-hexane extraction to constitute Extract II.

2.4.3 (c) Aqueous Extraction

The residues from methanol filtrations were further re-soaked using distilled water for 3 days (72 hours) in order to extract any remaining polar materials. The solutions were then filtered and subjected to freeze drying using Chemlabs Instrument Ltd (Edwards) located in the School of Biological Sciences, University of Nairobi. The freeze dried materials constitute the aqueous Extract III.

Figure 2.3: Extraction of plant material in a rotary evaporator

2.4.4 (d) Preparation of crude plants extracts

The plants powder was prepared as done traditionally. A 100g of plant extracts was boiled in 250 ml of water for 5 minutes. It was sieved to remove debris. This was done to ensure that the concentration used in treatment of mice was the same as in the treatment of humans. The resultant preparation was kept in the refrigerator at -4°C.

2.5. Experimental animals and parasites

2.5.1. Mice

Swiss white mice were used as definitive hosts of *S. mansoni (*Ogboli *et al.*, 2000*).* The mice were maintained at the Institute of Primate Research (IPR) rodent facility. The male and female adult mice, about 6 weeks of age, were used. They were housed at a temperature of about 25°C with 12 hours /12 hours darkness photoperiod and fed on mouse pellets (Laboratory chow, Unga Feed ® Co.) and water *ad libitum.* All the mice used had an average of 27- 30g.

2.5.2. Snails

Biomphalaria pfeifferi snails were collected from Mwea in Kirinyaga District. The snails were scooped out of water using a sieve attached to a long pole and then transferred from the sieve into water in plastic basins using a pair of forceps. They were transported in plastic containers lined with cotton wool to the at IPR Malacology laboratory. The snails collected were screened for cercaria under a strong light bulb (100watts) for 2h once every week for 5 weeks. Snail that were negative were housed in temperature a controlled

(25 -27°C) snail room. Plastic tanks were washed thoroughly using snail water (chlorine free water from IPR well). Sand and gravel from Mwea was sterilized by heating at 150°C for 12h then cooled and layered in plastic tanks. Then, snail water was poured in the tanks three quarter full. The screened snails were then transferred into the tanks for maintenance. The water was changed twice a week. Soft lettuce (dipped in hot water, cooled and dried in an oven) was added and fed to the snails. The tanks were aerated using *Daphnea*.

2.6 Hatching of *S. mansoni miracidia* from the eggs

A strain of *Schistosoma mansoni* originally obtained from humans and maintained in olive baboon (*Papio anubis*) at IPR was used in the study. Faecal samples from olive baboons with chronic *S. mansoni* infection were collected and placed in a plastic beaker. The faecal samples were thoroughly mixed with one litre of tap water in a plastic jar using a wooden spatula. The slurry was poured on a standard test sieve (Arthur Thomas Co. USA) with pore size of 600 µm placed on another test sieve of 250 µm into a collecting tray. The filtrate was transferred into urine jars while the faecal debris was discarded. Urine jars containing filtrate were filled with tap water and left to stand for 30 minutes in the dark. The supernatant was then be poured out and the pellet re-suspended in water and allowed to sediment in the dark. This procedure was repeated three times until the supernatant become clear. The clear supernatant was then poured out. The sediment was dispensed into a petri dish containing fresh water and then placed under a lamp of 100 watts for at least 1h to allow miracidia to hatch from the eggs.

2.7 Infection of snails with *S. mansoni* miracidia and shedding of infected snails

Using a Pasteur pipette with a rubber bulb 3-5 miracidia were picked from the petri dish under the dissecting microscope. The miracidia were dispensed into each well in a 24 well culture plate. The snails were then transferred individually from the snail tank into the wells and the plates covered with a cover to prevent the snails from crawling out. The plates were left for 30 minutes at room temperature to allow penetration after which snails were transferred into newly prepared snail tanks. Four weeks post infection the snails were covered with black clothes to prevent light from stimulating shedding of cercariae. Five weeks post infection snails were carefully removed from tanks using forceps and placed in 10 ml beakers containing fresh water. They were exposed to 100 watt lamp shielded with glass for 1-3h to shed cercariae. The cercariae suspension was then pooled together and counted in three 50 µm aliquots under the dissecting microscope. A volume containing 250 cercariae for challenge was then calculated.

2.8 Infection of mice with *S. mansoni* cercariae

At the start of bioassay (week 0) general anaesthesia was administered to the mice to produce loss of consciousness and suppression of reflex activity and muscle relaxation. A ratio based on volume of 3: 1 Ketamine and Rompun and (Agar, Holland) was used to provide a combined effect of anaesthesia. The anaesthesia dose of 0.02 ml /30g mouse body weight was injected intraperitoneally. The anaesthetized mice were shaved on the stomach area and arranged on a wooden rack as shown in (Figure 2.10). Cotton wool dipped in water was used to moisten the shaven area to allow easy penetration of cercariae. A 1cm metal ring was placed on the shaven area of each mouse, then, a

suspension constituting approximately 250 live cercariae were dispensed in the metal ring using a micropipette and a period of 30 minutes allowed for cercariae to penetrate into the mouse (Smithers and Terry, 1965).

Figure 2.4: Mice arranged on a wooden rack and cercariae dispensed in the ring to allow penetration into the mice.

2.9. Treatment of infected mice using plant extracts

For each plant extracts 150mg was dissolved in 10ml and 200 µl of each suspension was drawn. The plant extracts suspension was administered by dosing syringe which was placed in the oesophagus of the mouse slowly and treatment was repeated after 2 days. The positive control group received two doses of Praziquantel (PZQ) which is a reference drug at 900 mg/kg. For Infected control Swiss mice were treated with physiological saline.

2.10 Data Analysis

Data were expressed as mean ± standard error of means. Statistical analyses were done using Student's *t*-test for paired samples. ANOVA was used to compare the effective doses of the five plants extracts treatment in Swiss mice and 5% confidence limit was considered statistically significant. Sigma Prot (Systat software Inc.) were used for analysis of ANOVA and Student's t-test.

CHAPTER 3: Phytochemical analysis of the five plant extracts

3.1 Introduction

Plants have a long history of use on the African continent for the treatment of different diseases and complaints. In most African countries, up to 90% of the population still relies exclusively on plants as a source of medicine. The use of medicinal plants has gained much attention in the last decade, and among those plants commonly used as medication in folk medicine, various extracts have been the subject of many pharmacological studies (Artuso, 1997).

Plants are the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer *et al.*, 1999). Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants grow in different parts of the country. Thousands of species are known to have medicinal value and the use of different parts of several medicinal plants to cure specific ailments has been in vogue since ancient times.

Whereas medicinal plants have produced some very effective treatment for malaria as in case of artemisinin (Frederich *et al*, 2002), few attempts have been made to evaluate antischistosomal activity of such natural plants (Molgaard *et al*., 2001). The importance of plants as sources of natural product bioactive molecules lies not only in their pharmacological or chemotherapeutic effect but also in their role as template molecules for the production of new drugs (Phillipson, 1994).

The discovery of medicinal plants has usually depended on the experience of the populace based on long and dangerous self experiment. Progress over the centuries towards a better understanding of a plant derived medicine has depended on two factors that have gone hand in hand (Herborne, 1993).

One has been the development of increasingly strict criteria of proof that a medicine really does what it is claimed to do and the other has been the identification by chemical analysis of the active compound in the plant (Phillipson, 1994).

According to World Health Organization (2002a), more than 80% of the world's population relies on traditional medicines for their primary health care needs. The medicinal value of plants lies in some chemical substances that produce a definite physiological effect on the human body. The phytochemical research based on ethno-pharmacological information is generally considered an effective approach in the discovery of new effective agents from plants.

Medicinal plants therefore represent an important opportunity to rural communities in Africa, as a source of affordable medicine and as a source of income. Governments too need to be thinking about how to promote the benefits that medicinal plants have to offer, which may involve integrating herbal medicine into conventional healthcare systems. This raises important issues, such as regulation of traditional healers and ensuring certain standards are met (Koko *et al.*, 2005).

3.2 Phytochemical metabolites

Alkaloids are basic nitrogenous compounds with definite physiological and pharmacological activity. Alkaloid solution produces white yellowish precipitate when a few drops of Mayer's reagents are added (Siddiqui and Ali, 1997). Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent (Harbone, 1998).

Reducing sugar is any sugar that either has an aldehyde group or is capable of forming one in solution through isomerisation. This functional group allows the sugar to act as a reducing agent, include glucose, fructose, glyceraldehydes and galactose (Harbone, 1998)

Glycosides are compounds which upon hydrolysis give rise to one or more sugars (glycones) and a compound which is not sugar (aglycone or genine).

Terpenoids are a large and diverse class of naturally occurring organic chemicals similar to terpenes, derived from five-carbon isoprene units assembled and modified in thousands of ways. Most are multicyclic structures that differ from one another not only in functional groups but also in their basic carbon skeletons. These lipids can be found in all classes of living things, and are the largest group of natural products.(Siddiqui and Ali, 1997).

Steroid is a type of organic compound that contains a characteristic arrangement of four cycloalkane rings that are joined to each other. Hundreds of distinct steroids are found in plants, animals, and fungi. Steroids are used in medicine to treat many diseases.

Flavonoid, also referred to as bioflavonoid, is a polyphenol antioxidants found naturally in plants. They are secondary metabolites, meaning they are organic compounds that have no direct involvement with the growth or development of plants. Flavonoids are widely disbursed throughout plants and are what give the flowers and fruits of many plants their vibrant colors. They also play a role in protecting the plants from microbe and insect attacks. More importantly, the consumption of foods containing flavonoids has been linked to numerous health benefits. They are antioxidant, an indication that they biologically trigger the production of natural enzymes that fight disease (Siddiqui and Ali, 1997).

Tannins are widely applied to any large polyphenolic compound containing sufficient hydroxyls and other suitable groups such as carboxyl to form strong complexes with proteins and other macromolecules. The tannin compounds are widely distributed in many species of plants, where they play a role in protection from predation, and perhaps also as pesticides (Iyengar, 1995).

Saponins are essentially a type of chemical substance, with numerous structures that are discovered in natural sources. In certain types of plants, saponins come in great numbers. All saponins form soapy foam when shaken in a solution. Of particular interest is the use of saponins against the vector of the disease schistosomiasis (Togola *et al.*, 2008)

3.2 MATERIALS AND METHODS

3.2.1 Plants identification

The plants after collection were taken to National Museum of Kenya for identification as shown in Appendix I. The plants and their family and local names were used for the research (Table 3.1) as follows:

Table 3.1: The five plants used in the study with their family and local names

Family	Scientific name	Local name
Euphorbiaceae	*Bridelia micratha*	Mukoigo-Kikuyu
Euphorbiaceae	*Croton megalocarpus*	Mukinduri-Kikuyu
Labiatae	*Ocimum americanum*	Mutaa- Kamba
Aloaceae	*Aloe secundiflora*	Kiluma - Kamba
Compositae	*Sonchus luxurians*	Uthunga- Kamba

3.2.2 Identification tests to test the presence of various chemical constituents

Tests were done to find the presence of the active chemicals constituents such as alkaloids, glycosides, terpenoids and steroids, flavonoids, reducing sugar and tannin by the following procedures.

3.2.2.1 Alkaloid

To 0.5 ml of the extract solution was evaporated to dryness and 2% hydrochloric acid added in the residue heated on a boiling water bath. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation (Siddiqui and Ali, 1997).

3.2.2.2 Glycoside

To 0.5 ml of the extract solution in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid was added, and observed for a reddish brown coloration at the junction of two layers and bluish green color in the upper layer (Siddiqui and Ali, 1997).

3.2.2.3 Terpenoid and Steroid

To 4 mg of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Concentrated solution of sulphuric acid was added slowly and red violet color was observed for terpenoid and green bluish color for steroids (Siddiqui and Ali, 1997).

3.2.2.4 Flavonoid

Four milliliters of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of concentrated hydrochloric acid was added and red color was observed for flavonoids and orange color for flavones (Siddiqui and Ali, 1997).

3.2.2.5 Tannins

To 0.5 ml of extract solution, 1 ml of water and 1-2 drops of ferric chloride solution was added. Blue color was observed for Gallic tannins and green black for catecholic tannins (Iyengar, 1995).

3.2.2.6 Reducing Sugar

To 0.5 ml extract solution, 1 ml of water and 5-8 drops of Fehling's solution was added hot and observed for brick red precipitate (Siddiqui and Ali, 1997).

3.2.2.7 Saponins

The extract was diluted 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 presence of Saponins. The frothing was mixed with 3 drops of olive oil and shaken vigorously. Presence of Saponins results in formation of an emulsion (Siddiqui and Ali, 1997).

3.3 RESULTS

3.3.1 The Composition of five plant extracts

The composition of five plant extracts is shown on the Table 3.2.The Hexane and Dcm/ Meth extracts of the five plants were semi- solid or sticky composition while water extracts powder, pellets and semi- solid or sticky form.

Table 3.2: The state of five plant extracts

EXTRACT	STATE		
	H	**Dcm/Meth**	**Water**
Ocimum americanum	Semi- solid and sticky	Semi- solid and sticky	powder
Bridelia micrantha	Semi- solid	Semi- solid	pellet
Sonchus luxurians	Semi- solid	Semi-solid	powder
Croton megalocarpus	Semi- solid	Semi-solid	powder
Aloe secundiflora	Semi- solid and sticky	Semi-solid and sticky	Semi- solid and sticky

KEY:

H- Hexane extract

DCM/Meth- Dichloromethane/methanol extract

3.3.2 Phytochemical analysis of the five plant extracts

The phytochemical analysis to determine the presence of the active chemical constituents in the five plant extracts were carried out as shown below:

3.3.2.1 *Ocimum americanum* plant extract

The presence of the chemical constituent on the three extract in this group are shown on the **Table 3.2(A)**: Steroid, flavones and catecholics were common in the *Ocimum americanum* plant extracts while flavonoid, Gallic and saponins were absent.

3.3.2.2 *Bridelia micrantha* plant extract

The presence of the chemical constituent on the three extract in this group are shown on the Table 3.3 (B). Alkaloid, catecholics, glycosides, saponins and reducing sugar were present in all or two of the *Bridelia micrantha* plant extracts.

3.3.2.3 *Sonchus luxurians* plant extract

The presence of the chemical constituent on the three extract in this group are shown on the Table 3.4 (C): Glycosides, steroid and catecholic were present in all or two of *Sonchus luxurians* plant extracts.

3.3.2.4 *Croton megalocarpus* plant extract

The presence of the chemical constituent on the three extract in this group are shown on the Table 3.5 (D). Terpenoid, flavonoid and reducing sugar were present in all or two of *Croton megalocarpus* plant extracts.

3.3.2.5 *Aloe secundiflora* plant extract

The presence of the chemical constituent on the three extract in this group are shown on the Table 3.6 (E): Catecholic was present in two of *Aloe secundiflora* plant extract however, terpenoid, saponins and Gallic were absent in all plant extracts.

Table 3.2 (A): Presence of the active chemical constituents in *Ocimum americanum* plant extract

	Alkaloid	Glycosides	Terpenoid	Steroid	Flavonoid		Tannins		Reducing sugar	saponins
					Flavonoid	flavones	Gallic	catecholic		
OAH	-	-	-	+	-	-	-	-	-	-
OAD	-	-	-	+	-	+	-	+	Trace	-
OAW	+	+	+	-	-	+	-	+	+	-

Table 3.3 (B): Presence of the active chemical constituents in *Bridelia micrantha* plant extracts

	Alkaloid	Glycosides	Terpenoid	Steroid	Flavonoid		Tannins		Reducing sugar	saponins
					Flavonoid	flavones	Gallic	catecholic		
BMH	+	+	-	+	-	+	-	+	-	-
BMD	+	+	+	-	-	-	-	+	+	+
BMW	+	Trace	+	-	+	-	-	+	+	+

Key:
OAH- *Ocimum americanum* hexane extract
OAD- *Ocimum americanum* Dichloromethane/methanol extract
OAW- *Ocimum americanum* water extract
BMH- *Bridelia micrantha* hexane extract
BMD- *Bridelia micrantha* Dichloromethane/methanol extract
BMW- *Bridelia micrantha* water extract

Table 3.4 (C): Presence of the active chemical constituents in Sonchus luxurians plant extracts

	Alkaloid	Glycoside	Terpenoid	Steroid	Flavonoid		Tannins		Reducing sugar	saponins
					Flavonoid	flavones	Gallic	catecholic		
SLH	-	+	-	+	-	-	-	-	-	-
SLD	-	+	-	+	+	-	-	+	-	Trace
SLW	+	+	+	-	-	-	-	+	-	Trace

Table 3.5 (D): Presence of the active chemical constituents in *Croton megalocarpus* plant extract

	Alkaloi	Glycosid	Terpeno	Steroi	Flavonoid		Tannins		Reducing sugar	saponins
					Flavonoid	flavone	Gallic	catecholic		
CMH	-	-	+	-	+	-	-	-	-	-
CMD	-	-	+	-	+	-	-	-	+	-
CMW	+	+	+	-	-	+	-	-	+	+

Key:

SLH- *Sonchus luxurians* hexane extract

SLD- *Sonchus luxurians* Dichloromethane/methanol extract

SLW- *Sonchus luxurians* water extract

CMH- *Croton megalocarpus* hexane extract

CMD- *Croton megalocarpus* Dichloromethane/methanol extract

Table 3.6 (E): Presence of the active chemical constituents in *Aloe secundiflora* Plant extracts

	Alkaloid	Glycosides	Terpenoid	Steroid	Flavonoid		Tannins		Reducing sugar	saponins
					Flavonoid	flavones	Gallic	catecholic		
ASH	-	-	-	-	-	-	-	-	-	-
ASD	-	-	-	-	-	+	-	+	-	-
ASW	+	+	-	-	+	-	-	+	traces	-

Key

ASH- *Aloe secundiflora* hexane extract

ASD- *Aloe secundiflora* Dichloromethane/methanol

ASW- *Aloe secundiflora* water extract

3.4: DISCUSSION

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Wink, 1999).The medicinal effects of plant materials typically result from the secondary products present in the plant although, it is usually not attributed to a single compound but a combination of the metabolites.

Phytochemicals have been used as drugs for millennia. For example, Hippocrates may have prescribed willow tree leaves to abate fever. Salicin, having anti-inflammatory and pain-relieving properties, was originally extracted from the bark of the white willow tree and later synthetically produced became the staple over-the-counter drug aspirin (Molgaard *et al.*, 2001).

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. They are non essential nutrients, meaning that they are not required by the human body for sustaining life. It is a well-known fact that plants produce these chemicals to protect themselves and recent research demonstrates that they can also protect humans against diseases. There are more than thousand known phytochemicals (Koko *et al.*, 2005)

Presence of phytochemical compounds in these extracts illustrates the bioactive compounds in the extracts. Steroid, flavones and catecholics were common in the *Ocimum americanum* plant extracts while flavonoid, gallic and saponins were absent. Alkaloid, catecholics, glycosides, saponins and reducing sugar were present in all the *Bridelia micrantha* plant extracts. The above compounds able to bind physically to cell walls thereby preventing the adhesion of pathogens to human cell walls hence have antimicrobial activity (Nostro *et al.*, 2000).

Glycosides, steroid and catecholic were present in all *Sonchus luxurians* plant extracts. Terpenoid, flavonoid and reducing sugar were present in all *Croton megalocarpus* plant extracts.

Catecholic was present in two of *Aloe secundiflora* plant extract however, Terpenoid, Saponins and Gallic were absent. The most common compound in all the plant extracts was catecholics. Catecholics is tannin and tannins compounds are widely distributed in many species of plants, where they play a role in protection from predation, and perhaps also as pesticides.

Hundreds of distinct steroids are found in plants, animals, and fungi. Steroids are used in medicine to treat many diseases. Of particular interest is the use of saponins against the vector of the disease schistosomiasis. This includes a powerful antifeedant compound which is a highly modified limonoid from neem tree *Azadirachta indica* which is known as snail poison (Cioli *et al.*, 1995).

Herbal medicine is still the mainstay of about 75 - 80% of the whole population, and the major part of traditional therapy involves the use of plant extract and their active constituents (Akerere, 1993).

CHAPTER 4: Anti-Schistosomal activity of five plant extracts and different dosages for efficacious extracts

4.1. INTRODUCTION

Schistosomiasis, like many tropical diseases, is endemic in areas where poor living conditions and poverty are prevalent. Because these are water-borne species, populations that primarily rely on agriculture and fishing for their livelihood are at the highest risk of contamination. Often, individuals in these regions are also co-infected with other parasitic diseases such as malaria and hook worm. Women and children (peaking at age 10 to 19 years) are at high risk. Children play in water and women use water for their daily chores. Given the increased migration from rural to urban regions, the disease is spreading to urban areas and infecting swimmers. Due to increased ecotourism, the cases of schistosomiasis are being diagnosed more often in travelers (Engles et al., 2002).

Praziquantel is generally considered the drug of choice for treatment of Schistosomiasis (Hotez, 2009). The recommended dose is: Praziquantel, 60mg/kg of body weight for children up to 15 years old, and 50mg/kg of body weight for adults (King, 1989). Both drugs are administered as a single dose treatment. The treatment objective is to cure the disease and to prevent the evolution of infected patients to the chronic form of the disease. All confirmed cases should be treated (Utzinger *et al.*, 2003).

Many drugs have originated from biologically active plant chemicals, and many plants' medicinal uses can be attributed to various active chemicals found in them, there is a distinct difference between using a medicinal plant and a chemical drug. Drugs usually

consist of a single chemical, whereas medicinal plants can contain 400 or more chemicals (WHO, 2002a; Bah *et al.*, 2008).

Frequency of quote is an important indicator of the usage of the plants by the community. However, information obtained from the community may not always be reliable. It is possible that people may quote a particular plant more frequently since it is easily available, easily recognizable or resembles a certain disease (WHO, 2000).

With the tremendous increase in the global use of medicinal plants, several concerns regarding the efficacy and safety of the herbal medicines have also been raised (WHO, 1990). Hence it has become necessary to standardize the efficacy and safety measures so as to ensure supply of medicinal plant materials with good quality. The exact dosage of medicinal plants is often the weak point of traditional healers (Ramadan *et al.*, 2004).

The five plants used has no reference in literature but normally used as concoction to treat schistosomiasis in Machakos. Extracts using various solvent were tested for antischistosomal activity. It was also necessary to perform different dosages of the efficacious plants to ascertain the safer dose which will be safer to be used as a drug.

4.2. MATERIALS AND METHODS

4.2.1 Experimental Design

The mice that had been exposed to 250 cercariae of *S. mansoni* were divided into 18 groups with 6 mice each as per experimental design (Table 4.1). Mouse in a specified

group was marked using picric acid for identification during the fourth week of infection. On the sixth week, mice were treated with their respective plant extracts; The extracts were as follows: OAH- *Ocimum americanum* hexane extract, OAD- *Ocimum americanum* Dcm//methanol extract, OAW- *Ocimum americanum* water extract, OAC- *Ocimum americanum* crude, BMH- *Bridelia micrantha* hexane extract, BMD- *Bridelia micrantha* Dcm /methanol extract, BMW - *Bridelia micrantha* water extract, BMC- *Bridelia micrantha* crude, SLD- *Sonchus luxurians* , Dcm /methanol extract, SLW- *Sonchus luxurians* water extract, SLC- *Sonchus luxurians* crude.

CMW- *Croton megalocarpus* water extract, CMC- *Croton megalocarpus* crude, ASW- *Aloe secundiflora* water extract and ASC - *Aloe* secundiflora crude. CMH- *Croton megalocarpus hexane* and ASH- *Aloe secundiflora* hexane extracts were not soluble therefore were left out.

Mice in the positive control group were treated with Praziquantel (Biltricide) manufactured by Bayer- Germany. Distilled water was used as a carrier vehicle for the Praziquantel drug. Infected control Swiss mice were treated with physiological saline. Naïve mice were untreated. Perfusion for adult worm recovery was at week six and their livers were observed for gross pathology. The livers were preserved in 10% Formalin for histopathology.

