ANTI-SCHISTOSOMAL PROPERTIES OF THE WORMSEED PLANT, *CHENOPODIUM AMBROSOIDES* (FAMILY: *CHENOPODIACEA*) ON THE HUMAN PATHOGEN, *SCHISTOSOMA MANSONI* IN MICE

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Anti-Schistosomal Properties of the Wormseed Plant, *ChenopodiumAmbrosoides (*Family: *Chenopodiacea*) on the Human Pathogen, *Schistosoma Mansoni* in Mice

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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To my family, Bethel Church members, and friends for their patience, support and endurance during the period in which this study was carried out.

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LIST OF ABBREVIATIONS AND ACRONYMS

AME	Aqueous Methanol Extract
ANOVA	Analysis of Variance
Approx	Approximately
B cells	Bone marrow derived cells
BSA	Bovine Serum Albumin
cc	Cubic centimeter
CD	Cluster of differentiation (antigen cluster) on the cell
cm	Centimeter
CNS	Central nervous system
CP	Crude powder
DCM	Dichloromethane
DNA	Deoxyribonuclei acid
ELISA	Enzyme Linked Immunosorbent Assay
EPG	Eggs per gram
GIS	Geographical Information Systems
IFNγ	Interferon gamma
Ig	Immunoglobulin

IL	Interleukin
IPR	Institute of Primate Research
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KARI	Kenya Agricultural Research Institute
KDa	Kilo Daltons
KEMRI	Kenya Medical Research Institute
Kg	Kilogram
mg	Milligram
MHC	Major Histocompatibility Complex
Mm	Millimeter
ml	Milliliters
Na PCP	Sodium pentachloraplenate
NK	Natural killer cells
nm	Nanometers
^{o}C	Degree Celsius
P-value	Probability value
PBS	Phosphate buffered saline
pH	Negative Logarithm of Hydrogen ion concentration
PZQ	Praziquantel

R_f	Mobility Relative to front
RNA	Ribonuclei acid
rpm	Revolutions per minute
SEM	