Table 4.1: Experimental design for testing efficacy of plants extracts against S. *mansoni*

Plants extracts	WK0	WK4	WK6
OAH	I	T – 150mg\ kg	B/P
OAD	I	T – 150mg\ kg	B/P
OAW	I	T – 150mg\ kg	B/P
OAC	I	T – 150mg\ kg	B/P
BMH	I	T – 150mg\ kg	B/P
BMD	I	T – 150mg\ kg	B/P
BMW	I	T – 150mg\ kg	B/P
BMC	I	T – 150mg\ kg	B/P
SLD	I	T – 150mg\ kg	B/P
SLW	I	T – 150mg\ kg	B/P
SLC	I	T – 150mg\ kg	B/P
CMW	I	T – 150mg\ kg	B/P
CMC	I	T – 150mg\ kg	B/P
ASW	I	T– 150mg\ kg	B/P
ASC	I	T – 150mg\ kg	B/P
PZQ	I	T – 900mg/kg	B/P
IC	I	-	B/P
Naive	-	-	B/P

Key:

I – Infection with *S.mansoni* cercariae
T- Treatment with crude /plant extracts/
PZQ - Praziquantel
IC – Infected control
B- Sampling for blood
P- Perfusion for adult worm recovery, gross and histopathology
OAH- *Ocimum americanum* hexane extracts
OAW- *Ocimum americanum* water extract
OAD- *Ocimum americanum* Dcm/methanol extract
OAC- *Ocimum americanum* crude
BMH- *Bridelia micrantha* hexane extract
BMW- *Bridelia micrantha* water extract
BMD- *Bridelia micrantha* Dcm/methanol extract
BMC- *Bridelia micrantha* crude
ASC - *Aloe secundiflora* crude
SLD- *Sonchus luxurians* Dcm/methanol extract
SLW- *Sonchus luxurians* water extract
SLC- *Sonchus luxurians* crude
CMW- *Croton megalocarpus* water extract
CMC- *Croton megalocarpus* crude
ASW- *Aloe secundiflora* water extract

4.2.2. Parasitological Assay

4.2.2.1 Perfusion

At week 6 mice from each infected group were perfused to recover the adult worms using a modified method of Smithers and Terry (1965). Each mouse was anaesthesized with a dose of 0.02ml of ketamine/rompun mixture. A transverse mid ventral cut was made on the skin of the abdomen with a pair of scissors and the skin peeled off upwards and downwards. The abdominal wall was opened without cutting the viscera. A cut was made through the diaphragm. A cut was made on either side of xiphoid cartilage and the rib cage trimmed on either side to expose the heart. Care was taken not to puncture vessels next to xiphoid cartilage and also heart and lungs. The hepatic portal vein was incised and a perfusion needle containing perfusion fluid (0.85% sodium chloride and 1.5% sodium carbonate) inserted on the left ventricle of the heart and perfusion was carried out until the liver, lower limbs and mesenterics were clear. The perfusate was collected in a 20 cm glass petri dish container and transferred in a urine jar to settle.

4.2.2.2 Adult worm recovery

The adult worms were recovered using the method of Yole *et al.*, (1996). Urine jars with perfusate containing the recovered worms were topped to 1 litre with phosphate buffered saline (PBS). After the worms settled, the supernatant was sucked out, more PBS added and the settling procedure repeated three times. When the supernatant was clear, the worms were placed on a 10 cm petri dish containing PBS and the worms counted. After perfused mice were soaked on a plate dish containing PBS for extra worms to crawl out of the liver where they might have been trapped. The worm maturation, percentage worm

recovery and percentage worm reduction of adult worm for each group was calculated as shown below:

$$\text{Worm maturation} = \frac{\text{(Number of worms recovered in infected control)}}{\text{(Initial number of infecting cercariae)}} \times 100$$

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

$$\text{Percentage Worm reduction} = \frac{\text{(Mean of total worms in infected control} - \text{(Mean of total worms in experimental group)}}{\text{(Mean of total worms in infected control)}} \times 100$$

4.2.3. Gross and Histopathological Assessment

4.2.3.1 Gross Pathology

Gross pathology was focused on the general and overt appearance of the liver. Observation that was considered in the liver was inflammation, adhesions and presence of granulomas. Granulomas appear as numerous raised pinheads sized foci distributed over the surface of the liver lobes. Categorizing was done as following: 1-3 granulomas per lobe were considered few, 4-10 granulomas per lobe were considered moderate and ≥ 10 granulomas per lobe was considered severe.

4.2.3.2 Histopathological Assessment

After perfusion and recovery of worms, the liver was removed and preserved in 10% formalin for at least 2 weeks. A representative portion was obtained and washed in running water overnight to remove excess formalin. The tissues were then processed using an automatic tissue processor; then dehydrated sequentially in increasing concentrations of alcohols of 50%, 80%, 90%, and 96% at hourly stepped intervals. The tissues were then cleared off alcohol twice in two changes of Xylene. Infiltration with paraffin wax was done for 3 h in the paraffin wax oven set at 2^0 C below the melting point of wax (Baker *et al.* 1989). The tissues were then embedded in fresh molten paraffin wax and allowed to dry. The embedded tissues were sectioned at 0.7 µm thicknesses with a microtome and floated in warm water to spread out, then attached onto clean microscopic slides. After holding in hot oven for 15 m, the tissue sections were dewaxed in Xylene rehydrated and stained with haematoxylin and eosin dyes using standard histological procedures. The stained tissues were covered slipped with DPX, dried and examined microscopically for granulomas. Granulomas sizes were measured. The mean size was calculated from average of width and length (Farah *et al.*, 2000).

4.2.4 Treatment of infected mice using different dosages

The plant extracts which were found to be more efficacious were: *Ocimum americanum* hexane, water and *Bridelia micrantha* water extracts. Different doses of the plant extracts of *Ocimum americanum* hexane, water and *Bridelia micrantha* water extracts were prepared using different concentrations : 150mg , 300mg and 600 mg per kg each and thereafter 200 µl of each suspension was drawn. The plant extracts suspension was

administered by dosing syringe which was placed in the oesophagus of the mouse and given twice on alternating days. The experimental design is shown in Table 4.2. Infection of Snail, infection of Mice, Perfusion, Gross and Histopathology was carried out as described in Chapter 2 (section 2.7. and 2.8) and this Chapter (section 4.2.2. and 4.2.3.)

Table 4.2: Experimental design for different dosage

Plants extracts	WK0	WK4	WK6
OAH	I	T(150mg/kg) (300mg/kg) (600mg/kg)	B/P
OAW	I	T(150mg/kg) (300mg/kg) (600mg/kg)	B/P
BMW	I	T(150mg/kg) (300mg/kg) (600mg/kg	B/P
PZQ	I	T(900mg/kg)	B/P
IC	I	-	B/P
Naïve	-	-	B/P

KEY:
PZQ-Praziquantel
OAH- *Ocimum americanum* hexane extract
OAW- *Ocimum americanum* water extract
BMW- *Bridelia micrantha* water extracts

IC- Infected control
150mg- low concentration
300mg- medium concentration
600mg –High concentration
B- Bleeding
P- Perfusion

4.3. RESULTS

4.3.1 Worm recovery and reduction

Worm reduction percentages of PZQ were (75.2%) as shown on Table 4.3 and Figure 4.1. There was no significant difference between PZQ and the following plant extracts groups ($p > 0.05$): OAH (68.7%), BMW (63.4%), OAW (61.6%), OAC (52%) and BMH (48.7%).The plant extracts were similar to PZQ indicating high worm reduction percentage therefore able to protect the animal from parasitic infection. On the other hand PZQ compared with the other groups; OAD (48.3%), CMW (47.7%), SLD (46.3%), BMD (42%), CMC (38.7 %), SLW (36.3 %), ASW (34.2%), ASC (30.9%), BMC (30.6), SLC (24.2) and the difference was significant (Anova; $p < 0.001$). When IC was compared with PZQ, OAH, BMW, OAW, OAC and BMH showed significant difference (Anova; $p < 0.001$). However there was no significant difference between IC and OAD, SLD, BMD, CMW, SLW, ASW, SLC, ASC and BMC treatment groups (Anova; $p > 0.05$). These plant extracts were similar to IC indicating low worm reduction percentage therefore they were unable to protect against infection. Similar results were obtained when the experiments were repeated (Appendix 4).

Table 4.3: Mean number of worms, recovery and reduction percentage

		Mean number of worms recovered per				
Treatment Group	Dose mg/kg	Total males	Total Females	Total worms **Mean ± SE**	% Worm recovery	% Worm reduction
PZQ	900X2	10.8±0.47	6.8 ± 1.43	17.6 ± 1.9	24.8	75.2
OAH	150X2	14.3±1.45	7.7 ± 0.88	22.0 ± 2.33	31.3	68.7
OAD	150 X2	20.3±3.84	16.3 ± 2.91	36.6 ± 6.75	51.7	48.3
OAW	150 X2	14.0 ± 2.16	13.0 ± 3.24	27.0± 5.4	38.4	61.6
OAC	150 X2	19.8 ±4.85	14.0 ± 3.76	33.8 ±8.61	48.0	52.0
BMH	150X2	23.5 ± 3.52	12.8± 1.68	36.3 ±5.2	51.3	48.7
BMD	150 X2	22.8 ± 2.14	18.0 ± 3.54	40.8 ± 5.68	58.0	42.0
BMW	150 X	13.8 ± 0.63	12.0 ± 6.52	25.8 ± 7.15	36.6	63.4
BMC	150 X2	28.0 ± 3.39	20.8 ± 3.17	48.8 ± 6.56	69.4	30.6
SLD	150 X2	21.0 ± 4.46	16.8 ± 4.53	37.8 ± 8.99	53.7	46.3
SLW	150 X2	21.3 ± 1.93	23.3 ± 4.78	44.6 ± 6.71	63.4	36.6
SLC	150 X2	29.8 ± 1.70	23.5 ± 3.23	53.3 ± 4.93	75.8	24.2
CMW	150 X2	20.0 ± 2.35	16.8 ± 2.75	36.8 ± 5.1	52.3	47.7
CMC	150 X2	24.3 ± 3.33	18.8 ± 1.80	43.1 ± 5.13	61.3	38.7
ASW	150 X2	30.0 ± 5.57	16.3 ± 1.20	46.3 ± 6.77	65.8	34.2
ASC	150 X2	27.2 ± 3.10	21.4 ± 2.14	48.6± 5.24	69.1	30.9
IC	-	41.8±5.65	29.0 ± 4.22	70.8 ± 9.87	-	

Key:
PZQ- Praziquantel;
OAH- *Ocimum americanum* hexane extract
OAD- *Ocimum americanum* Dcm//methanol extract
OAW- *Ocimum americanum* water extract
OAC- Ocimum americanum crude
BMH- *Bridelia micrantha* hexane extract
BMD- *Bridelia micrantha* Dcm/methanol extract
BMW- *Bridelia micrantha* water extract
BMC- Bridelia micrantha crude
SLD- *Sonchus luxurians* Dcm//methanol extract
SLW- *Sonchus luxurians* water extract
SLC- *Sonchus luxurians* crude
CMW- *Croton megalocarpus* water extract
CMC- *Croton megalocarpus* crude
ASW- *Aloe secundiflora* water extract
ASC - *Aloe secundiflora* crude
IC – infected control

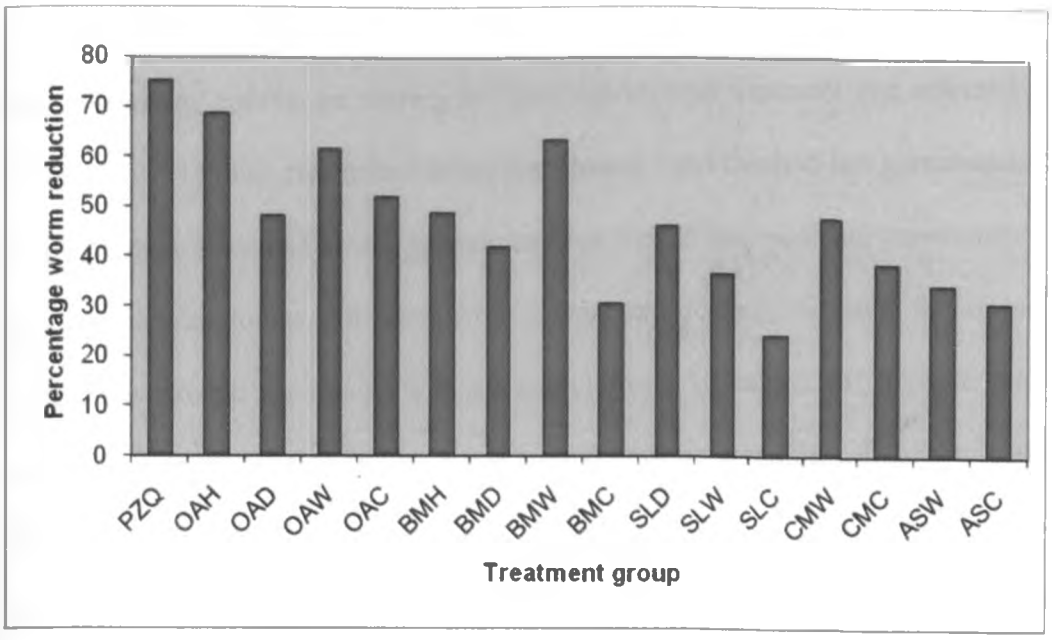


Figure 4.1: Percentage worm reduction of the five plant extracts

Key:
PZQ- Praziquantel
OAH- *Ocimum americanum* hexane extract
OAD- *Ocimum americanum* Dcm/methanol extract
OAW- *Ocimum americanum* water extract
OAC- Ocimum americanum crude
BMH- *Bridelia micrantha* hexane extract
BMD- *Bridelia micrantha* Dcm/methanol extract
BMW- *Bridelia micrantha* water extract
BMC- Bridelia micrantha crude
SLD- *Sonchus luxurians* Dcm//methanol extract
SLW- *Sonchus luxurians* water extract
SLC- *Sonchus luxurians* crude
CMW- *Croton megalocarpus* water extract
CMC- Croton megalocarpus crude
ASW- *Aloe* secundiflora water extract
ASC - *Aloe* secundiflora crude

4.3.2 Gross pathology

Gross pathology results are shown in Table 4.4 in both treatment and infected control groups, In PZQ group, two mice had no granulomas and two had few granulomas. In the IC group, three mice had severe granulomas one mouse had moderate granulomas. Naïve mice had no granulomas. All extracts of *Ocimum americanum* had either few or moderate granulomas except one mouse with no granulomas. All extracts of *Bridelia micrantha* had few, moderate, severe and only one animal with no granuloma. In all the extracts of *Sonchus luxurians* had either moderate, severe except one mouse with few granulomas. In all the extracts *of Croton megalocarpus* had either moderate or severe granulomas. All the extracts of *Aloe secundiflora* had either moderate or severe granulomas. The trend in granuloma pathology was: IC>AS>CM>SL>BM>OA>PZQ for both treatment and control groups. The highest were IC, AS and CM groups while the least were PZQ, OA and BM respectively. Similar results were obtained when the experiment was repeated (Appendix 4).

Table 4.4: Liver gross pathology in different treatment groups and controls

Plant extracts	No. of mice	Granuloma classification for all the groups				Adhesions		Inflam mation	
		None	Few	Moderate	Severe	AP	AA	I	NI
PZQ	4	3(75)	1(25)	-	-	+		+	
OAH	4	1(25)	3(75)	-	-	+		+	
OAD	4	-	2(50)	2(50)	-	+		+	
OAW	4	-	3(75)	1(25)	-	+		+	
OAC	4	-	1(25)	3(75)	-	+		+	
BMH	4	-	1(25)	3(75)		+		+	
BMD	4	-	-	2(50)	2(50)	+		+	
BMW	3*	1(33.3)	2(66.7	-	-	+		+	
BMC	3*	-	-	1(33.3)	2(66.7)	+		+	
SLD	4	-	1(25)	2(50)	1(25)	+		+	
SLW	4	-	-	2(50)	2(50)	+		+	
SLC	4	-	-	1(25)	3(75)	+		+	
CMW	4	-	-	3(75)	1(25)	+		+	
CMC	3*	-	-	1(33.3)	2(66.7)	+		+	
ASW	4	-	-	2(50)	2(50)	+		+	
ASC	4	-	-	2(50)	2(50)	+		+	
IC	4	-	-	1(25)	3(75)	+		+	
Naive	2	-	-	-	-	-		-	

Key:

PZQ- Praziquantel

OAH- *Ocimum americanum* hexanes extract

I- Inflammation

OAD- *Ocimum americanum* Dichloromethane/methanol extract NI- No Inflammation

OAW- *Ocimum americanum* water extract

OAC- *Ocimum americanum* crude

BMH- *Bridelia micrantha* hexane extract

BMD- *Bridelia micrantha* Dichloromethane/methanol extract

BMW- *Bridelia micrantha* water extract

BMC- *Bridelia micrantha* crude

AP-adhesion present

AA- adhesion absent

SLD- *Sonchus luxurians* Dichloromethane/methanol extract

SLW- *Sonchus luxurians* water extract

SLC- *Sonchus luxurians* crude

CMW- *Croton megalocarpus* water extract

CMC- *Croton megalocarpus* crude

ASW- *Aloe* secundiflora water extract

ASC - Aloe secundiflora crude

IC – infected control

* - one mouse died in each of the groups

4.3.3 Histopathology

4.3.3.1 Granuloma sizes of the five plant extracts

Granuloma sizes in were measured using micrometer inserted in a microscope. Granuloma size was calculated as average of its length and breadth (Farah *et al.*, 2000) as shown in Figure 4.2. Granuloma sizes for all the groups were subjected to ANOVA Test. When Granuloma sizes of PZQ was compared with OAH, BMW, OAW, OAC, OAD, CMW and BMH treatment groups there was no significant difference (Anova; $p>0.05$). On other hand when PZQ was compared with IC, BMD, CMC, SLC, SLD, SLW, ASC and ASW there was significant difference (Anova; $p< 0.001$). When IC was compared with PZQ, OAH, BMW, OAW, OAC and OAD there was significant difference (Anova; $p< 0.001$). However there were no significant difference between IC and SLC, SLD, SLW, ASC, ASW, CMC, CMW, BMD, BMC treatment groups (Anova; $p>0.05$). When the experiment was repeated the similar results were obtained (Appendix 4).

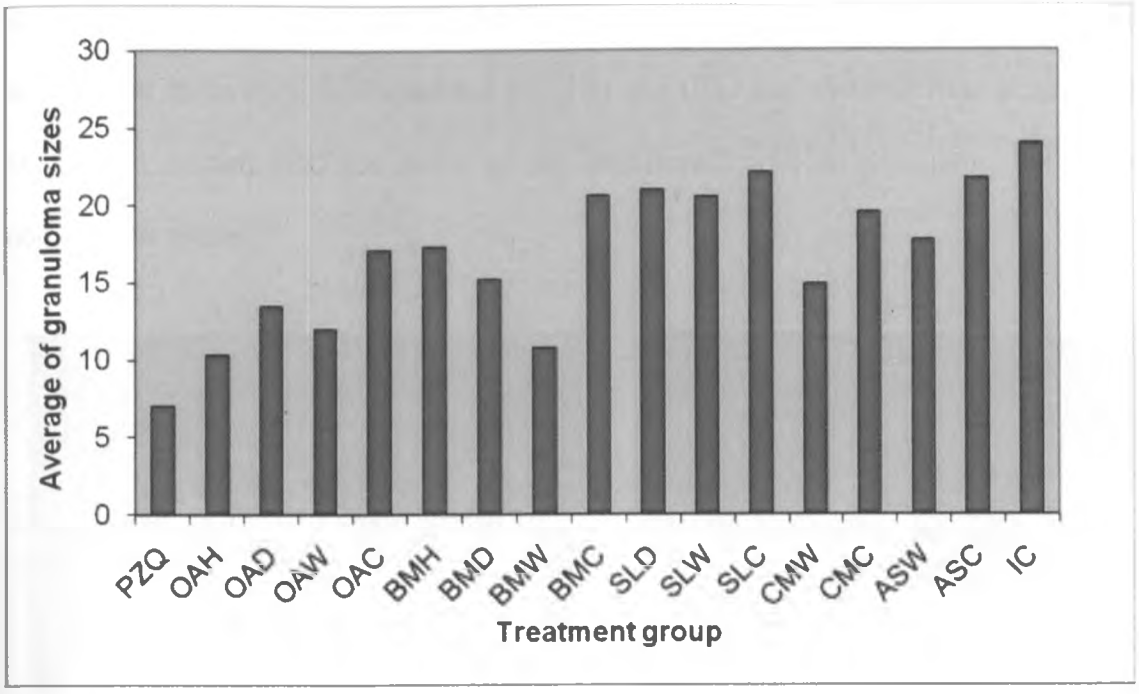


Figure 4.2: Size of granuloma in liver tissue

Key:

PZQ- Praziquantel

OAH- *Ocimum americanum* hexane extract

OAD- *Ocimum americanum* Dichloromethane/methanol extract

OAW- *Ocimum americanum* water extract

OAC- *Ocimum americanum* crude

BMH- *Bridelia micrantha* hexane extract

BMD- *Bridelia micrantha* Dichloromethane/methanol extract

BMW- *Bridelia micrantha* water extract

BMC- Bridelia micrantha crude

SLD- *Sonchus luxurians* Dichloromethane/methanol extract

SLW- *Sonchus luxurians* water extract

SLC- *Sonchus luxurians* crude

CMW- *Croton megalocarpus* water extract

CMC- *Croton megalocarpus* crude

ASW- *Aloe secundiflora* water extract

ASC - *Aloe secundiflora* crude

IC – infected control

4.3.3.2 Photomicrographs of liver tissues sections from mice

In Figure 4.3 (A), (B), (C) and (D) the liver tissues sections were showing a granuloma with one to three eggs. In Figure 4.4 (A), (B) and (C) was showing liver sections of OAH plant extract, PZQ and naïve mouse (uninfected) with no granuloma, but intact normal liver tissue.

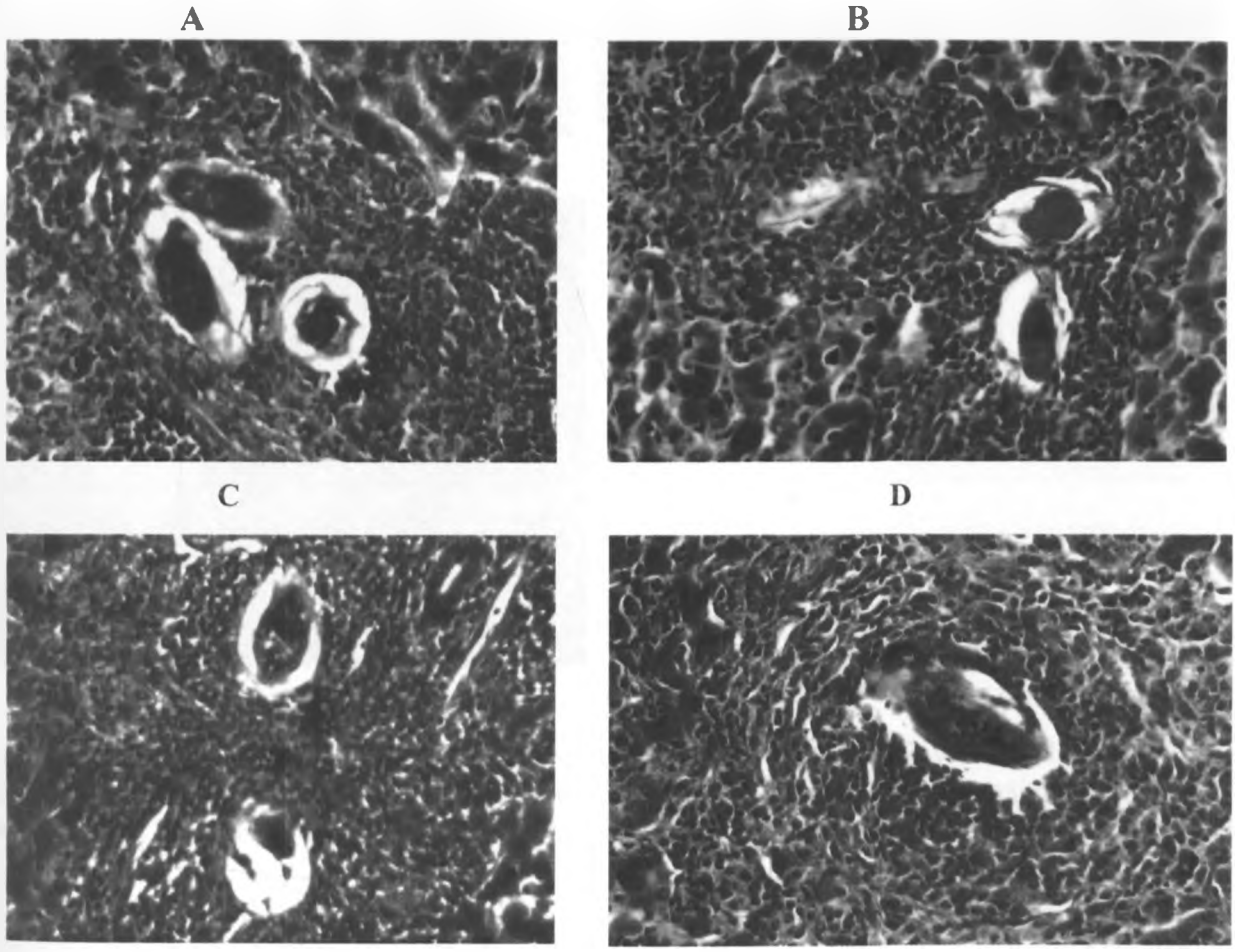


Figure 4.3: Photomicrographs of liver tissue sections (A) from IC group showing a granuloma with three eggs (B) from ASC group showing a granuloma with two eggs (C) from SLC group showing a granuloma with two eggs (D) from CMW group showing a granuloma with one egg. (X 400)

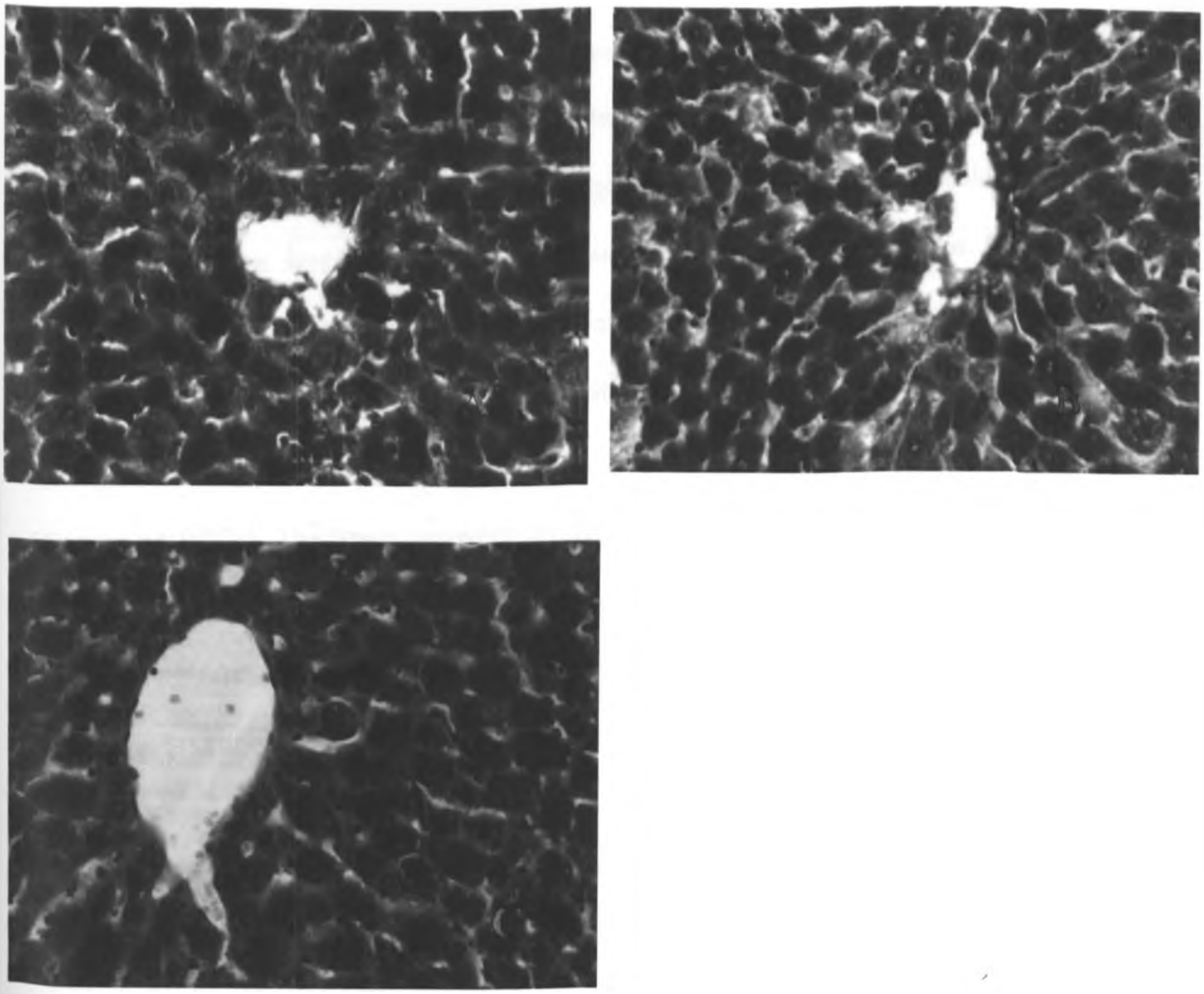


Figure 4.4: Photomicrographs of liver tissues sections (A) from mice treated with OAH showing liver tissue (B) from PZQ treatment group showing liver tissue (C) from naive group showing normal liver tissue (X 400)

4.3.4 Different dosages for eefficacious plant extracts

4.3.4.1 Worm recovery and reduction percentages for efficacious plant extracts

The total means worm recovery and percentage worm reduction are in Table 4.5 and Figure 4.5. There was no significant difference between PZQ and the following treatment groups (Anova; $p > 0.05$): OAH - 300mg), BMW -300mg, OAH – 150, -300mg (OAW). On the other hand PZQ compared with other groups; OAW-600mg, BMW – 150mg, BMW – 600mg, OAW-150 mg and OAH-600mg the difference was significant (Anova; $p < 0.001$). The worm recovery for IC when compared with PZQ, OAH-300 mg, BMW-300mg, OAH- 150mg and OAW-300 mg showed significant difference (Anova; $p < 0.001$). However, there was no significant difference between IC and OAW-600mg/kg, BMW – 150mg, BMW – 600mg, OAW-150 mg and OAH-600mg treatment groups (Anova; $p > 0.05$). The highest worm reduction was PZQ, OAH-300 and BMW - 300 while the lowest was OAW-600, BMW- 150, BMW- 600 and OAW-150 respectively. When the experiment was repeated the similar results were obtained (Appendix 4).

4.5: Mean number of worms and recovery percentage

		Mean number of worms recovered per group (**mean ± SE**)				
Treatment Group	Dose mg/kg	Total males	Total Females	Total worms Mean ± SE	% Worm recovery	% Worm reduction
PZQ	900X2	12.3±3.46	4.5±1.06	16.8±4.52	20.7	79.3
OAH	150X2	22.3±4.84	14.5±3.04	36.8±7.88	45.4	54.6
OAH	300 X2	16.0±2.48	11.0±2.07	27.0±4.55	33.3	66.7
OAH	600 X2	25.6±5.01	22.0±4.0	47.6±9.01	58.8	41.2
OAW	150 X2	25.3±4.20	19±5.01	44.3±9.21	54.9	45.1
OAW	300X2	22.7±5.84	16.7±4.76	39.4±10.6	48.6	51.4
OAW	600 X2	27.4±2.66	23±3.27	50.4±5.93	62.2	37.8
BMW	150 X2	22.5±5.63	18.3±5.37	40.8±11.0	50.3	49.7
BMW	300X2	17.7±4.04	12.0±3.41	29.7±7.45	36.7	63.3
BMW	600 X2	30±5.40	20.0±2.76	50±8.16	61.7	38.3
IC		49.0±5.64	32±4.84	81±10.5	-	

KEY:

PZQ-Praziquantel

OAH- *Ocimum americanum* hexane extract

OAW- *Ocimum americanum* water extract

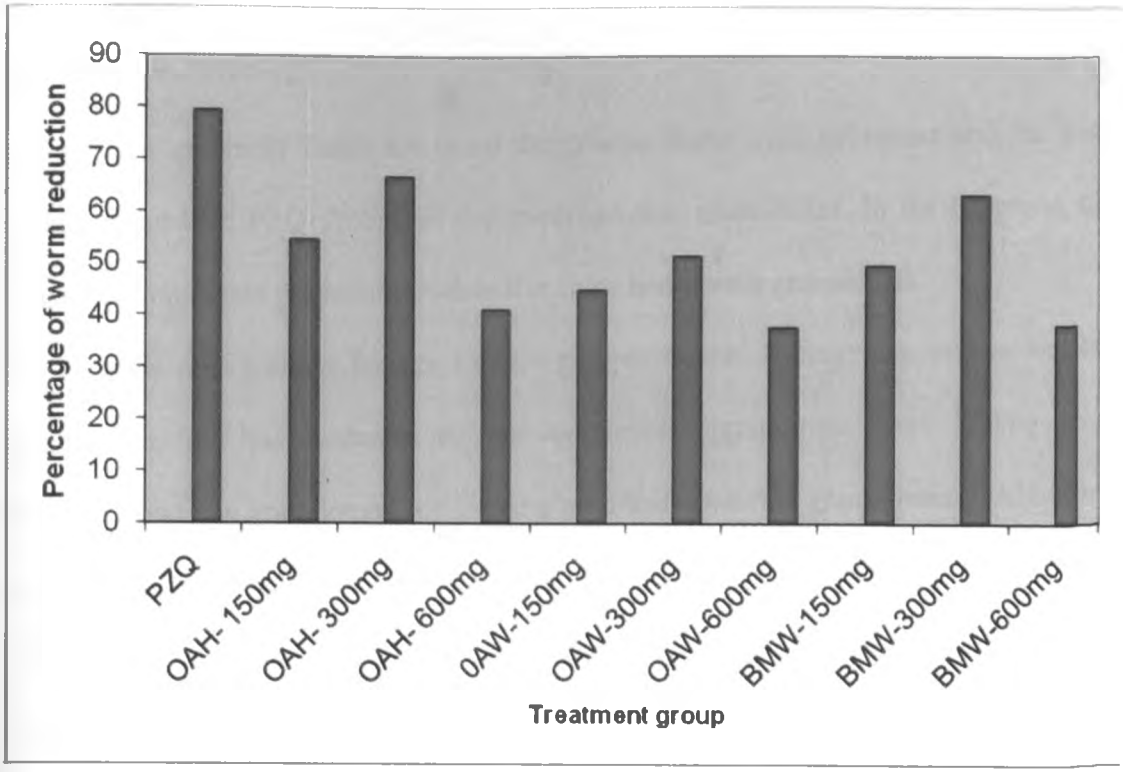
BMW- *Bridelia micrantha* water extracts

IC - Infected Control

150mg- low concentration

300mg- medium concentration

600mg –High concentration



4.5: Percentage of worm reduction for efficacious plant extracts

KEY:

PZQ-Praziquantel

OAH- *Ocimum americanum* hexane extract

OAW- *Ocimum americanum* water extract

BMW- *Bridelia micrantha* water extracts

150mg- low concentration

300mg- medium concentration

600mg –High concentration

4.3.4.2 Gross pathology

Results are shown in Table 4.6 in all the groups, there were adhesions and the livers were inflamed. In PZQ group, all the mice had few granulomas. In the IC group, one mouse had moderate granulomas while five mice had severe granulomas.

In *Ocimum americanum hexane extract* groups; OAH- 150mg: one mouse had few granulomas, four had moderate and one had severe granulomas. OAH- 300mg group: five mice had few granulomas while one mouse had moderate granulomas; OAH-600mg group: three mice with moderate and three mice with severe granulomas.

In *Ocimum americanum* water extract groups; OAW- 150mg: three mice had moderate granulomas and three had severe granulomas. OAW- 300mg group had five mice with moderate granulomas while one mouse had severe granulomas.OAW-600mg group; had three mice with moderate and three mice with severe granulomas.

Bridelia micrantha water extracts groups; BMW - 150mg four mice had moderate and one had severe granulomas. BMW - 300mg group had two mice with few granulomas while four mice had moderate granulomas. BMW -600mg group had four mice with moderate and one mouse with severe granulomas. The highest granuloma pathology in decreasing order was IC<OAW-600<OAW-150<BMW-600<BMW-150<OAH-600<OAH-150<BMW-300<OAH-300<PZQ. Similar results were obtained on repeated experiment (Appendix 4).

Table 4.6: Gross pathology of liver tissue with treatment with efficacious plant extracts

Plant extracts	No. of mice	Granuloma classification for all the groups				Adhesions		Inflammation	
		None	Few %	Moderate %	Severe %	AP	AA	I	NI
PZQ 900mg	4*	-	4(100)	-	-	+		+	
OAH 150mg	6	-	1(16.7	4(66.7)	1(16.7)	+		+	
OAH 300mg	6	-	5(83.3	1(16.7)	-	+		+	
OAH 600mg	6	-	2(33.3	3(33.3)	1(16.7)	+		+	
OAW 150mg	6	-	-	3(50)	3(50)	+		+	
OAW 300mg	6	-	-	5(83.3)	1(16.7)	+		+	
OAW 600mg	5*	-	-	3(60)	3(60)	+		+	
BMW 150mg	5*	-	-	4(80)	1(20)	+		+	
BMW 300mg	4*	-	2(50)	2(50)	-	+		+	
BMW 600mg	4*	-	-	2(50)	2(50)	+		+	
IC	6	-	-	1(16.7)	5(83.3)	+		+	

Key:
None (no granuloma)
Few (1-3 granuloma per lobe)
Moderate (1-3 granuloma per lobe)
Severe (1-3 granuloma per lobe)
OAW- *Ocimum americanum* water extract
AP – Adhesions present
BMW- *Bridelia micrantha* water extracts
150mg- low concentration

I – Inflammed
NI – Not inflammed
AA - Adhesions Absent
PZQ-Praziquantel
OAH- *Ocimum americanum* hexane extract

300mg- medium concentration
600mg –High concentration
* - mice died

4.3.4.3 Histopathology for efficacious plant extracts

4.3.4.3.1 Sizes of granuloma in liver tissue

Granuloma size was calculated as average of its length and breadth (Farah *et al.*, 2000) as shown in Figure 4.6. Granuloma sizes for all the groups were subjected to ANOVA test. When PZQ was compared with treatment groups; OAH-300, BMW- 300, OAW-300 and OAH- 150 groups there were no significant difference (Anova; $p > 0.05$). PZQ compared to IC, OAW-150, BMW-150, OAW-600, and OAH -600 and BMW-600 groups there were significant difference (Anova; $p < 0.001$). IC compared to PZQ, OAH- 300, BMW-300, OAW-300 and OAH- 150, OAW-150, there was significant difference (Anova; $p < 0.001$). IC compared to treatments groups; BMW-150, OAW-600, OAH -600 and BMW-600 groups there was no significant difference (Anova; $p > 0.05$).

4.4: DISCUSSION

Following perfusion, different treatment groups had different worm recoveries. Praziquantel had the lowest worm count while infected control had the highest worm count. The plant extracts which were significant similar to Praziquantel (Anova; $p > 0.05$) were OAH BMW, OAW, CMW, CMC and BMH. OAH, BMW and OAW had high percentage worm reduction when compared with other groups. OAH, BMW, OAW, were close to PZQ in terms high worm reduction demonstrating that they were able to protect the mice from schistosomiasis infection. This is an indication that the three plant extracts were able to invoke protection in Swiss mice leading to higher worm reduction. These results indicated that OAH, BMW and OAW are possible drug candidates.

There was significant difference (Anova; $p<0.001$) between, PZQ and the other treatment groups; OAC, OAD, SLD, BMD, SLW, ASW, SLC, ASC and BMC implying that they were not protective. PZQ has good efficacy against the adult *S. mansoni* worm (Utzinger *et al.*, 2003). Owing to PZQ high efficacy against all five human schistosome species, good tolerability and ease of administration as a single oral dose, it has become the drug of choice for the treatment and morbidity control of schistosomiasis (Utzinger *et al.*, 2003; WHO, 2002b).

The worm reduction for IC when compared with PZQ, OAH, BMW, OAW, CMW and BMH showed significant difference (Anova; $p< 0.001$). The extracts therefore showed antischistosomal activities. There was no significant difference between IC and OAC, OAD, SLD, BMD, SLW, ASW, SLC, ASC and BMC treatment groups (Anova; $p>0.05$).

These groups had high worm burden therefore low worm recovery which means, not protective. This study showed that most of the crude products had high worm burden which meant that they could not protect the mice from the *Schistosoma* infection. The plant extracts which was closest to PZQ in protective activity were OAH, BMW, and OAW indicating their antischistosomal activities.

Gross pathology was focused on the general and overt appearance of the liver. Observations that were considered in the liver were inflammation, adhesions and presence of granulomas. A granuloma is as a result of an inflammatory reaction in body tissues. It is characterized by a central accumulation of mononuclear cells, primarily macrophages, with a surrounding rim consisting of lymphocytes and fibroblasts. The granulomas evolve with stimulation of mononuclear cells from a variety of cytokines (Woodard, 1982). In Schistosomiasis mansoni, granulomas are formed around eggs trapped in tissues, especially the liver and the colon. The number of granulomas is correlated with number of eggs, which is in turn correlated with number of worms.

Granulomas appeared as numerous raised pinheads sized foci distributed over the surface of the liver lobes. All the livers observed in the all the groups had adhesions. This shows that the body was fighting an infection. All the livers were also inflamed which implies a cell mediated immunity activity. The liver of PZQ compared with IC showed PZQ with few or no granuloma which means that there were few or no worms. On other hand the results of IC indicated moderate and severe granuloma due to heavy worm burden. In the PZQ treated group and *Ocimum americanum* extract some mice had no granuloma.

Absence of granuloma is very crucial in schistosome infection as it is an indication of lack of disease/ pathology resulting from an inflammatory response. For the *Bridelia micrantha* extracts, BMW group had reduced pathology compared with the rest of the group. The low worm counts contributed to reduced liver disease/ pathology. Liver of mice treated with *Sonchus luxurians, Croton megalocarpus* and *Aloe secundiflora* plant extracts showed either moderate or severe granuloma. IC and SLC group had the worst pathology; mice from these groups had severe granulomas.

The best plant extracts with no or few granulomas were OAH, BMW and OAW which is supported by high worm reduction and reduced gross pathology.

The sizes of granuloma of the five plant extracts varied in sizes. When IC was compared with PZQ, there was a significant difference. There was no significant difference when PZQ group was compared with OAH, BMW, OAW, OAC, OAD, BMD, CMW and BMH treatment groups. This means that those treatment groups were able to fight infection, in similar manner to PZQ. When IC was compared with plant extracts; OAH, BMW OAW and OAC there was significant difference. It means that these plants extracts were dissimilar to IC group. When IC was compared to the following plant extracts; SHC, SHD, SHW, ASC, ASW, CMC, CMW, BMD, BMH, BMC there was no difference This means that the extracts were not protective in terms of granuloma size reduction.

The observation from these results indicated that the following plant extracts; OAH, BMW and OAW were closer to PZQ in percentage worm reduction (PZQ (75.2%), OAH

(68.7%), BMW (63.4%) and OAW (61.6%) and in size of granuloma PZQ (7.05 μm), OAH (10.3 μm), BMW (10.8 μm) and OAW (11.9 μm).

Plant extracts have become a source of hope as a wide group of medicinal plant preparations are available that have been used over the centuries almost exclusively on the basis of empirical evidence. Hence, it has become necessary to revisit the importance of these herbal medicines. Increasing interest by multinational pharmaceutical companies and domestic manufacturers of herbal-based medicines is contributing to a significant economic growth of the global medicinal plants sector. The exact dosage of medicinal plants is often the weak point of traditional healers. This was the basis of this experiment: to determine which was the best dosage (150mg, 300mg and 600mg) of the three efficacious plants, *Ocimum americanum* hexane extract (OAH), *Ocimum americanum* water extract (OAW) and *Bridelia micrantha* water extract (BMW)

The three efficacious plants extract were *Ocimum americanum* hexane extract (OAH), *Ocimum americanum* water extract (OAW) and *Bridelia micrantha* water extract (BMW) were selected because they showed high worm reduction, good gross pathology and small granuloma sizes which was significantly similar to PZQ.

Plant extracts were administered to the mice in different dosages (150mg/ml, 300mg/ml and 600mg/ml per kg). When PZQ (79.3%) was compared with the following plant extracts: OAH - 300mg/ml (66.7%), BMW -300mg/ml (63.3%), OAH – 150 mg/ml (54.6%), and OAW 300mg/ml (51.4%) in terms of worm reduction percentage were

similar. This is indication that the extracts were able to fight the infection owing to high worm reduction. On the other hand PZQ compared with other treatment groups; OAW-600mg/ml, BMW – 150mg/ml, BMW – 600mg/ml, OAW-150 mg/ml and OAH-600mg/ml and IC there was significant difference indicating high worm recovery. The dosages concentration which was more effective was 300mg/ml in OAH and BMW plant extracts which gave worms reduction of 66.7% and 63.3% respectively. They attained the WHO criteria of 40% protection.

All the livers observed in the all the groups had adhesions .This shows that all these livers were fighting an infection. PZQ compared with IC showed few or no granulomas, while IC showed moderate and severe granulomas which were an indication of heavy worm burden due to the fact that the animals were unprotected from the infection. In the 150mg/ml concentration of all the three plant extracts the gross pathology was showing either moderate or few animals with severe granulomas. PZQ and OAH - 300 mg/ml and BMW -300 mg/ml plant extracts showed few granulomas which was an indication that were able to protect the animals compared to other different dosages. The high dosage of 600mg/ml of all the three plant extracts was showing severe granuloma. This is an indication that the extracts work best when ionized. This means the higher the concentration the fewer the active ions and therefore the less the effectiveness.

PZQ (8.9 μm) has small granulomas as compared with IC (25.8 μm) showing that PZQ reduced the granuloma sizes, resulting to reduced disease. The granulomas of 150mg group of BMW and OAW were large and similar to IC. OAH-300mg/ml (12.5), BMW-

300/ml (11 μm), OAW-300 mg/ml (14.9 μm) and OAH- 150 mg/ml (16.1 μm) groups were significantly similar to PZQ in terms of small granuloma sizes. On the other hand the granuloma sizes of OAW -150(20.8 μm), OAW-600 (19.2 μm), OAH -600 (17.5 μm) and BMW-600 (21.0 μm) groups was significantly similar to IC in term of the sizes.

The results indicated that the plant extracts; OAH-300mg/ml, BMW- 300/ml induced cytokine responses similar to PZQ, which caused reduction of granulomas, and hence reduction of the disease. In addition, they had high worm reduction, and reduced gross pathology, similar to PZQ. The two plant extracts were selected for immunological assays.

CHAPER 5: Immunological responses of mice after treatment with efficacious plant extracts

5.1. INTRODUCTION

Schistosomiasis causes a range of morbidities, the development of which seems to be influenced to a large extent by the nature of the induced immune response and its effects on granuloma formation and associated pathologies in target organs. Field studies in endemic areas, combined with animal experiments, have led to the view that host genetics, infection intensity, *in utero* sensitization to schistosome antigen and co infection status all influence the development of the immune response and, so, disease severity. Two main clinical conditions are recognized in *S. mansoni*-infected individuals' acute schistosomiasis and chronic schistosomiasis (Pearce and MacDonald, 2002).

5.1.1: Acute schistosomiasis (TH1 disease)

Acute schistosomiasis in humans is a debilitating febrile illness (Katayama fever) that can occur before the appearance of eggs in the stool and which is thought generally to peak between 6 and 8 weeks after infection (Pearce and MacDonald, 2002). During acute illness, which is less well studied than chronic disease, there is a measurable level of tumour-necrosis factor (TNF) in the plasma, and peripheral-blood mononuclear cells (PBMCs) produce large quantities of TNF, interleukin-1 (IL-1) and IL-6. (Cleric and Shearer, 1994).

Granuloma formation occurs in response to chronic inflammatory stimuli. This pattern of inflammation is initiated and maintained by sensitized T helper 1 (TH1) cells

(Romagnani, 2000). Notably, cytokine production by PBMCs after stimulation with parasite antigen reflects a dominant T helper 1 (TH1), rather than TH2, response. Presumably, in the natural progression of the disease, the developing egg-antigen induced TH2 response down regulates the production and effector functions of these pro-inflammatory mediators; the production of IL-10 during this period has a crucial role in this process (Vennervald Dunne *et al.*, 2004; Wynn *et al.*, 1998).

An examination of disease in mice has shown that inability to develop a TH2 response to regulate the initial pro-inflammatory response that is associated with acute schistosomiasis is lethal. This first became apparent when C57BL/6 IL4 mice were infected with *S. mansoni*. Coincident with the onset of parasite egg production in these animals, a condition that was similar to severe acute schistosomiasis in humans developed, which was characterized by cachexia and significant mortality (Lucey, 1993).

These mice developed relatively normal hepatic granulomas, but pathological changes in the intestine were more evident in the absence of IL-4 (Bica, 2000). The non-haemorrhagic lesions on the mucosal surface were associated with the inefficient passage of eggs into the lumen. This process was accompanied by detectable levels of lipopolysaccharide in the plasma, perhaps owing to the translocation of intestinal bacteria (Pearce and MacDonald, 2002).

Analyses of the immune responses of infected IL4 mice showed that there was a correlation between elevated levels of nitric oxide (NO) and disease severity. Treatment

with uric acid, which is a peroxyradical scavenger, had marked ameliorative effects, which indicates that a combination of reactive oxygen and nitrogen intermediates might have a role in acute disease (Bonneau *et al.*, 1993).

5.1.2: Chronic schistosomiasis (TH2 disease)

Chronic disease is graded according to severity. The most serious form is a life-threatening hepatosplenic disease, which is usually accompanied by severe hepatic and periportal fibrosis, portal hypertension and portosystemic shunting of venous blood. Although TH2 responses seem to have a crucial role in modulating potentially life-threatening disease during the initial stages of schistosomiasis, prolonged TH2 responses contribute to the development of hepatic fibrosis and chronic morbidity (Doenhoff *et al.*, 1988).

The main TH2 cytokine that is responsible for fibrosis is IL-13. So, *schistosome* infected mice in which IL-13 is either absent , ineffective (IL-4 receptor α-chain-knockouts; IL4rα) or neutralized by treatment with soluble IL-13Rα2–Fc, fail to develop the severe hepatic fibrosis that normally occurs during infection, which leads to prolonged survival of these mice (Asahi *et al.*, 1999).

The mechanism by which IL-13 is able to promote fibrogenesis has been elucidated in a series of studies. These findings might have implications beyond schistosomiasis for the possible use of IL-13 blocking therapies in other fibrotic diseases. Mediators that are associated with TH1 responses, such as interferon-γ (IFN-γ), IL-12, TNF and NO can

prevent IL-13-mediated fibrosis. Infection intensity is one factor that can affect the severity of chronic schistosomatic disease, perhaps particularly in children. However, it seems to be more important whether or not an infected individual is genetically predisposed to disease (Pearce and MacDonald, 2002).

5.1.3: The importance of a balanced TH response

Interestingly, the severe disease is not related to increased parasite burden, but, rather, seems to be linked to the immunological consequences of the absence of TH2 cytokines. So, 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 mediators. Research has showed that IL-10 is the primary regulator of pro-inflammatory responses. Its absence results in increased disease severity during schistosomiasis. Consistent with this, during infection, individuals developing highly polarized TH1 responses had a lethal acute wasting condition that seemed to be an exaggerated form of the disease. Severe disease, with excessive TH2 responses, and marked mortality during the chronic stages of infection was associated with increased granuloma size and fibrosis (Bica, 2000).

IL-10 may have an important regulatory role in schistosomiasis, preventing the development of excessive TH1- and TH2-mediated pathologies (Pearce and MacDonald, 2002 and Wynn *et al.*, 1995). The T helper 1 (TH1) and TH2 dichotomy was first shown in murine CD4+ lymphocytes clones. These cells could be differentiated in terms of the cytokines they secrete. The TH1 subsets produce interleukin 2 (IL-2,) interferon gamma

(IFN-γ) and lymphotoxin. TH2 subsets produce IL-4, IL-5, IL-6 IL-10 and IL-13 (Cleric and Shearer, 1994). It is now apparent that in the mouse model, both Type 1 and Type 2 cytokines can orchestrate granuloma formation though with differences in the size of the lesions induced (Chikunguwo and Stadecker, 1991; Cheever, 1997).

A type 2 response results in formation of larger granulomas size which is an indicator of morbidity. Larger lesions are detrimental and small ones ideal (Amiri et al., 1992). Small lesions are due to compromise between egg sequestration and tissue pathology. Production of a Type 1 rather than Type 2 cytokines during infection is preferable in mice (Brunet *et al.*, 1998). IL- 12 promotes type 1 responses while limiting Type 2 responses. This accounts for the reduced granuloma and fibrosis. IFN-γ has been shown to play a role in resistance to *S. mansoni* by stimulating macrophage to produce IL-12 or nitric acid (Vasconcelos and Pearce, 1996 and Wynn *et al.*, 1995; Dunne *et al.*, 1995).

5.1.4: Induction of TH2 responses in mice by schistosomes

An inability to make TH2 responses renders individuals acutely sensitive to infection with schistosomes and highly susceptible to intestinal helminth infections. The egg stage of the schistosoma is responsible for inducing the TH2 response during infection. By contrast, the worms themselves seem to be poor inducers of a TH2 response. As for certain other helminth products, schistosome eggs or soluble antigens that are derived from the eggs induce an intense TH2 response without the need for additional adjuvant (Boros and Warren, 1970).

It has been shown that carbohydrates on egg antigens are integral to this process and, specifically, that a polylactosamine sugar (lacto-*N*-fucopentaose III) acts as a TH2 adjuvant. The emerging role of carbohydrates as factors that are important for the induction of the immune response during schistosomiasis opens up the possibility that innate pattern-recognition receptors that identify carbohydrates might have a crucial role in the induction of a TH2 response. A wide range of C-type lectin receptors which are expressed on the surface of dendritic cells (DCs) indicates various candidates that could be involved in the innate recognition of antigens from pathogens that initiate a TH2 response. It remains to be seen whether Toll-like receptors (TLRs) which have crucial roles in the recognition of viral, bacterial, fungal and protozoal organisms, and in the development of TH1 immune response exist for the recognition of helminth antigens and/or have any role in the induction of TH2 responses (Chacon, 2000).

TH2-response-inducing pathogens stimulate dendritic cells to produce IL-4, which then promotes TH2-response development. However, DCs do not need to produce IL-4 to direct TH2 development, because egg-antigen-pulsed IL4 DCs induce excellent TH2 responses. Several cytokines other than IL-4 have been implicated in TH2 development, but, on closer examination, have been found to be of minimal importance for the expression of this type of immune response during schistosomiasis. IL-6 can direct the development of IL-4-producing T cells. However, IL-6 does not have a main role during the development of TH2 responses to schistosome eggs *in vivo*, although it might be involved at some level in the regulation of IFN-γ and IL-12 production (Pearce and MacDonald, 2002).

5.1.5: Humoral responses

Schistosoma infection stimulates production of antibodies; IgG1, IgG2, IgG4, IgA, IgE and IgM (Hahan *et al.,* 1991). There is evidence that some antibodies mostly IgG2 and IgG4 block or compete with protective antibodies, IgG, IgA and IgE (Hagan *et al.,* 1998). It has been shown that IgM monoclonal antibodies (MAb) that recognize surface carbohydrate determinants shared between schistosomula cercariae and miracidia block antibody or complement dependent killing of schistosomula *in vitro* (Yi *et al.*, 1986).

This suggests that the interaction of the IgM to the live schistosomulum surface results in the formation of antigen-antibody complex, which reduces the efficacy of binding of other antibodies. Resistance towards schistosomiasis in humans is associated with a Th 2 response with enhanced IgE production against parasite antigens. IgE participates in antibody dependent cell mediated cytotoxicity (ADCC) response that is the main mechanism of killing parasites (Hagan *et al.,* 1991).

5.2 MATERIALS AND METHODS

5.2.1 Experimental Design and Sampling schedule

The sampling schedule was as follows; At week O the Swiss white mice were infected with cercariae and treated with the two efficacious plant extacts *Ocimum americanum*-300mg/kg (OAH) and *Bridelia micrantha*-300mg/kg (BMW) at WK 4. At WK 6, the efficacious groups OAH and BMW and control groups (IC and PZQ) were sampled for

serum for antibody ELISA. Spleens and lymph nodes were obtained to prepare cell suspension for cell culture in order to obtain supernatant for cytokine analysis. Another identical set of mice per group were perfused for worm recovery and livers observed for gross pathology. Tissues were preserved for histopathology.

5.2.2: Sampling of mouse blood, Spleen and Lymph nodes

5.2.2.1: Blood

At Wk 6 for naïve, all treated groups and infected control, mice from each group were anaesthesitized as previously described in Chapter 2. The right ventricle was located and 1 ml syringe inserted into it with the bevel facing downwards. Blood was sucked in small jerks in order to create vacuum and prevent the heart from collapsing. The whole volume of blood collected was dispensed into microfuge (eppendorf) tube and left on the bench for 1h to clot. Thereafter, the clotted blood was stored in at 4 C, overnight, and then processed for serum.

5.2.2.2: Preparation of serum

The clotted blood was spun at high speed of (700 rpm) for 10 minutes in a microfuge. The clear supernatant (serum) was then retrieved and transferred to a clean/ sterile epperdorf tube and stored at -20°C until use for antibody detection using ELISA.

5.2.2.3: Preparation of spleen cells

The peritoneum was opened by cutting the stomach wall horizontally and vertically to expose the abdominal organs. The spleen could be seen through the stomach wall. Sterile

forceps was used to remove the spleen, which was placed into incomplete medium (RPMI 1640, 0.1% Gentamycin, 5×10^{-5} Beta mercaptoethanol) in a Petri dish. The spleen from each mouse was transferred on sterilized wire gauze, in a sterile petri dish containing incomplete media in a sterile culture hood. A 10 ml syringe piston was used to squash the spleen. A Pasteur pipette was used to disperse the cells. The cells were then dispensed in 15 ml tubes and incomplete RPMI 1640 added up to 10 ml mark. The cells were centrifuge at 450g for 10 mins at RT, supernatant discarded and pellet resuspended. Washing was repeated two times. 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 assay. Lymphocytes were counted using a haemocytometer. Cell suspension was made up to 3×10^{6} cells per ml in complete medium.

5.2.2.4: Preparation of Mesenteric lymph node cells

After obtaining blood for serum preparation, the abdominal skin was gently peeled downwards up to the intestine and the Mesenteric lymph nodes located at the various intestinal loops and close to the superior mesenteric artery, one of the major blood vessels that supply the intestines and lower abdominal organs with blood and oxygen.

Using a watch maker's forceps, the layer covering the lymph node was removed and it popped out. The lymph node was carefully removed without attached fat and placed in a small 5cm Petri-dish containing incomplete medium (RPMI 1640, 0.1% Gentamycin, 5×10^{-5} Beta mercaptoethanol). The cells were washed in incomplete media by centrifugation

two times at 450g, room temperature for 10 minutes. The supernatant was discarded and the pellet resuspended in an incomplete media and the wash up in procedure repeated. After the 2nd wash, the supernatant was discarded and cells resuspended in 1ml completed medium (incomplete medium + 10% foetal calf serum).The other procedure was the same as the spleen (See 6.2.2.3).

5.2.3: Culture supernatant

Flat-bottomed 48-well microtitre plates were used for culture and 6 x 10^5 cells were dispensed in each well. Duplicate wells were set for each regime. Negative control had only medium and cells. Positive control had 1 µg per well of Concanavalin A. Test wells had 10 µg per well of SWAP and 0- 3 hr release protein (See Appendix 2). The total volume of culture medium per well was 400 µl. The plates were incubated at 37^0C at 5% CO_2 for 48 hours for Con A and 72 hours for the other Antigens, supernatants were collected at specified times and stored at - 20^0C until use.

5.2.3.1*:* Interferon gamma ELISA

ELISA plates (Nunc-Immuno ™ plate marxi sorp ™ surface, Denmark) were coated with 50 µl/well of 1 µg/ml solution of monoclonal antibody IFN-γ, in PBS, pH7.2 and incubated overnight at 4°C. The plate was washed six times with PBS- Tween 20 (100µl/well) and blocked by adding 100µl/well of PBS with 0.05% Tween 20 (PBS-Tween) containing 0.1% BSA and incubated for 1 h at 37^OC. After six times washes, 50µl/well of culture supernatants (either spleen /lymph nodes) were added. Recombinant mouse IFN-γ serially diluted was used as the standard. The plates were washed six times with PBS Tween and 50µl/well of Biotinylated monoclonal antibody IFN-γ at 0.5 µl/ml

in incubation buffer was added and incubated for 1h at RT. After incubation, the plates were washed six times and 50µl/well of streptavidin-HRP diluted 1:1000 in incubation buffer added and incubated for 1h at 37^{O}C. After washing, 50µl of the substrate (TMB microwell peroxide substrate Kirkeguard and Perring laboratories USA) was added and the plate incubated in the dark for 30 minutes. Colour develops depending on the strength of binding. The reaction was stopped by using 50µl 1% SDS (Figure 6.1). The optical density was read at 630nm using ELISA reader (Figure 5.1)

5.2.3.2*:* Interleukin – 5

Immulon 4 ELISA (Nunc-Immuno ^{TM} plate marxi sorp ^{TM} surface, Denmark) was coated with 50 µl/ml of 5 µl/ml solution of monoclonal antibody IL-5, in PBS, pH7.2 and was incubated overnight at 4 ^{o}C. The procedure was as for IFN-γ. (5.2.3.1)

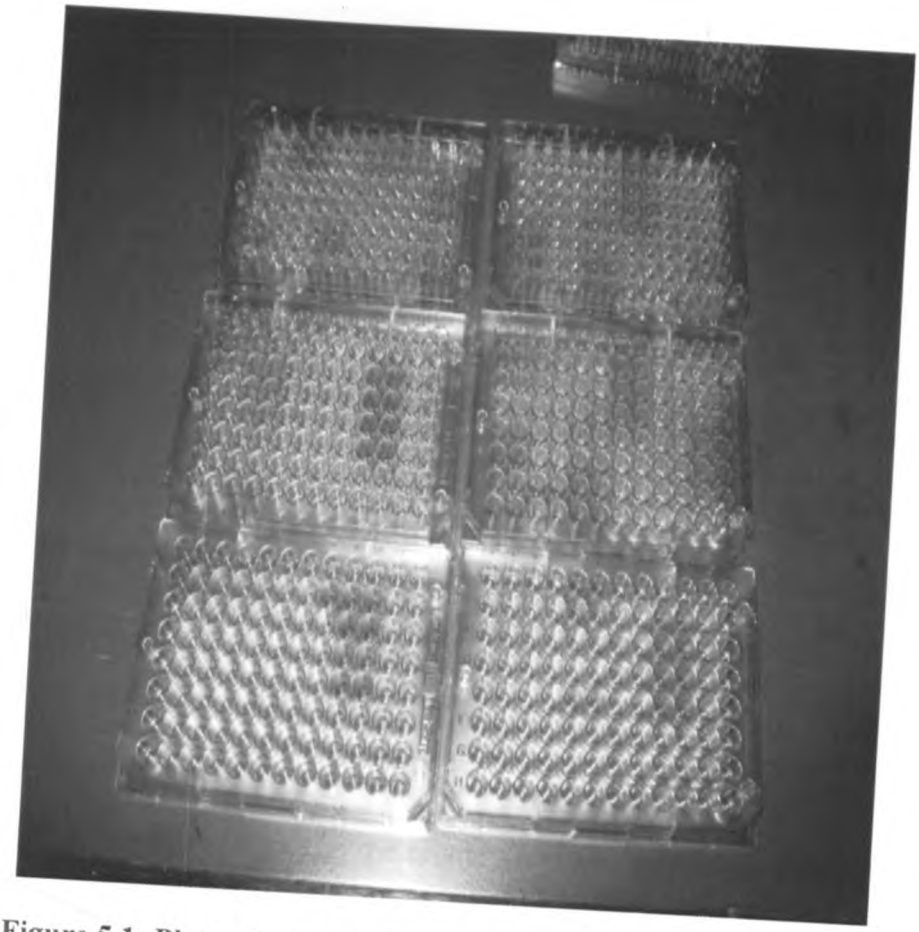


Figure 5.1: Plates showing colour changes after ELISA reaction

5.2.4 Enzyme Linked Immunosorbent Assay (ELISA) for Schistosome Specific IgG

The ELISA plate (Nunc-Immuno ^{TM} plate marxi sorp ^{TM} surface, Denmark) was coated with 50µl of 10 µg/ml SWAP, 0-3 hr release antigen diluted in carbonate/bicarbonate buffer, pH 9.6. It was incubated overnight at 4°C to allow the antigen to bind onto the wells. The excess antigen was dispensed off on a blotting paper. Then 100 µl of 3 % BSA in PBS /Tween 20 (0.05 %) per well was added to block the free sites. Blocking prevent binding of non-specific proteins. The plate was then incubated for one hour at 37°C and washed six times with PBS /tween 20 (0.05%). The serum samples were diluted in 0.5% BSA/PBS/tween: 1:20. The blank (0.5% BSA /PBS /tween) was prepared. Fifty µl of the diluted serum samples and the blank were added in duplicate to the plate. The plates were incubated for 2h at 37°C to allow any antibodies present to bind to the antigens after which the plates were washed six times. Fifty µl of the conjugate Goat anti-mouse immunoglobulin G Horse radish peroxidase (HRP) diluted 1:2000 was added in each well. The plates were incubated for 1 h at 37°C to allow binding of the secondary antibody to the primary one and washed six times. Fifty µl of the substrate (TMB microwell peroxide substrate Kirkeguard and Perring laboratories USA) was added and the plate incubated in the dark for 30 minutes. Colour develops depending on the strength of binding. The plates were read at 630nm on an ELISA reader, Marxi Kinetic Microplate reader (Molecular Devices, Palo Alto, England).

5.3. RESULTS

5.3.1 Worm recovery and reduction of two efficacious extracts

The efficacious plant extracts which were determined from Chapter 4 were *Ocimum americanum*-300mg/ml (OAH) and *Bridelia micrantha*-300mg/ml (BMW). The worm recovery was determined by perfusion of the mice treated with 2 efficacious extracts and their infected controls PZQ and IC infected with S. *mansoni* according to the method of Yole *et al.*, 1996 and worm reduction calculated.

The worm recovery and percentage worm reduction of PZQ, OAH and BMW was shown in Table 6.1. There was no significant difference between (OAH) and (BMW) when compared with PZQ (t- test; $p > 0.05$). OAH has closest worm reduction to PZQ. This was significantly different when IC was compared with PZQ, OAH and BMW (t-test; $p < 0.05$). When the experiment was repeated the similar results were obtained (Appendix 5).

Table 5.1: Mean number of worms and recovery percentage

		Mean number of worms recovered per group (mean ± SEM)				
Treatment Group	Dose mg/kg	Total males	Total Females	Total worms Mean ± SEM	% Worm recovery	% Worm Reduction
PZQ	900X2	14.8±2.9	10.3±2.3	25.1±5.2	41.3	58.7
OAH	300X2	16±2.2	9.6±2.06	25.6±4.26	42.2	57.8
BMW	300X2	22.4± 1.7	16.2± 2.2	38.6±3.9	63.5	36.5
IC	-	36.8±3.5	24±3.4	60.8±6.9	-	

KEY:
PZQ – Praziquantel
OAH- *Ocimum americanum* Hexane extract
BMW- *Bridelia micrantha* Water extract
IC - Infected control
Dose – mg (plant extract / PZQ) kg x number of doses

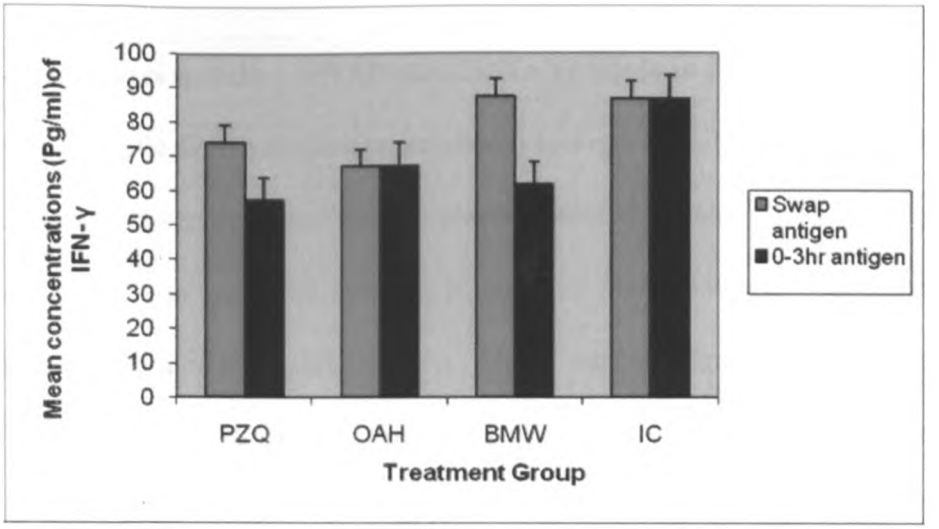
5.3. 2: Cytokines responses of mice after treatment with two efficacious plant extracts

5.3.2.1: Interferon gamma (IFN-γ) responses

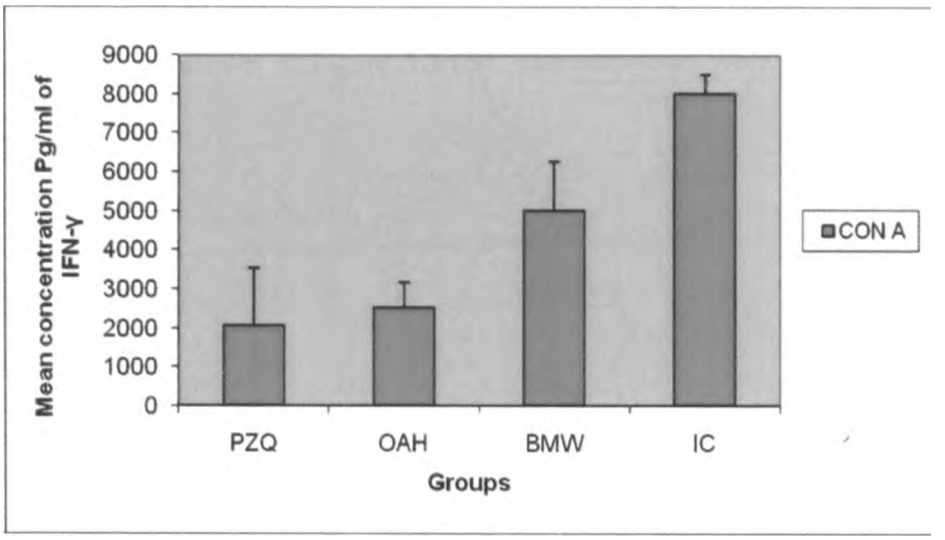
5.3.2.1.1: IFN-γ responses to 0-3hr, SWAP and CONA antigens in Lymph nodes cells

The 0-3hr antigen specific IFN-γ responses are shown in Figure 5.2 (A): PZQ compared to OAH-300mg, BMW-300mg and IC showed no significant difference (t- test; $p > 0.05$) however the responses were higher for IC. IC compared to the two plant extracts showed no significant difference (t- test; $p > 0.05$). The SWAP antigen specific IFN-γ responses are shown in Figure 6.5 (A): PZQ compared to OAH, BMW and IC showed no significant difference (t- test; $p > 0.05$), however the responses was higher for BMW. There was no significant difference between IC and the two plant extracts OAH and BMW (t- test; $p > 0.05$).

CONA antigen specific IFN-γ responses are as shown in Figure 5.2 (B): The response was high because CONA stimulate any lymphocyte (non-specific pattern) and is therefore used to confirm the cells are alive. When the experiment was repeated the similar results were obtained (Appendix 5).



A



B

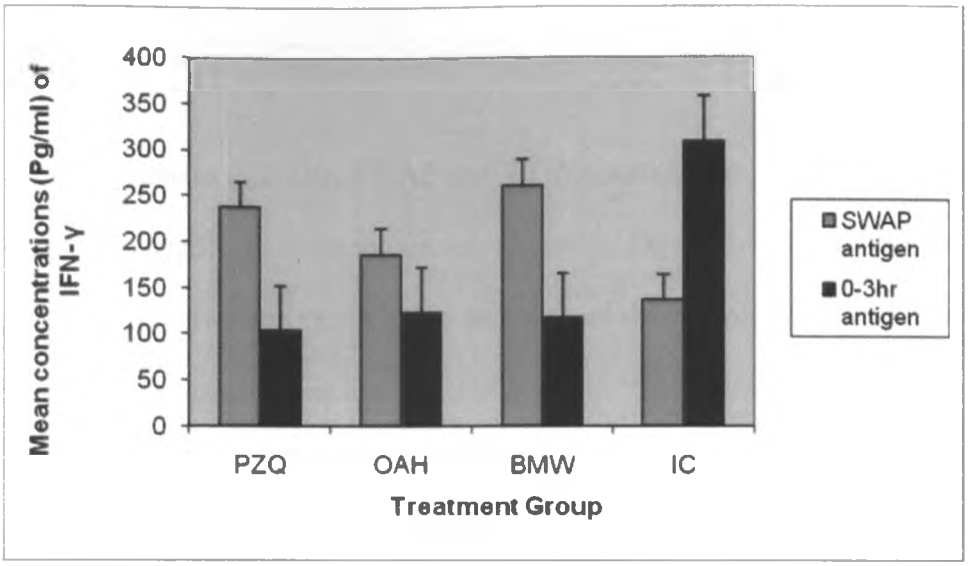
Figure 5.2: IFN - gamma responses in Lymph node cells stimulated with SWAP & 0- 3hr (A) and with CONA antigen (B)

Key:
PZQ – Praziquantel
OAH- *Ocimum americanum* Hexane extract
BMW- *Bridelia micrantha* Water extract
IC - Infected control

5.3.2.1.2: IFN-γ responses to 0-3hr, SWAP and CONA antigens in Spleen cells

The 0-3hr antigen specific IFN-γ responses are shown in Figure 5.3 (A): There was no significant difference between PZQ and the two plants extract OAH and BMW (t- test; $p > 0.05$). The same result was indicated between IC the two plants extracts. The SWAP antigen responses are shown in Figure 5.6 (A): There was no significant difference between PZQ and OAH, BMW (t- test; $p > 0.05$). However IC and PZQ indicated significant difference (t- test; $p < 0.05$). On the other hand, IC with OAH and BMW plant extracts indicated no significant difference (t- test; $p > 0.05$).

The CONA responses are shown in Figure 5.3 (B): The responses were high showing cells were alive.



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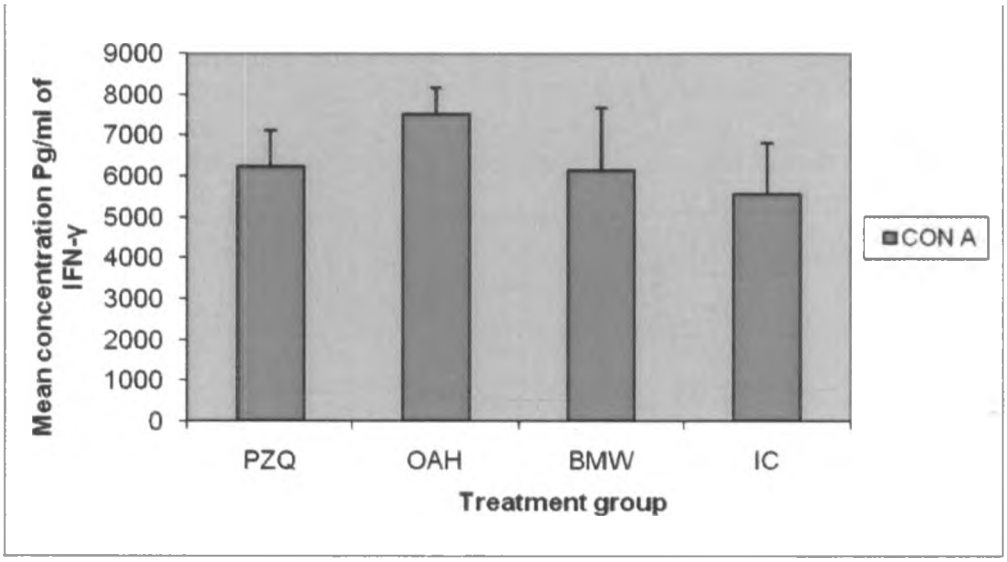
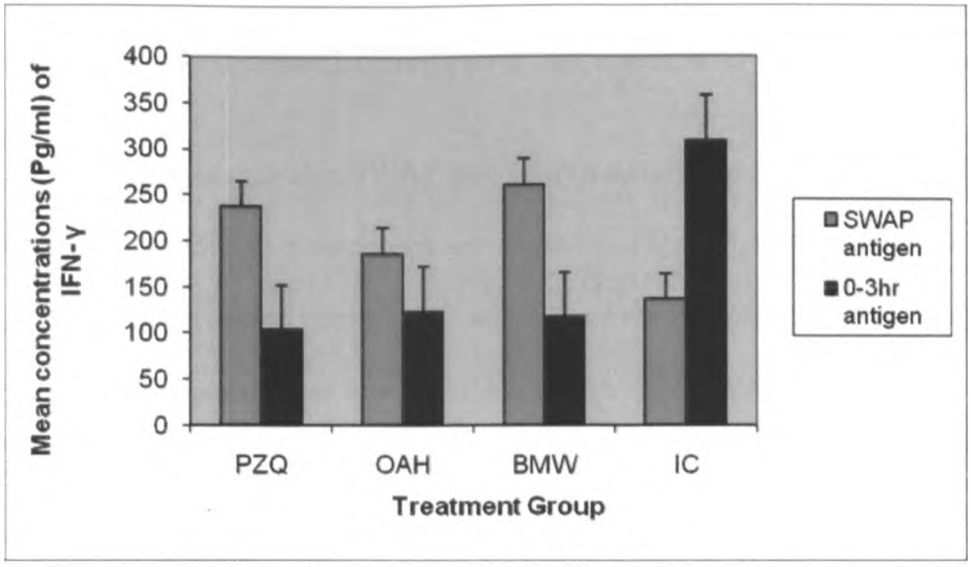


B

Figure 5.3: IFN – gamma responses in Spleen cells stimulated with SWAP & 0-3hr (A) and with CON A antigens (B)

Key:
PZQ – Praziquantel
OAH- *Ocimum americanum* Hexane extract
BMW- *Bridelia micrantha* Water extract
IC - Infected control



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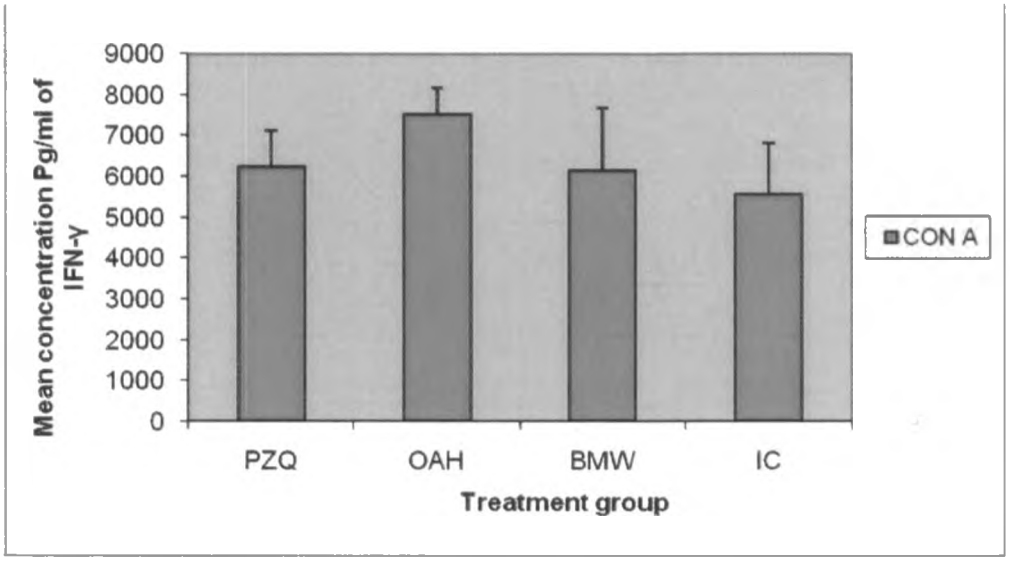


B

Figure 5.3: IFN – gamma responses in Spleen cells stimulated with SWAP & 0-3hr (A) and with CON A antigens (B)

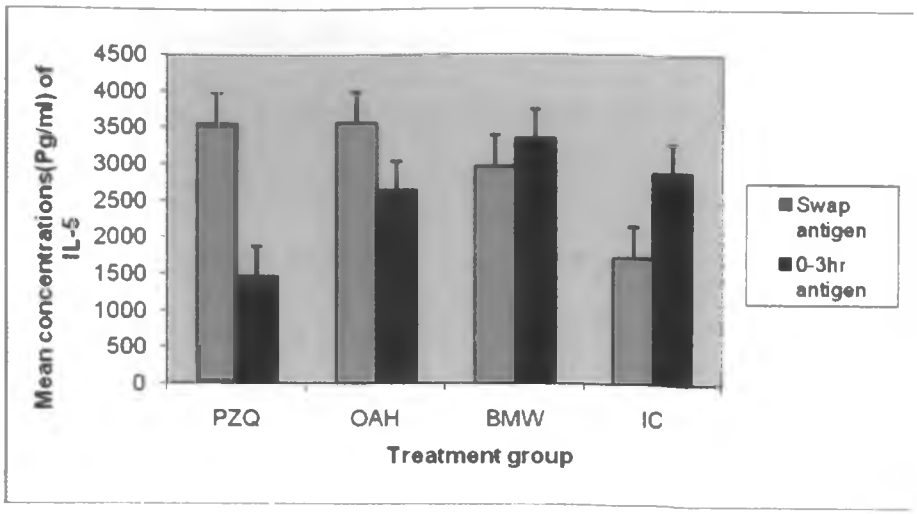
Key:
PZQ – Praziquantel
OAH- *Ocimum americanum* Hexane extract
BMW- *Bridelia micrantha* Water extract
IC - Infected control

5.3.2.2: Interleukin – 5 responses in lymph node and spleen cells

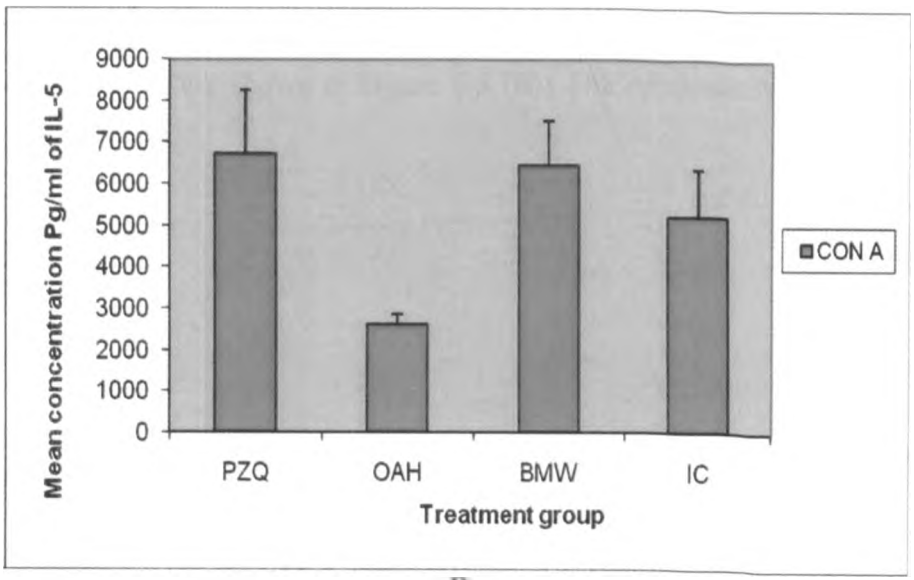
5.3.2.2.1: IL-5 responses to 0-3hr, SWAP and CONA antigen in Lymph nodes cells

The 0-3hr antigen specific IL-5 responses are shown in Figure 5.4 (A). There was no significant difference between controls (PZQ and IC) and the two plant extracts (t- test; $p > 0.05$), however the responses was low for BMW group. The SWAP antigen responses are shown in Figure 6.4 (A): There was significant difference between PZQ and IC (t-test; $p < 0.05$). On the other hand there was no significant difference between PZQ and the two plant extracts (t- test; $p > 0.05$).

The CONA antigen responses are shown in Figure 5.4 (B): The responses were high showing cells were alive.



A



B

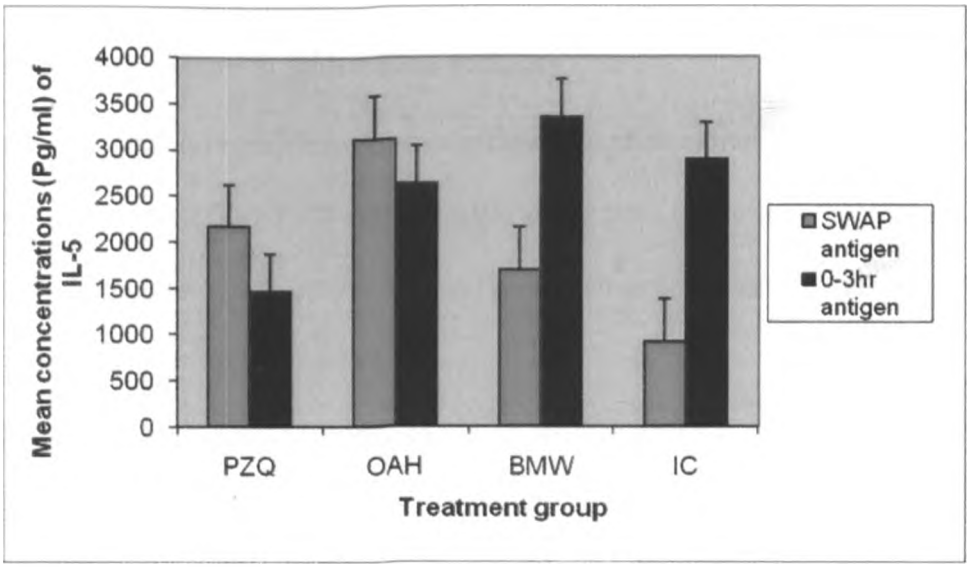
Figure 5.4: IL-5 responses in Lymph node cells stimulated with SWAP & 0-3hr (A) and with CON A antigens (B)

Key:
PZQ – Praziquantel
OAH- *Ocimum americanum* Hexane extract
BMW- *Bridelia micrantha* Water extract
IC - Infected control

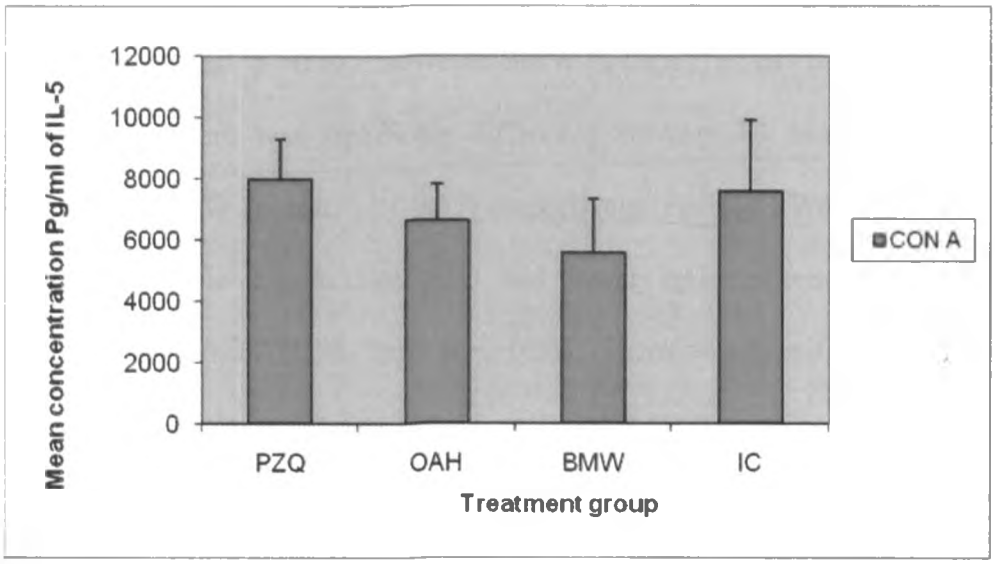
5.3.2.2.2: IL-5 responses to 0-3hr, SWAP and CONA antigen in Spleen cells

The 0-3hr antigen specific IL-5 responses are shown in Figure 5.5 (A): There was significant difference between PZQ and OAH, BMW and IC (t- test; $p < 0.05$). However there was no significant difference between IC and two plants extract OAH and BMW (t-test; $p > 0.05$) .The SWAP antigen responses are shown in Figure 5.5 (A): There was no significant difference between PZQ and the two plants extracts OAH and BMW (t- test; $p > 0.05$), On the other hand there was no significant difference between IC and BMW (t-test ; $p > 0.05$).However there was significant difference between IC and OAH (t- test ; $p < 0.05$).

The CONA responses are shown in Figure 5.5 (B**):** The responses were high showing cells were alive.



A



B

Figure 5.5: IL-5 responses in Spleen cells stimulated with SWAP& 0-3hr (A) and CON A antigens (B)

Key:
PZQ – Praziquantel
OAH- *Ocimum americanum* Hexane extract
BMW- *Bridelia micrantha* Water extract
IC - Infected control

5.3.3: Antibody responses to schistosome antigens

Schistosoma- specific IgG responses in two efficacious plant extracts groups (OAH and BMW) and controls (PZQ and IC) were assayed using both 0-3 hr and SWAP antigens using ELISA the IgG level was determined and presented as absorbance (O.D) at 630 nm.

5.3.3.1: Schistosome specific response to 0-3 hr antigen and SWAP antigen

The 0-3 hr specific IgG responses are shown in Figure 5.6. PZQ had the lowest IgG response which was significantly different to infected control (t- test; $p< 0.05$). However there was no significant difference between PZQ and the two plant extracts OAH-300mg and BMW- 300mg (t- test; $p >0.05$). However BMW had a higher OD than the PZQ and OAH respectively. There was significant difference between IC and the two plant extracts OAH and BMW (t- test; $p < 0.05$) respectively. For the SWAP specific IgG responses are shown in Figure 5.6: PZQ had lower IgG responses which were significantly different from IC (t- test; $p < 0.05$). There was significant difference between PZQ the two plants extracts OAH and BMW (t- test; $p< 0.05$) .However BMW had a higher OD than OAH and PZQ. IC compared with OAH and BMW treatment group showed significant difference (t- test; $p < 0.05$). When the experiment was repeated the similar results were obtained (see Appendix 5).

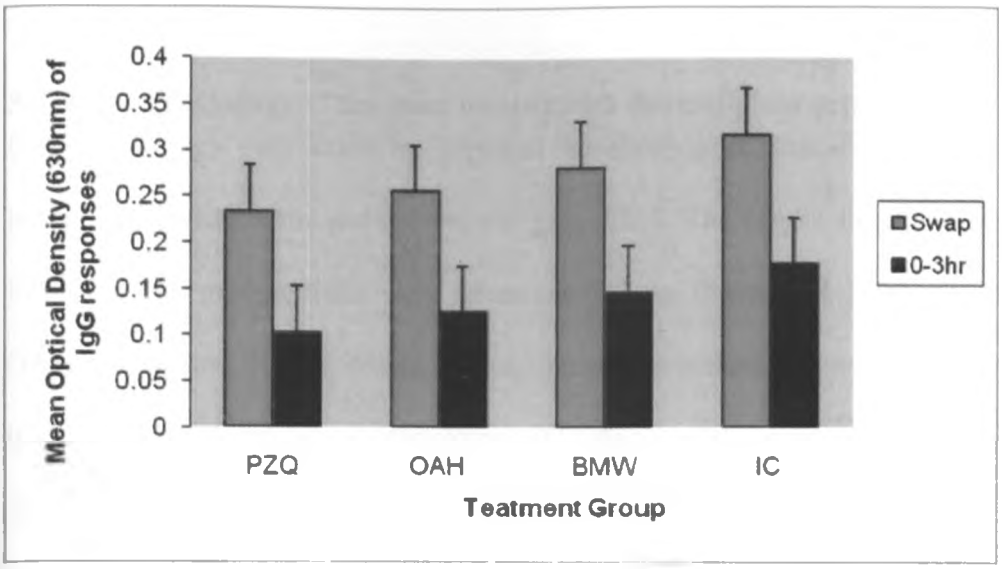


Figure 5.6: SWAP and 0-3hr Antigens specific IgG responses

Key:
PZQ – Praziquantel
OAH- *Ocimum americanum* Hexane extract
BMW- *Bridelia micrantha* Water extract
IC - Infected control

5.3.4 Gross pathology of the mice treated with the two plant extracts

Gross pathology was done by physical observation of the liver surface to detect inflammation; adhesions and presence of granuloma. The results are shown in Table 5.2. In all the four groups, there were adhesions and the livers were inflamed. In the PZQ, OAH- 300mg and BMW- 300mg groups, showed no presence of or few granuloma while IC had moderate and severe granuloma. IC group had the most severe gross granuloma pathology, while PZQ had the least pathology.OAH was similar to PZQ with low pathology.

Table 5.2: Gross pathology of liver tissues

Plant extracts	No. of mice	Granuloma classification for all the groups				Adhesions		Inflammation	
		None %	Few %	Moderate %	Severe %	AP	AA	I	NI
PZQ	6	2(33.3%)	4(66.7%)	-	-	+		+	
OAH 300Mg	6	1(16.6%)	4(66.7%)	-	-	+		+	
BMW 300Mg	5 *	1(20%)	4(80%)	-	-	+		+	
IC	6	-	-	4(66.7%)	2(33.3%)	+		+	

Key:
None (no granuloma)
Few (1-3 granuloma per lobe)
Moderate (1-3 granuloma per lobe)
Severe (1-3 granuloma per lobe)
AP – Adhesions present
AA - Adhesions Absent
* - One mice died
I – Inflamed
NI – No inflammation
PZQ – Praziquantel
OAH- *Ocimum americanum* Hexane extract
BMW- *Bridelia micrantha* Water extract
IC - Infected control

5.3.5. Histopathology of the mice treated with the two plant extracts

5.3.5.1 Granuloma sizes from liver of infected mice

Granuloma sizes in diameter were measured using micrometer in a microscope as shown in the Figures 5.7. Granuloma sizes all the groups were subjected to T- test. PZQ compared with OAH-300mg/ml and BMW- 300mg/ml groups showed no significant difference (t- test; $p > 0.05$). When IC group was compared with plant extracts, OAH, BMW and PZQ there were significant difference (t- test; $p < 0.001$). The IC group had the highest number of granuloma.

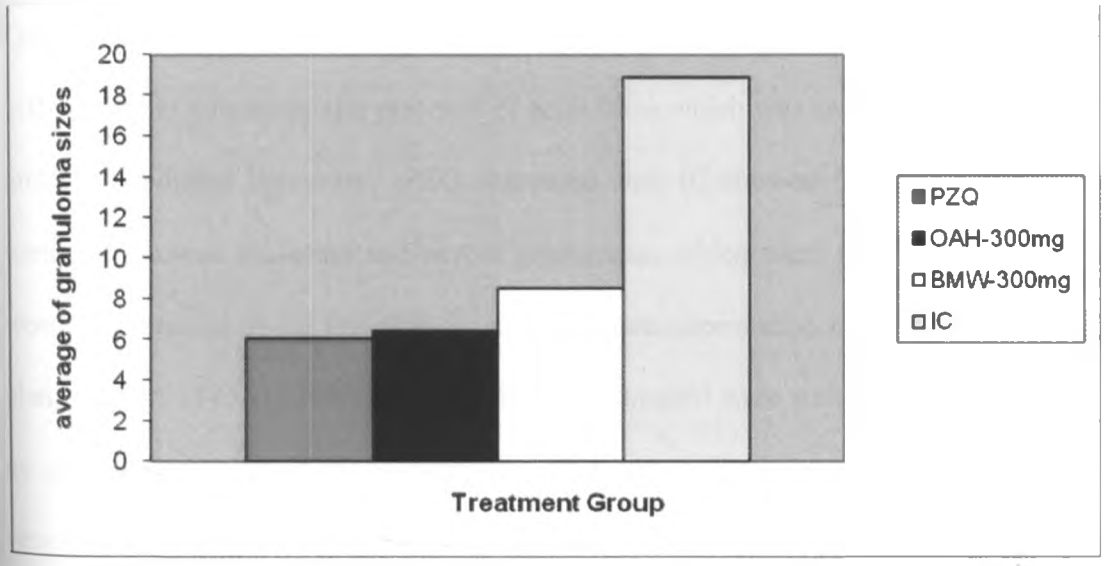


Figure 5.7: Granuloma sizes of liver tissue

Key:
PZQ – Praziquantel
OAH- *Ocimum americanum* Hexane extract
BMW- *Bridelia micrantha* Water extract
IC - Infected control

5.4: DISCUSSION

This experiment had only two efficacious groups and two controls PZQ and IC. The efficacious groups were OAH-300 mg/ml and BMW- 300mg/ml groups which were selected from dosage experiment. The worm reduction value for was PZQ (58.7%), OAH (57.8%) and BMW (36.5%), which was similar to results from the dosage experiment. When the two efficacious groups were compared with PZQ they were significantly similar in worm recovery and reduction. This was a confirmation that they were close to PZQ in reducing worms.

Gross pathology was done by physical observation of the liver surface to detect inflammation; adhesions and presence of granuloma which was an indication of infection and cell mediated immunity. PZQ compared with IC showed few or no granulomas, while IC showed moderate and severe granulomas which were an indication of heavy worm burden due to the fact that the animals were unprotected from the infection. The plant extracts of OAH-300mg/ml and BMW- 300mg/ml were similar to PZQ in terms of no or few granuloma in gross pathology. This means that the two extract groups were very effective in reducing gross pathology at a similar level to PZQ. IC group showed moderate and severe granuloma which was an indication that the animal was not protected from the infection.

Granuloma sizes OAH-300mg/ml (6.4μm) and BMW- 300mg/ml (8.4 μm) groups was significant similar to the PZQ (6.0 μm), however significantly different to IC (18.8 μm) group. This means that the two plant extracts were effective in reducing granuloma sizes.

The results obtained from the two extracts, OAH and BMW, shown them to be protective in terms of worm reduction, gross pathogy and histopathology which are in agreement with results of previous chapters.

The macrophages and dendritic cells that capture antigens in both the spleen and the lymph node present antigens to T and B cells, consequently initiate an immune response. The spleen is an immunologic filter of the blood and the major function of the lymph nodes is to filter antigen from the lymph.

The study therefore sought to assess if the lymphocytes from spleens and Mesenteric lymph node of mice infected with *S. mansoni* and treated with OAH and BMW expressed TH1 or TH2 profile as defined by IFN -γ or IL-5. Sandwich ELISA was used to measure the levels of IFN -γ or IL-5.

Interferon gamma is important for both innate and specific immunity with immunostimulatory and immunomodulatory effects (Schoenburn and Wilson, 2007). The host immune response to *S. mansoni* is a T-cell dependent process. The TH-1 type of response is the response which is directed against early stages of the parasitic infection which is an important induction of the cell mediated immunity to S. mansoni (Pearce & MacDonald, 2012).

Cytokine responses, both IFN and IL5, to Con A antigen in both spleen and mesenteric lymph nodes was high confirming that the cells were viable.

SWAP and 0-3hr immune responses for both lymph node cells and spleen were significantly different between PZQ and IC. When PZQ was compared with OAH-300mg, BMW-300mg s no significant difference was observed in both lymph nodes and spleen. The plant extract with high IFN responses was BMW extract for both spleen and lymph node cells. This implies that BMW was able to produce the TH-1 which is important profile for cell mediated immunity which would fight the infection. However IC had higher IFN responses for lymph node cells than the rest of the groups. This was probably due to high worm antigen concentration in circulation due to higher number of worms. Interferon-γ is used to treat chronic granulomatous disease (Todd and Goa, 1992).

PZQ and the treatment groups, BMW and OAH, were able to protect the mice from infection and produce cell mediated immunity. IC group produced high response due to high number of worm.

IL-5 is an interleukin produced by T helper-2 cells and mast cells. Its functions are to stimulate B cell growth and increase immunoglobulin secretion. It is also a key mediator in eosinophil activation (Dubcquol *et al.*, 1994).

The production of eggs brings about the most dominate responses that occur at the acute stage of the disease. IL-5 express TH-2 response which is mediated by CD4+ cells that are highly polarized with production of large amounts of IL-4; IL-5 andIL-13 among other cytokines (Chacon, 2000).

In this study, all extracts induced production of IL-5. When PZQ was compared with IC for both lymph node and spleen cells the response for 0-3hr antigen was high, while with SWAP antigens the response was low. When PZQ was compared with BMW and OAH there was no significant difference but OAH was higher in response. From the observation the OAH was able to generate more IL-5 compared with the rest of the groups. However, for the SWAP antigens responses PZQ and OAH were high compared with BMW and IC in that order. From the observation PZQ and OAH was generating more IL-5 responses than other groups.

IL-5 is able to the elimination antibody bound parasites through the release of cytotoxic granule proteins. Given that eosinophils are the primary IL-5-expressing cells this is an important fact in that eosinophils play an important role in regulating parasitic infections (Dubucquoi et al., 1994). PZQ, OAH and BMW had high IL-5 responses, implying that this cytokines was involved in reducing worm burdens in these groups. However, IC too had high IL-5 levels, which did not translate in reduced worm counts. IL- 5 in IC could have occurred due to stimulation of high levels of schistosome worm antigens.

Schistosome infection stimulate IgG antibodies which together with neutrophils, eosinophil and macrophage participate in antibody dependent cellular cytotoxicity (ADCC) plays an role in damaging schistosomule stage (Hagan *et al.*,1998). Serum antibody analysis was done using 0-3hr and SWAP proteins antigens.

PZQ had lower IgG responses which were significantly different from IC. PZQ and the two plant extracts OAH-300mg and BMW- 300mg were significantly similar; however BMW had higher IgG responses. This is an indication that BMW and OAH plant extract were able to fight infection using humoral immunity in a similar manner to PZQ, with BMW being better.

IC group had the highest IgG responses when compared with all the other groups. The high response in IC may be associated to high worm burden and resulting to more antigens being released inducing non-specific response, which was not protective.

0-3hr IgG responses were lower compared to SWAP. This is expected since adult worms produce larger amounts of antigens compare to the early schistosomule stage.

The results indicated that the plant extracts; OAH and BMW were close to PZQ in terms of worm reduction, gross pathology and histopathology. However the OAH plant extracts was closest to PZQ in worm reduction percentage (PZQ-58.7% and OAH – 57.8%) and gross pathology was showing either few or no granuloma. The granuloma sizes of OAH plant extract was also close to PZQ (PZQ- 6.4 while OAH – 6.7μm). From the observation PZQ and OAH were able to generate more IL-5 responses which was an indication of being able express TH-2 response which is mediated by CD4+ cells. However BMW plant extract was able to produce high Interferon gamma and IgG responses compared with PZQ and OAH. This was an indication that the BMW plant extract was able to fight infection using both humoral and cell mediated immunity.

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CHAPTER 6: *Invitro* activity showing cercaricidal and wormicidal effect

6.1. INTRODUCTION

Schistosomiasis is a global health problem caused by several species of schistosome blood flukes. The initial stage of infection is invasion of human skin by a multicellular larva, the cercariae. The initiation of infection of the human host by schistosome parasites involves penetration of skin by a multicellular larva (0.1 mm) called a cercaria (ae). Cercariae have forked tails that propel them through fresh water. Depending upon their specific vertebrate host, cercariae can respond to a variety of stimuli, including motion, light, and shadow, chemical gradients, and heat. Upon contact with human skin, cercariae are stimulated by the lipid on the surface of skin to begin penetration (Dorsey *et al.*, 2002).

Initial penetration involves mechanical entry into the superficial, cornified layer of skin, which presents little barrier in the aquatic environment. However, further entry requires degradation of intercellular bridges between epidermal cells, the dermal/epidermal basement membrane, and the extracellular matrix of the dermis. Ultimately the larvae, which have now shed their tails and are called schistosomula, enter small vessels in the superficial dermis where they complete their life cycle. Proteins secreted by cercariae play key roles in facilitating skin invasion and evading the immune response of the host (Skelly and Shoemaker, 2000).

Microscopic and biochemical analyses have identified three potential sources of proteins released by cercariae. First, a carbohydrate-rich surface glycocalyx is released upon

entry. This glycocalyx protects the organism from osmotic shock in fresh water but is a potent activator of complement and must be jettisoned prior to entry into the bloodstream (Skelly and Shoemaker, 2000). Second, proteolytic secretions are produced by the set of two groups of acetabular "glands," which are in fact clusters of cells with cytoplasmic processes extending into the anterior end of the organism. Acetabular cells release their contents beginning at the earliest stages of skin invasion and, at least in human skin, well into the superficial dermis. The escape glands, a third potential source of secretions, appear to release their contents late in invasion as the organisms enter dermal vessels (Fusco *et al.*, 1988).

Once inside the human body, the worms penetrate the wall of the nearest vein and travel to the liver where they grow and sexually mature. Mature male and female worms' pair and migrate either to the intestines or the bladder where egg production occurs. One female worm may lay an average of 200 to 2,000 eggs per day for up to twenty years. Most eggs leave the blood stream and body through the intestines. Some of the eggs are not excreted, however, and can lodge in the tissues. It is the presence of these eggs, rather than the worms themselves that causes the disease (Gryseels, 2006).

6.2 MATERIALS AND METHODS

6.2.1 Cercaricidal effect of plant extracts on cercariae

Three concentrations of plant extract (150, 300 and 600 µg /ml) of the five plants: *Sonchus luxurians*, *Ocimum americanum*, *Bridelia micrantha*, *Croton megalocarpus and Aloe secundiflora* were prepared (Anjaneyulu, 1977) as shown in Table 6.1:

Table 6.1: Concentrations of various plant extracts on effect of cercariae and worms.

Extracts weight (µg)	Amount of water (ml)	Concentration (µg/ml)
1500	10	150
3000	10	300
6000	10	600

Two milliliter of the prepared concentration of each plant extract was dispensed in a well of 24 cell culture plate containing an aliquote of 20 cercariae. Two replicates for each concentration was made. Each preparation was observed under a dissecting microscope for cercariae motility at the following time points: 5, 10, 20, 30, 45 and 60 minutes. Immobile cerceriae were enumerated and recorded at every point, however, when all cercariae were immobile before 1 h, the experiment was terminated at that specific point. At the end of each experiment, iodine was added for clarity in counting of the total number of cercariae as a confirmation of accuracy of the counting procedure.

6.2.2 Wormicidal effect of efficacious plant extracts on worms

Three concentrations of plant extracts of the two plants: *Ocimum americanum* hexane, water and *Bridelia micrantha* water extracts (150, 300 and 600 µg/ml was prepared the same as shown in Table 6.1. Before the experiment started the worms were subjected to the following chemicals to confirm their viability, 10% sodium chloride, 10% formalin, 70% Alcohol and diluted Jik. Within 1 minute the worms were dead and it was easy to tell the difference between dead and live worm. Two milliliter of the prepared concentration of each plant extract was dispensed in a well of 24 cell culture plates containing 5 male and 5 female *S. mansoni.* The procedure was as for cercaricidal (6.2.2).

6.3. RESULTS

6.3.1 Cercaricidal effect of the five plant extracts on cercariae

The cercaricidal effects of plant extracts are shown in Table 6.2. The results were the duplicate observations of lowest (150µg/ml), moderate (300µg/ml) and maximum (600µg/ml) concentration at different time intervals.

In *Bridelia micrantha* extract at 150µg/ml, 300 µg/ml and 600µg/ml concentrations within 5-15 minutes the cercariae were active. Within 30-1 h cercariae showed slow movement and were all at base for all the three concentrations. By 1 hour for the 150µg/ml and 300 µg/ml concentrations cercariae were showing slow movement, in addition 600µl/ml concentration the cercariae heads were separated. The plant extract with highest cercaricidal effect in the group was DCM/ Meth extract.

In *Ocimum americanum* extract for 150µg/ml concentration all the Cercariae were active within 5minute to 1 hr. However for 300µg/ml and 600µg/ml concentrations within 15-30 minutes cercariae showed slow movement and were all at the base except in the hexane extract which cercariae was active. However within 1 hour for 150µg/ml concentration cercariae showed slow movement, in addition 300µg/ml and 600µg/ml concentrations cercariae were dead. The plant extract with highest cercaricidal effect in the group was DCM/ Meth extract.

In *Sonchus Luxurians* extract for all the concentration within 5Min - 1hr cercariae were active. However for the DCM/ Meth extract for 300µg/ml and 600µg/ml concentrations within 30 minutes the cercariae were at the base, in addition within 1h were showing slow movement but in 600µg/ml concentration the head had separated from the tail. The plant extract with highest cercaricidal effect in the group was DCM/ Meth extract.

In *Aloe secundiflora* water extract for all the concentrations within 5 minute to 1 h cercariae were active. Only water extracts was used for the experiment because other extracts could not dissolve, this also implied to *Croton megalocarpus* extracts.

In *Croton megalocarpus* water extract for all the concentrations within 30 minutes cercariae were active. However after 30 minute to 1h cercariae showed slow movement.

In all experiments the plant extract with the highest cercaricidal effects were *Bridelia micrantha, Ocimum americanum and Sonchus Luxurians* DCM/ Meth extracts. Water extracts of all the plant showed no effect. When the experiment was repeated the similar results were obtained (Appendix 6).

Table 6.2: Different concentrations of plant extracts on cercariae

Plant	Time in	Various concentrations		
	Minute	150µg	300µg	600µg
BMW	5	√	√	√
	15	√	√	√
	30	√	SM	SM
	60	SM	SM	SM
BMD	5	√	√	√
	15	√	√	√
	30	SM	SM	SM
	60	SM	×	×
BMH	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	SM	SM	SM
OAW	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	√	√	√
OAD	5	√	√	√
	15	√	√	√
	30	SM	SM	SM
	60	SM	×	×
OAH	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	SM	X	X
SLW	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	√	SM	SM
SLD	5	√	√	√
	15	√	√	√
	30	√	SM	SM
	60	×	×	×
ASW	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	√	√	√
CMW	5	√	√	√
	15	√	√	√
	30	√	SM	SM
	60	SM	SM	SM

Key:
BMW- *Bridelia micrantha* water extract
BMD- *Bridelia micrantha* Dichloromethane /methanol extract
BMH- *Bridelia micrantha* hexane extract
OAW- *Ocimum americanum* water extract
OAD- *Ocimum americanum* Dichloromethane/methanol extract
Key:
BMW- *Bridelia micrantha* water extract
BMD- *Bridelia micrantha* Dichloromethane /methanol extract
BMH- *Bridelia micrantha* hexane extract
OAW- *Ocimum americanum* water extract

× - Dead

SM- Slow movement

OAD- *Ocimum americanum* Dichloromethane/methanol extract
OAH- *Ocimum americanum* hexane extract
SLW- *Sonchus luxurians* water extract
SLD- *Sonchus luxurians* Dichloromethane /methanol extract
CMW- *Croton megalocarpus* water extract
ASW- *Aloe* secundiflora water extract
OAH- *Ocimum americanum* hexane extract
SLW- *Sonchus luxurians* water extract
SLD- *Sonchus luxurians* Dichloromethane /methanol extract
CMW- *Croton megalocarpus* water extract
ASW- *Aloe* secundiflora water extract
√ - Active

6.3.2 Wormicidal effect of the plant extracts on worms

The Wormicidal effects of plant extract on worms are shown on Table 6.3. The results were the duplicate observations of lowest (150µg/ml), moderate (300µg/ml) and maximum (600µg/ml) concentration at different time intervals. In 150µg concentration for *Ocimum americanum* hexane, water and *Bridelia micrantha* water extracts at five minutes time intervals the worms were alive. However at fifteen minutes for the same concentrations they were dead. For all plants in 300µg/ml and 600µg/ml concentrations at five minutes to one hour the worms were dead. The worms in the perfusion fluid which served as a control were alive and well. When the experiment was repeated the similar results were obtained (Appendix 6).

Table 6.3: Wormicidal effect of different concentrations of plant extracts on worms

Plant Extracts	Time in minutes	Various concentrations		
		150µg	**300µg**	**600µg**
OAH	5	√	×	×
	15	×	×	×
	30	×	×	×
	60	×	×	×
OAW	5	√	×	×
	15	×	×	×
	30	×	×	×
	60	×	×	×
BMW	5	√	×	×
	15	×	×	×
	30	×	×	×
	60	×	×	×
Control worm in perfusion fluid	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	√	√	√

Key:

OAH- *Ocimum americanum* hexane extract
OAW- *Ocimum americanum* water extract
BMW- *Bridelia micrantha* water extract
√ - Active
× - Dead

6.4: DISCUSSION

Schistosomes (also known as blood flukes) are digenetic trematodes. A characteristic feature of members in this family is that mature female worms are more slender than the male worms and are normally carried by the males in a ventral groove known as the gynaecophoric canal, which is formed by ventrally flexed lateral outgrowths of the male body. Schistosomes utilize aquatic snails as intermediate hosts for the completion of their life cycles. The transmission depends on absolutely the presence of intermediate snail hosts in bodies of water. Man gets infected by coming into contact with water infested with cercariae released into water by snails (Beltran & Boissier, 2008).

The cercaricidal effects of the five plant extracts was tested in an in -Vitro study using different dosages timed from 0-1hour. Cercariae in 150µg/ml and 300 µg/ml concentrations of *Bridelia micrantha* extracts were showing slow movement after 1 h, while in 600µg/ml concentration the cercariae heads were separated. The plant extract with highest cercaricidal effect in the group was DCM/ Meth extract.

Ocimum americanum extracts at 150µg/ml concentration cercariae showed slow movement by 1 h. On the other hand, the Cercariae were dead 300µg/ml and 600µg/ml by 1 h. The plant extract with the highest cercaricidal effect in the group was DCM/ Meth extract.

For *Sonchus Luxurians* extract at 300 ug/ml concentration, cercariae showed low movement but in 600µg/ml concentration for DCM/ Meth extract the head cercariae were

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSION

7.1 General discussion

Phytochemical studies of the plant preparations are necessary for standardization, which helps in understanding the significance of phytoconstituents in terms of their observed activities. The phytochemical screening of the compounds present in the efficacious plant extracts were; alkaloid, catecholics, glycosides, saponins, reducing sugar, steroid, flavones and catecholics. Most of them have therapeutic value.

From the study, when the two efficacious plants extract, OAH and BMW, were compared with PZQ, they were significantly similar in low worm recovery and high worm reduction. This was an indication that they were close to PZQ in fighting the parasitic infection compared to other plant extracts.

Gross pathology was done by physical observation of the liver surface to detect inflammation, adhesions and presence of granuloma which was an indication of infection and cell mediated immunity. The plant extracts of OAH and BMW were similar to PZQ in terms of no or few granuloma in gross pathology. This means that the two extract groups were very effective in protecting the animal from pathological effect of the parasite eggs. IC group showed moderate to severe granuloma, which is an indication that the animal was not protected from the pathological effect of the eggs.

Granuloma sizes of OAH and BMW plant extracts groups were significantly similar to the PZQ in size, however significantly different to IC (infected control group). This

means that the two plant extracts contain substances which were able to reduce sizes of granulomas, leading to protection of the animal against the infection. However, crude plants were unable to protect the animals from the infections which was indicated by high worm counts, increased gross pathology and large sizes of granuloma. When mice were subjected to different concentrations of the efficacious extracts, the best dosage was 300mg/ml. This was indication by high worm reduction, reduced pathology, histopathology and high immunological responses.

BMW extract had high IFN-γ responses for both spleen and lymph node cells. This implied that treatment group was able to produce the TH-1 which is an important profile for cell mediated immunity in fighting the infection. Other studies show that Interferon-γ has been used to treat chronic granulomatous disease (Todd and Goa, 1992).

In this study, all extracts were able to produce IL-5 for both lymph node and spleen cells. The response was high for BMW, OAH and PZQ (positive control). That meant that PZQ, OAH and BMW were able to eliminate antibody-bound parasites through the release of cytotoxic granule proteins. Eosinophils are the primary IL-5-expressing cells therefore they play an important role in regulating parasitic infections (Dubucquoi, 1994).

BMW plant extract induced higher IgG responses compared to OAH and PZQ. This is an indication that it was able to fight parasitic infections using the humoral arm of immunity. The SWAP antigens produced better IgG responses compared to 0-3 hr antigen.

Ocimum americanum hexane and water and *Bridelia micrantha water* extracts were able to kill the worms within five minutes. However when subjected to cercariae the extracts were unable to kill cercariae. The study showed the plant extracts with the highest cercaricidal effects were *Bridelia micrantha, Ocimum americanum and Sonchus Luxurians* DCM/ Meth extracts and the effect was between 30 to 60 minutes. This is an indication that plant extracts had different effects on the different stages of *Schistosoma mansoni*, cercariae which develop in the intermediate host, the snail, and worms, which develop in the mammalian definitive host.

7.2 Conclusion

1. Alkaloids, catecholics, flavonoids, saponins, reducing sugar and steroids, were present in OAH and BMW and had therapeutic value.

2. OAH and BMW plant extracts were significantly similar to PZQ in terms of worm reduction and reduced pathology, an indication that the extracts were able to fight the parasitic infection.

3. OAH and BMW plant extracts were able to generate IL-5, IFN- γ and IgG responses which were an indication that the extracts were able to fight infection using both humoral and cell mediated immunity.

4. OAH and BMW plant extracts were able to show wormicidal effects.

5. The best dosage for OAH and BMW plant extracts was 300mg/ml and this was indicated by high worm reduction, reduced pathology, histopathology and high immunological responses.

6. The two plant extracts *Ocimum americanum* hexane and *Bridelia micrantha water* extracts were proven to be efficacious as they were very close to PZQ which is the drug used for treating schistosomiasis which is world wide health problem.

7. Therefore the two plant extracts have shown to be possible future candidates for drug development for schistosomiasis.

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Appendix 1: Plant identification

NATIONAL MUSEUMS OF KENYA

WHERE HERITAGE LIVES ON

The East African Herbarium
P.O. Box 45166 00100 Nairobi, Kenya
Telephone: 3743513, 3742131/4 ext 2274
Fax: 3741424
E-Mail: botany@museums.or.ke

REF: NMK/BOT/CTX/1/2

31st May 2011

Naomi Waigajo
P.O. Box 3837-00200
NAIROBI

Dear Naomi,

PLANT IDENTITY

The specimen you have brought to us has been identified as follows:

Family

1. Euphorbiaceae - *Bridelia micrantha* (Hochst.) Baill. Mukoigo Kikuyu
2. Aloaceae - *Aloe secundiflora* Engl. Kiluma Kamba
3. Labiatae - *Ocimum americanum* L. Mutaa Kamba
5. Euphorbiaceae - *Croton megalocarpus* Hutch. Mukinduri Kikuyu
6. Compositae - *Sonchus luxurians* (R.E.Fr.) C.Jeffrey Uthunga Kamba

Yours sincerely,

Dr. Itambo Malombe
Ag.Head, Botany Department

Appendix 2: Antigens preparation

Schistosome Worm Antigen Preparation (SWAP)

Adult *S. mansoni* worms were obtained from infected mice perfused at week five post-infection (see 2.13). The worms were placed in a tube containing PBS, and sonicated (23 kHz, 16 μm amplitude) for 10 minutes and the homogenate centrifuged for 1 hour at 100,000 g, at 4 °C to obtain the soluble protein. The concentration of protein was assayed by Bio-Rad method of Bradford (Bradford, 1976). This method utilized bovine serum albumin, BSA, (Bio-Rad Co.) as a standard protein. The optical densities at 595nm, of serial dilution of BSA were read using a Cecil spectrophotometer. Optical densities of different dilutions of the SSP were obtained at the same wavelength as BSA dilutions. The concentration of SSP dilution was then calculated using the equation of the curve given by the spectrophotometer (Cecil Instruments CE 6600R, England). The protein was aliquoted and sterilized by exposure to UV light (10 minutes, 5 cm from a 30 watt ultra violet OSRAM bulb) before use in *in vitro* assays. The aliquots were stored at – 20 °C. The Protein concentration was adjusted to 100 μg/ml in PBS before use in *in vitro* assays.

Preparation of 0-3 hr release protein

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 as described by Ramolho-pinto *et al., (*1974). The heads were isolated on discontinuous percoll gradient and washed two times in complete media. Two concentrations of Percoll (Sigma Co. Sweden) were prepared; one which was 70% Percoll mixed with 9 ml of RPMI 1640 media). The other set consisted of 45% Percoll (9 ml of Percoll mixed with 11 ml of

RPMI 1640). A drop of Hepes (Flow Laboratories, Scotland) was added into each tube to keep the pH constant. The 45% Percoll was layered over the 70% Percoll to make a discontinuous gradient. Cercariae suspension was chilled for one hour at 4^0 C. They settled at the bottom of the beaker. Excess water from the chilled cercariae suspension was sucked out using a Pasteur pipette and the cercariae resuspended. The cercariae suspension was placed in chilled glass tubes and centrifuged for 10 seconds at 450g.

Glucose was made up to 5% in double distilled water. After centrifugation, the supernatant was sucked out of the glass tubes containing cercariae and the 0.5 ml glucose added. The suspension was shaken on a vortex for 90 seconds to separate the heads from the tails of the chilled cercariae. The separated heads and tails of cercariae were dispensed gently on the Percoll gradient using a Pasteur pipette and centrifuged at 450g, for 10 min. The heads formed a band at the top of the interface of the gradient.

The heads were aspirated and washed three times in complete media. The heads were re-suspended in complete media and transferred to the Bijou tubes and incubated at 37 ^{0}C, in the presence of 5% carbon dioxide for 0-3 hr release protein. After 3 hr of incubation, the schistosomula (head) suspension was centrifuged (10 minutes at 450g, at 37 ^{0}C). The supernatant was obtained. This contains the proteins released by penetrating schistosomula between 0-3 hr of penetration

Concanavalin A (Con A)

Concanavalin A is a plant lectin which combines with sugar moieties on the surface of the lymphocytes inducing mitosis. It stimulates mitosis in lymphocytes regardless of their antigenic specificity. Responding cells undergo blast transformation prior to mitosis.

If cultured in the presence of radio-labelled thymidine the cells incorporate thymidine into their DNA so that proliferation responses can be quantified.

A commercially prepared concanavalin A (Sigma Cell Culture) was dissolved in sterile PBS to make a concentration of 1 mg/ml. The solution was sterilized using 0.45 μm Nalgene disposable filter (Nalgene Co. USA) and stored at - 20 °C. It was used at a concentration of 1 μg per well in *in vitro* assays.

APPENDIX 3: Preparation of Media and its components

RPMI 1640 preparation from powder (wash)

Measure 1L of distilled water into 1L beaker.

Tear the sachet containing the powder for 1L.

Pour it into the beaker.

Add 2 grams of sodium bicarbonate into the mixture.

Stir by magnetic stirer until all is completely dissolved.

Bring the PH between 7.2 – 7.4

Filter sterilizes using the 0.5 l filter of 0.45 μm in culture hood.

Upon filtering in the hood add 10ml of both L-glutamine and β mecapto ethanol.

Also add 4 mls of commercially made gentamycin.

The media is now ready for use.

Store it at 4°C.

For complete media (C10) from the wash media just remove 50ml of wash from ½L and replace it with 50 ml of fetal calf serum.

This is ready for cutting cells.

L – GLUTAMINE 200MM/ML PREPARATION

Weigh 1.4610 grams of L. glutamine in a weighing boat.

Put this in a clean beaker.

Measure 50ml of Double distilled water.

Add the 50ml into the beaker containing the L-glutamine.

Mix thoroughly on a magnetic stirrer until the L–glutamine dissolves completely.

Filter sterilizes using the 0.45μm filter.

Aliquot in 5 ml and store at –20°C.

β- MERCAPTO ETHANOL PREPARATION

Take the β-mercapto ethanol bottle and open it in a normal fume hood with an outlet.

Take 15µl of β-mercapto ethanol – Dispense this into a 50 ml tube containing 45 ml sterile phosphate buttered saline.

Mix by shaking the tube.

Filter sterilizes using 0.2 µm or 0.45 µm filter.

Aliquot 5 ml, in Bijou and store at –20°C.

APPENDIX 4- Replica Experiments for Chapter 4

Table 1: Mean number of worms and recovery percentage

		Mean number of worms recovered per group **(mean ± SE)**				
Treatment Group	Dose mg/kg	Total males mean	Total females mean	Total worms Mean ± SE	% Worm recovery	% Worm reduction
PZQ	900X2	13.0±1.08	10.5±2.06	23.5±3.14	30.8	69.2
OAH	150X2	13.3±3.53	11.3±1.33	24.7±4.81	32.4	67.6
OAD	150 X2	21.0±1.78	23.3±2.87	44.3±4.65	58.1	41.9
OAW	150 X2	20.0±2.89	14.0±3.0	34.0±5.89	44.6	55.4
OAC	150 X2	21.7±1.20	18±4	39.7±5.2	52.0	48
BMH	150X2	20.7±5.84	17.7±4.84	38.4±10.68	50.3	49.7
BMD	150 X2	24.5±2.66	21±2.27	45.5±4.93	59.6	40.4
BMW	150 X2	14.0±1.47	12.3±1.25	26.3±4.93	34.5	65.5
BMC	150 X2	33.7±5.54	30.7±4.61	64.4±10.15	84.4	15.6
SLD	150 X2	25±1.53	19.0±2.0	44.0±3.53	57.7	42.3
SLW	150 X2	27.3±4.56	21±5.05	48.3±9.61	63.3	36.7
SLC	150 X2	29.8±2.78	20.3±3.28	51.1±6.06	66.9	33.1
CMW	150 X2	21.0±4.16	15±1.53	36.0±5.69	47.2	52.8
CMC	150 X2	22.8±1.44	14.3±2.43	37.1±3.87	48.6	51.4
ASW	150 X2	29.0±5.37	21.0±3.58	50.0±8.95	65.5	34.5
ASC	150 X2	32.3±6.77	24.0±3.21	56.3±9.98	73.8	26.2
IC	-	42.0±5.15	34.3±3.85	76.3±9.0	-	-

Key:

PZQ- Praziquantel
OAH- *Ocimum americanum* hexane extract
OAD- *Ocimum americanum* Dichloromethane/methanol extract
OAW- *Ocimum americanum* water extract
OAC- *Ocimum americanum* crude

BMH- *Bridelia micrantha* hexane extract
BMD- *Bridelia micrantha* Dichloromethane/methanol extract
BMW- *Bridelia micrantha* water extract
IC – infected control

BMC- *Bridelia micrantha* crude
SLD- *Sonchus luxurians* Dichloromethane/methanol extract
SLW- *Sonchus luxurians* water extract
SLC- *Sonchus luxurians* crude
CMW- *Croton megalocarpus* water extract
CMC- *Croton megalocarpus* crude
ASW- *Aloe* secundiflora water extract
ASC - *Aloe* secundiflora crude,

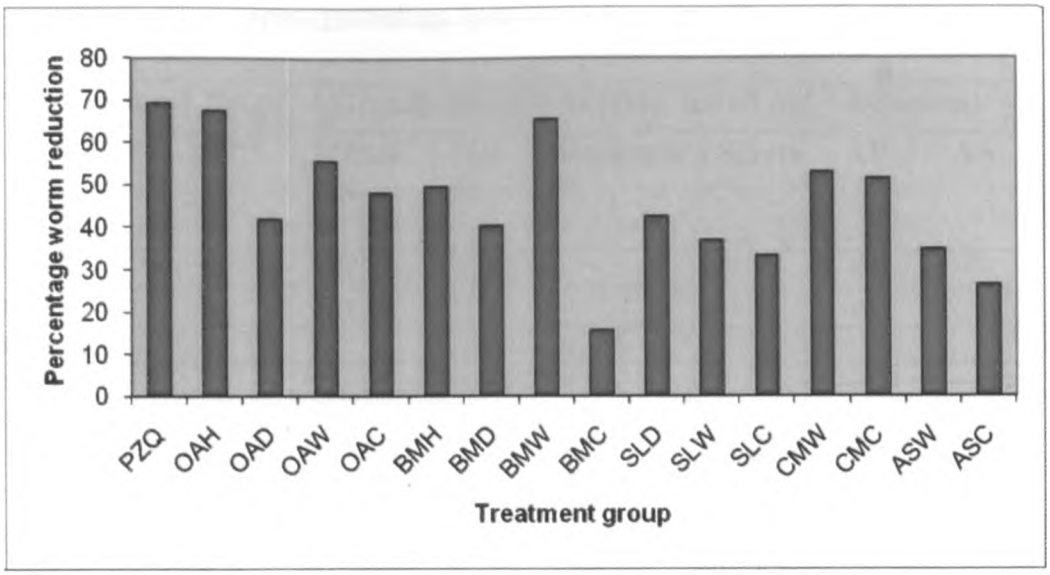

Figure 1: Percentage of worm reduction

Key:

PZQ- Praziquantel
OAH- *Ocimum americanum* hexane extract
OAD- *Ocimum americanum*
Dichloromethane/methanol extract
OAW- *Ocimum americanum* water extract
OAC- *Ocimum americanum* crude

BMH- *Bridelia micrantha* hexane extract
BMD- *Bridelia micrantha*
Dichloromethane/methanol extract
BMW- *Bridelia micrantha* water extract
BMC- *Bridelia micrantha* crude
SLD- *Sonchus luxurians*
Dichloromethane/methanol extract
SLW- *Sonchus luxurians* water extract
SLC- *Sonchus luxurians* crude
CMW- *Croton megalocarpus* water extract
CMC- *Croton megalocarpus* crude
ASW- *Aloe* secundiflora water extract
ASC - *Aloe* secundiflora crude
IC – infected control

Table 2: Liver gross pathology in different treatment groups and controls

Plant extracts	No. of mice	Granuloma classification for all the				Adhesions		Inflammation	
		None %	Few %	Moderate %	Severe %	AP	AA	I	NI
PZQ	4	2(50)	2(50)	-	-	+		+	
OAH	4	1(25)	3(75)	-	-	+		+	
OAD	4	-	1(25)	3(75)	-	+		+	
OAW	4	-	2(50)	2(50)	-	+		+	
OAC	4	-	1(25)	3(75)	-	+		+	
BMH	4	-	-	2(50)	2(50)	+		+	
BMD	4	-	-	2(50	2(50)	+		+	
BMW	3	-	2(66.	1(33.3)	-	+		+	
BMC	3	-	-	3(66.7)	1(33.3)	+		+	
SLD	4	-	-	3(75)	1(25)	+		+	
SLW	4	-	-	2(50)	2(50)	+		+	
SLC	4	-	-	1(25)	3(75)	+		+	
CMW	4	-	-	2(50)	2(50)	+		+	
CMC	3	-	-	1(33.3)	2(66.7)	+		+	
ASW	4	-	-	2(50)	2(50)	+		+	
ASC	4	-	-	1(25)	3(75)	+		+	
IC	4	-	-	1(25)	3(75)	+		+	
Naïve mice	2	-	-	-	-	-		-	

Key:

PZQ- Praziquantel
OAH- *Ocimum americanum* hexane extract
OAC- *Ocimum americanum* crude
BMH- *Bridelia micrantha* hexane extract
BMD- *Bridelia micrantha* Dichloromethane/methanol extract
BMW- *Bridelia micrantha* water extract
BMC- *Bridelia micrantha* crude
SLD- *Sonchus luxurians* Dichloromethane/methanol extract
OAD- *Ocimum americanum* Dichloromethane/methanol extract
OAW- *Ocimum americanum* water extract
SLW- *Sonchus luxurians* water extract
SLC- *Sonchus luxurians* crude
CMW- *Croton megalocarpus* water extract
CMC- *Croton megalocarpus* crude
ASW- *Aloe* secundiflora water extract
ASC - *Aloe* secundiflora crude
IC – infected control

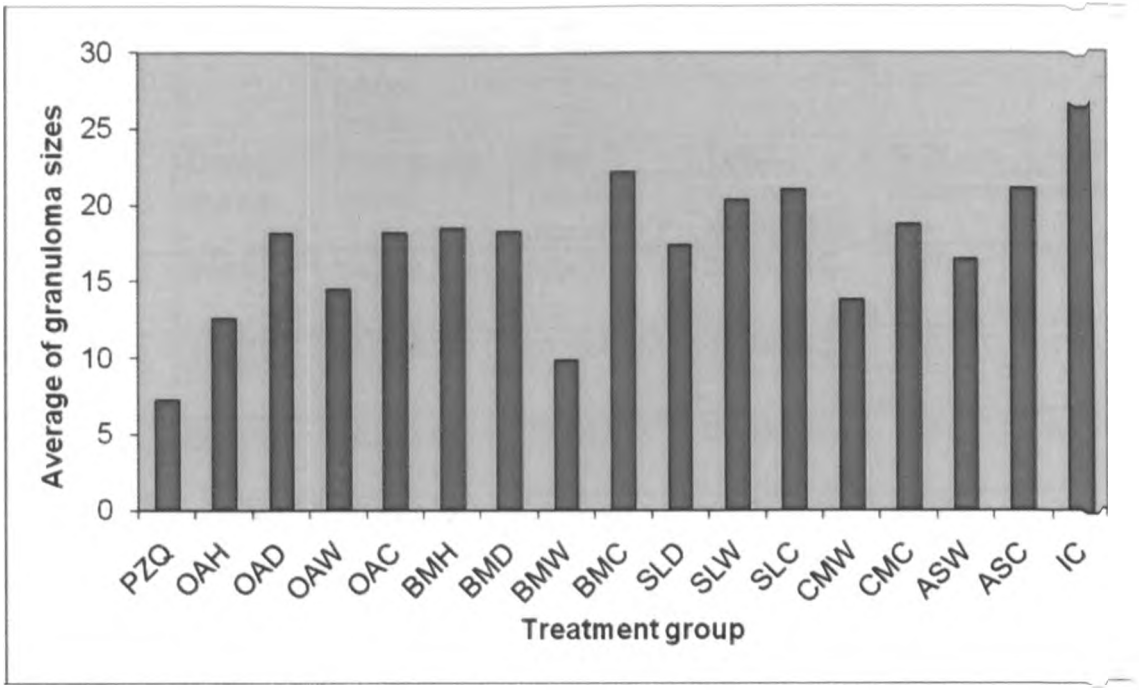

Figure 2: size of granuloma in the liver tissue for five plant extracts

Key:

PZQ- Praziquantel
OAH- *Ocimum americanum* hexane extract
OAD- *Ocimum americanum* Dichloromethane/methanol extract
OAW- *Ocimum americanum* water extract
OAC- *Ocimum americanum* crude

BMH- *Bridelia micrantha* hexane extract
BMD- *Bridelia micrantha* Dichloromethane/methanol extract
BMW- *Bridelia micrantha* water extract
BMC- *Bridelia micrantha* crude
SLD- *Sonchus luxurians* Dichloromethane/methanol extract
SLW- *Sonchus luxurians* water extract
SLC- *Sonchus luxurians* crude
CMW- *Croton megalocarpus* water extract
CMC- *Croton megalocarpus* crude
ASW- *Aloe* secundiflora water extract
ASC - *Aloe* secundiflora crude
IC – infected control

Table 3: Mean number of worms and recovery percentage for efficacious plant extracts

		Mean number of worms recovered per group (mean ± SE)				
Treatment Group	Dose mg/kg	Total males means	Total Females means	Total worms Mean ± SE	% Worm recovery	% Worm reduction
PZQ	900X2	14.0±3.76	8.0±2.12	22.0±5.88	26.7	73.3
OAH	150X2	24.2±4.76	17.5±4.06	41.7±8.82	50.5	49.5
OAH	300 X2	16.0±3.47	13.3±2.25	29.3±5.72	35.5	64.5
OAH	600 X2	29.5±5.28	22.3±4.8	51.8±10.1	62.8	37.2
OAW	150 X2	28.8±3.67	28.5±4.64	57.3±8.31	69.4	30.6
OAW	300X2	21.6±2.84	28.0±5.04	49.6±7.88	60.1	39.9
OAW	600 X2	33.4±5.31	28±4.47	61.4±9.78	74.4	25.6
BMW	150 X2	33.0±5.77	26.8±4.93	59.8±10.7	72.5	27.5
BMW	300X2	17.7±3.54	16.7±2.93	34.4±6.47	41.7	58.3
BMW	600 X2	31± 4.33	28± 4.8	59.0±9.13	71.5	28.5
IC		50.2±6.52	32.3± 4.88	82.5±11.4		

KEY:
PZQ-Praziquantel
OAH- *Ocimum americanum* hexanes extract
OAW- *Ocimum americanum* water extract
BMW- *Bridelia micrantha* water extracts
150mg- low concentration
300mg- medium concentration
600mg –High concentration

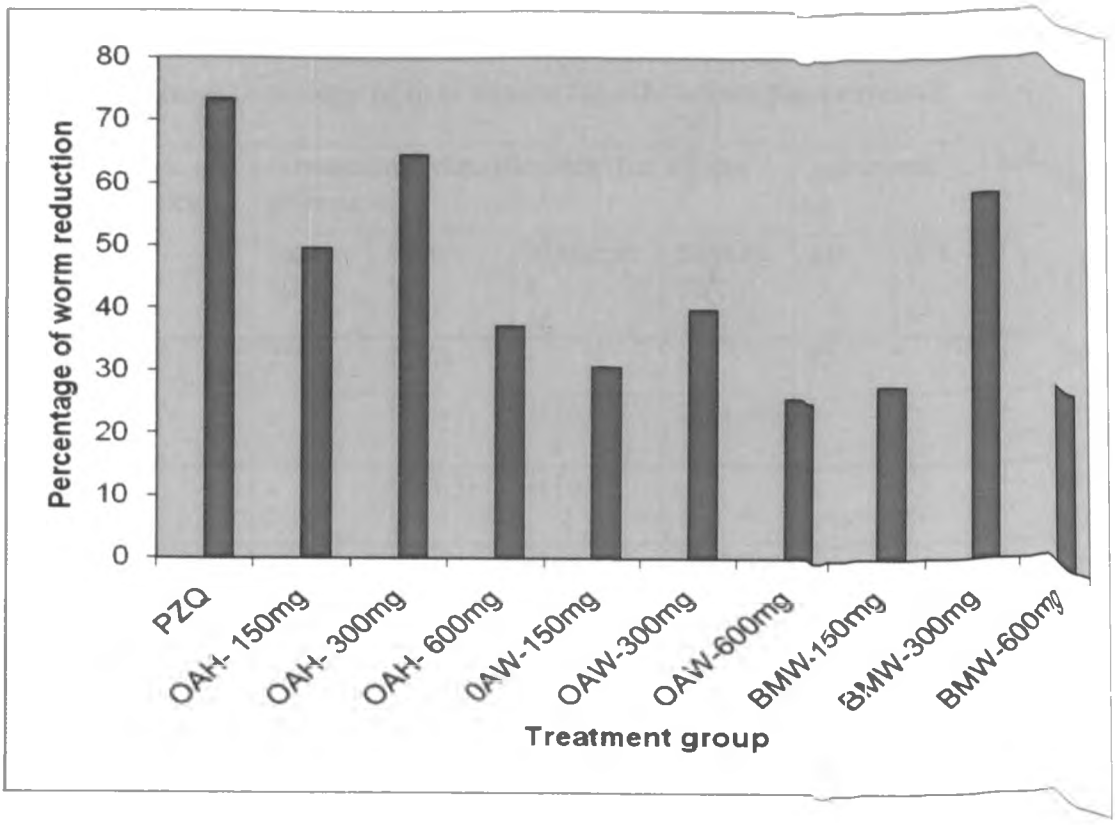

Figure 3: Percentage of worm reduction for efficacious plant extract

KEY:
PZQ-Praziquantel
OAH- *Ocimum americanum* hexane extract
OAW- *Ocimum americanum* water extract
BMW- *Bridelia micrantha* water extracts
150mg- low concentration
300mg- medium concentration
600mg –High concentration

Gross pathology

Table 4: Gross pathology of liver tissues for efficacious plant extracts

Plant extracts	No. of mice	Granuloma classification for all the groups				Adhesions		Inflammation	
		None %	Few %	Moderate %	Severe %	AP	AA	I	NI
PZQ	4	2(50)	2(50)	-	-	+		+	
OAH 150Mg	6	-	1(16.7)	3(50)	2(33.3)	+		+	
OAH 300Mg	6	-	5(83.3)	1(16.7)	-	+		+	
OAH 600Mg	6	-		4(66.7)	2(33.3)	+		+	
OAW 150Mg	6	-	-	4(66.7)	2(33.3)	+		+	
OAW 300Mg	6	-	-	4(66.7)	2(33.3)	+		+	
OAW 600Mg	5	-	-	2(40)	3(60)	+		+	
BMW 150Mg	5	-	-	3(60)	2(40)	+		+	
BMW 300Mg	4	-	2(50)	2(50)	-	+		+	
BMW 600Mg	4	-	-	2(50)	2(50)	+		+	
IC	6	-	-	1(16.7)	5(83.3)	+		+	

Key:

None (no granuloma)
Few (1-3 granuloma per lobe)
Moderate (1-3 granuloma per lobe)
Severe (1-3 granuloma per lobe)
AP – Adhesions present
OAH- *Ocimum americanum* hexane extract

BMW- *Bridelia micrantha* water extracts

150mg/ml- low concentration

300mg/ml- medium concentration

600mg/ml –High concentration

I – Inflamed
NI – Not inflamed
AA - Adhesions Absent
PZQ-Praziquantel

OAW- *Ocimum americanum* water extract

Granuloma sizes

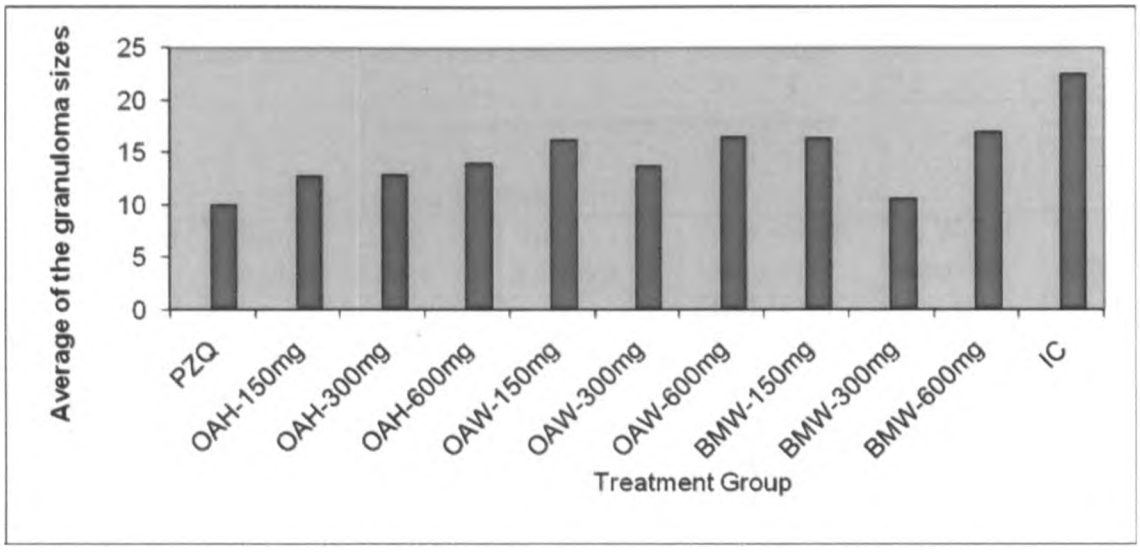

Figure 4: Size of granuloma in the liver tissue for efficacious plant extracts

Key:

PZQ-Praziquantel

OAH- *Ocimum americanum* hexane extract

OAW- *Ocimum americanum* water extract

BMW- *Bridelia micrantha* water extracts

150mg/ml- low concentration

300mg/ml- medium concentration

600mg/ml –High concentration

APPENDIX 5: Replica Experiments for Chapter 5

Immunological responses of efficacious plant extracts

Table 1: Mean number of worms and recovery percentage

		Mean number of worms recovered per group **(mean ± SEM)**				
Treatment Group	Dose Mg/kg	Total males	Total Females	Total worms **Mean ± SEM**	% Worm recovery	% Worm reduction
PZQ	900X2	15±2.5	10.2±1.19	25.2±3.69	41.3	58.7
OAH	300X2	16±2.1	12±1.6	28±3.7	45.9	54.1
BMW	300X2	23± 3.18	17± 1.9	40±5.1	65.6	34.3
IC	-	37±4.3	24±4.7	61±9.0	-	

KEY:

PZQ – Praziquantel

OAH- *Ocimum americanum* Hexane extract

BMW- *Bridelia micrantha* Water extract

IC - Infected control

Dose – mg (plant extract / PZQ) kg x number of doses

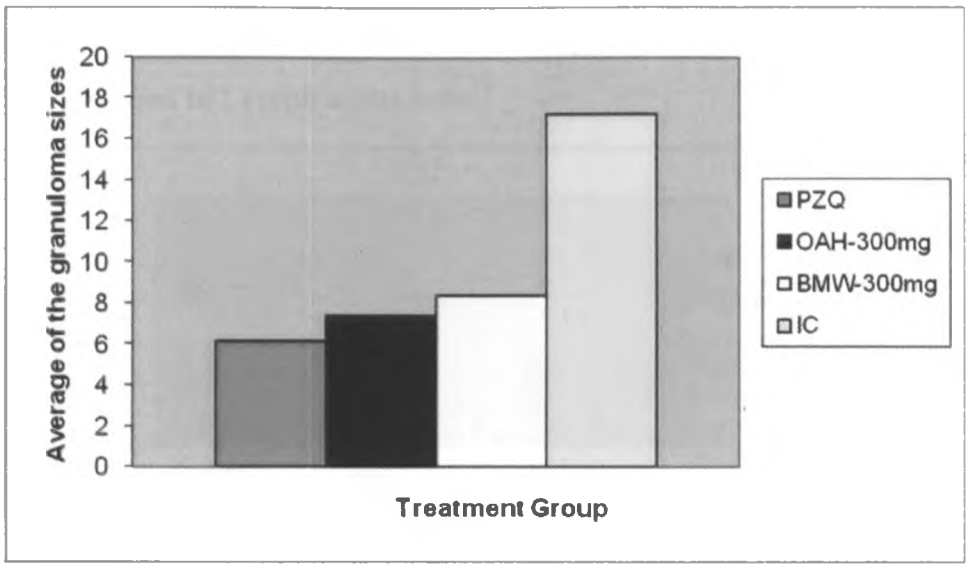

Figure 1: Percentage of worm Reduction

KEY:
PZQ – Praziquantel

OAH- *Ocimum americanum* Hexane extract

BMW- *Bridelia micrantha* Water extract

Cytokines responses

IFN- gamma in Lymph nodes cells

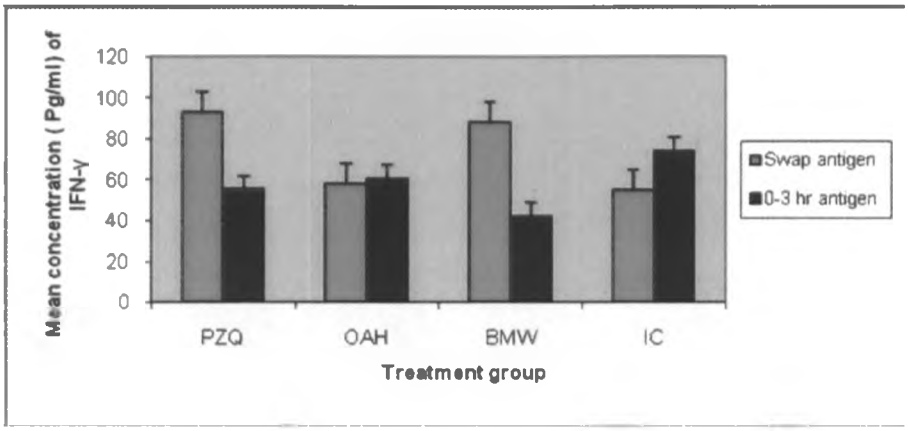

Figure 2: IFN - gamma in Lymph nodes cells stimulated with SWAP antigen

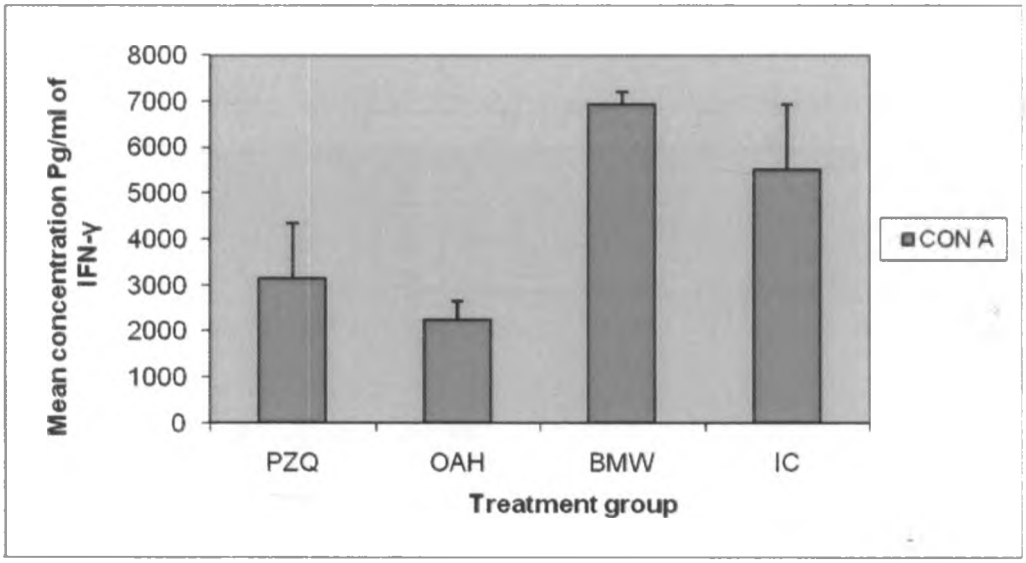

Figure 3: IFN - gamma in Lymph nodes cells stimulated with CON A antigen

IFN- gamma in spleen cells

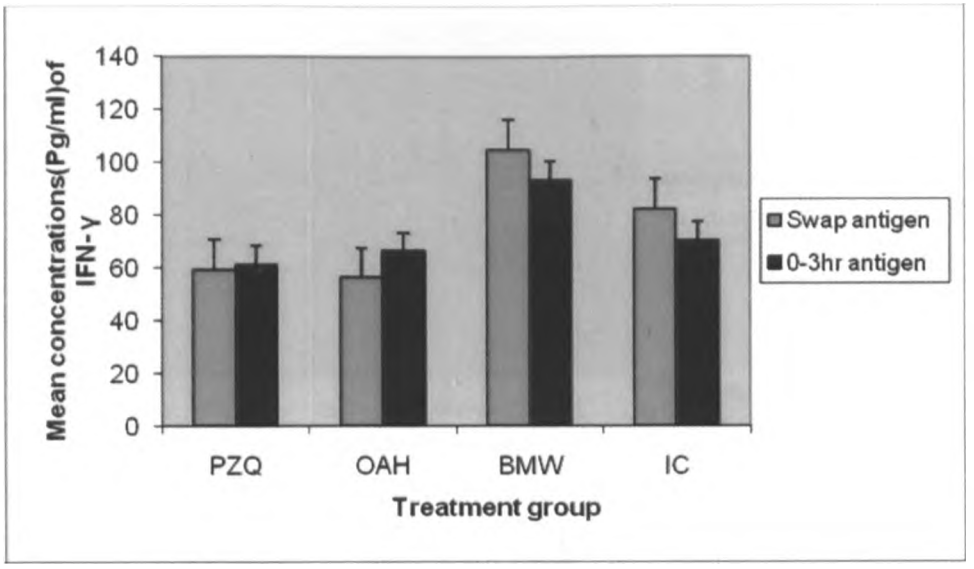

Figure 4: IFN - gamma –in Spleen cells stimulated with SWAP & 0-3hr antigen

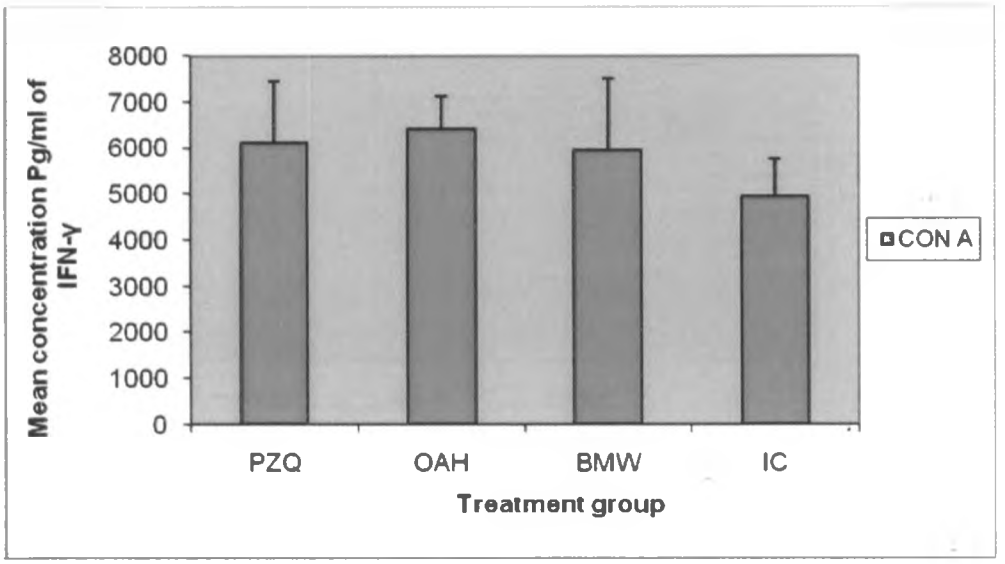

Figure 5: IFN - gamma in Spleen cells stimulated with CON A antigen

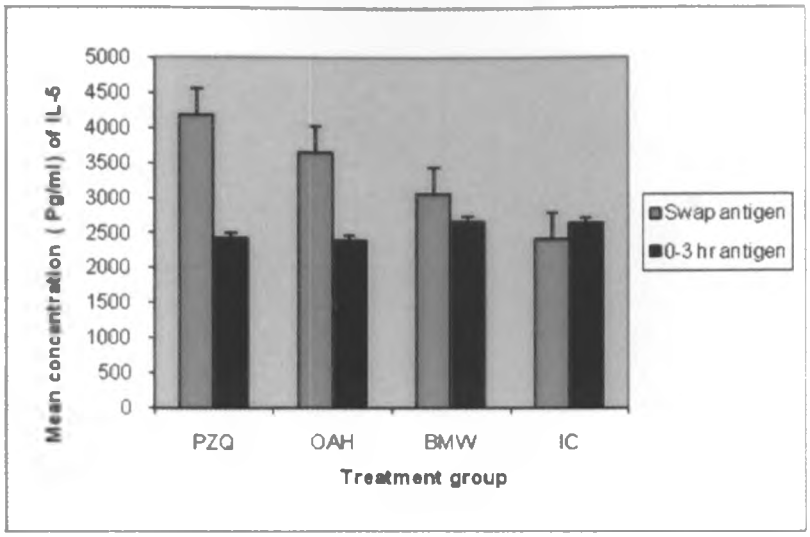

Figure 6:IL-5 in Lymph nodes stimulated with SWAP& 0-3hr antigen

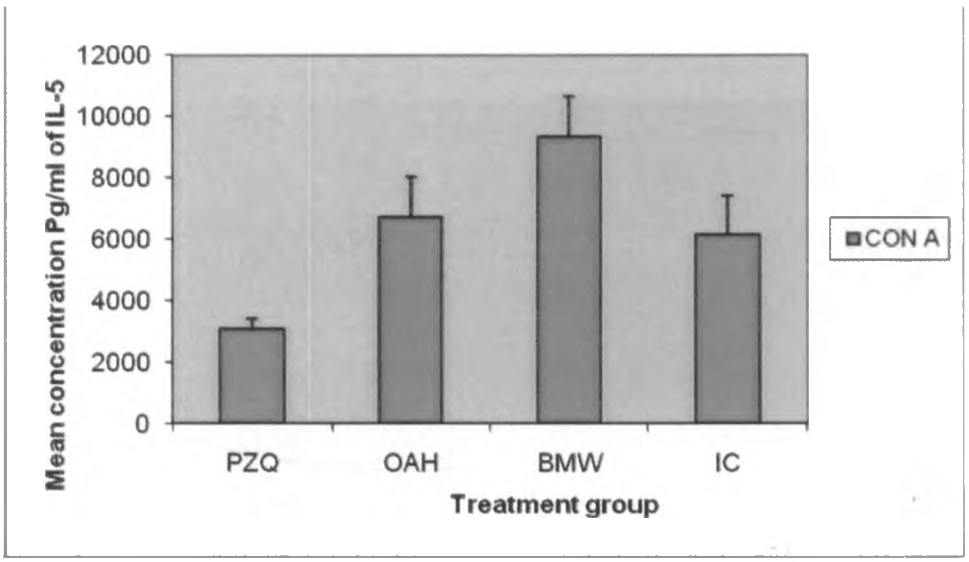

Figure 7: IL-5 in Lymph node cells stimulated with CON A antigen

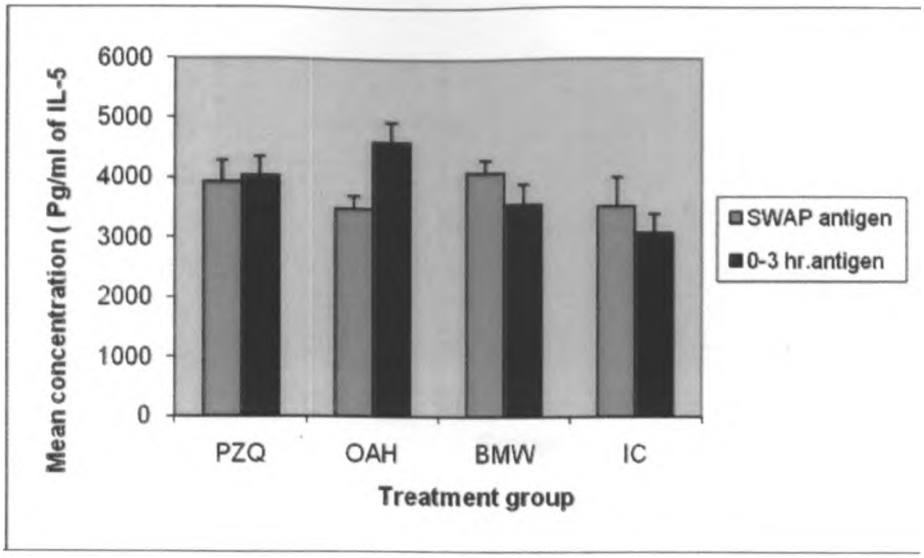

Figure 8: IL-5 in Spleen cells stimulated with SWAP& 0-3hr antigen

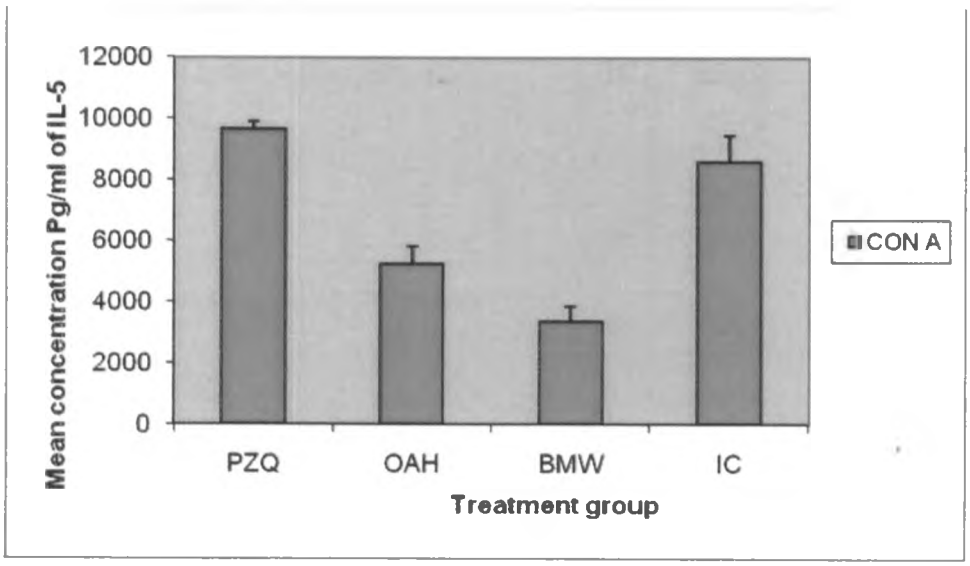

Figure 9: IL-5 in Spleen cells stimulated with CON A antigen

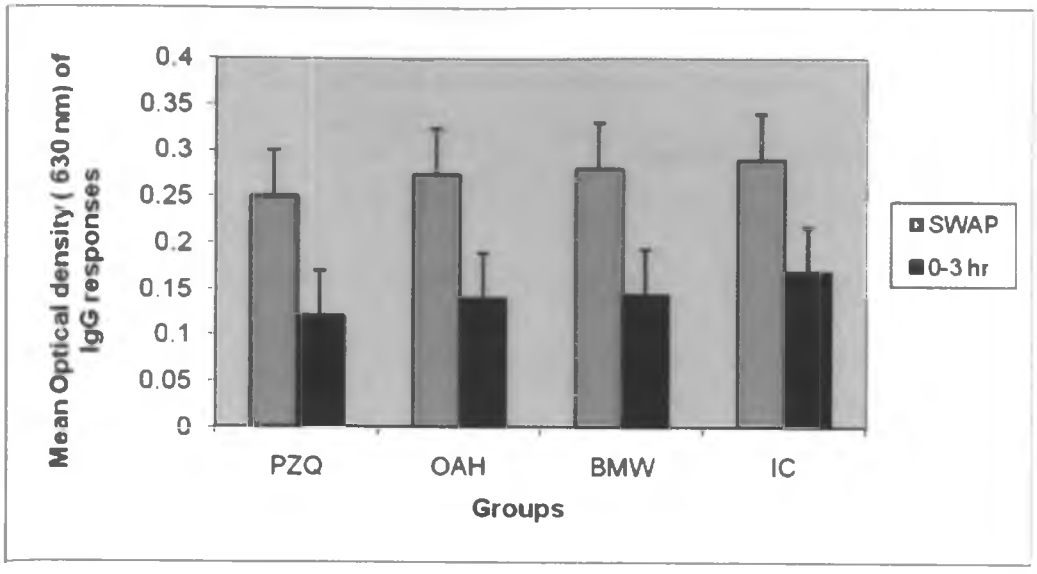

Figure 10: SWAP and 0-3hr Antigens specific IgG responses

Key:

PZQ – Praziquantel

BMW- *Bridelia micrantha* Water extract

OAH- *Ocimum americanum* Hexane extract

IC - Infected control

Table 2:Gross pathology of liver tissues

Plant extracts	No. of mice	Granuloma classification for all the groups				Adhesions		Inflammation	
		None %	Few %	Moderate %	Severe %	AP	AA	I	NI
PZQ	6	4(66.7%)	2(33.3)		-	+		+	
OAH 300Mg	6	1(16.7)	5(83.3)	-	-	+		+	
BMW 300Mg	5	-	5(100%)	-	-	+		+	
IC	6	-	-	4(66.7)	2(16.7)	+		+	

Key:
None (no granuloma)
Few (1-3 granuloma per lobe)

Moderate (1-3 granuloma per lobe)
Severe (1-3 granuloma per lobe)

AP – Adhesions present
AA - Adhesions Absent

I – Inflamed
NI – No inflammation

PZQ – Praziquantel

OAH- *Ocimum americanum* Hexane extract

BMW- *Bridelia micrantha* Water extract

IC - Infected control

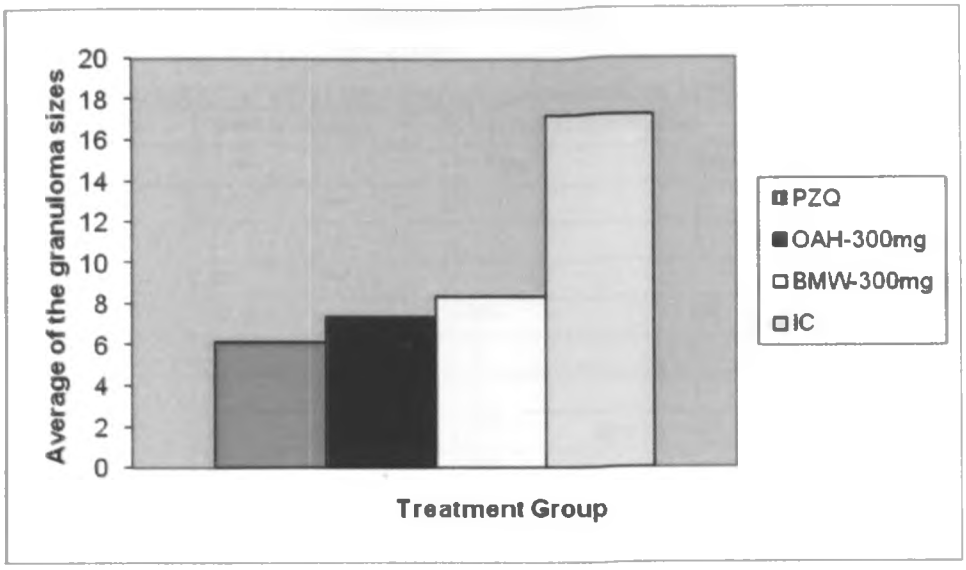

Figure 11: Granuloma sizes of liver tissue

Key:

PZQ – Praziquantel,

OAH- *Ocimum americanum* Hexane extract,

BMW- *Bridelia micrantha* Water extract,

IC - Infected control

APPENDIX 6: Replica Experiments for Chapter 6- Invitro activity showing cercaricidal and wormicidal effect

Table1: Cercaricidal effect on different concentrations of plant extracts

Plant Extracts	Time in minutes	Various concentrations		
		150µg	300µg	600µg
BMW	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	SM	SM	SM
BMD	5	√	√	√
	15	√	√	√
	30	SM	SM	SM
	60	SM	SM	×
BMH	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	SM	SM	SM
OAW	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	√	√	√
OAD	5	√	√	√
	15	√	√	√
	30	SM	×	×
	60	SM	×	×
OAH	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	SM	×	×
SLW	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	√	√	√
SLD	5	√	√	√
	15	√	√	√
	30	√	SM	SM
	60	SM	×	×
ASW	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	√	√	√
CMW	5	√	√	√
	15	√	√	√
	30	√	SM	SM

	60	Slow movement	Slow movement	Slow movement

Key:

BMW- *Bridelia micrantha* water extract
BMD- *Bridelia micrantha* Dichloromethane /methanol extract
BMH- *Bridelia micrantha* hexane extract
OAW- *Ocimum americanum* water extract
OAD- *Ocimum americanum* Dichloromethane/methanol extract
OAH- *Ocimum americanum* hexane extract
SLW- *Sonchus luxurians* water extract
SLD- *Sonchus luxurians* Dichloromethane /methanol extract
CMW- *Croton megalocarpus* water extract
ASW- *Aloe* secundiflora water extract
√ - Active
× - Dead
SM- Slow movement

Wormicidal effect on different concentrations of plant extracts

Plant Extracts	Time in minutes	Various concentrations		
		150µg	300µg	600µg
OAH	5	√	×	×
	15	×	×	×
	30	×	×	×
	60	×	×	×
OAW	5	√	×	×
	15	×	×	×
	30	×	×	×
	60	×	×	×
BMW	5	√	×	×
	15	×	×	×
	30	×	×	×
	60	×	×	×
Control worm in perfusion fluid	5	√	√	√
	15	√	√	√
	30	√	√	√
	60	√	√	√

Key:

OAH- *Ocimum americanum* hexane extract

OAW- *Ocimum americanum* water extract

BMW- *Bridelia micrantha* water extract

√ - Active

× - Dead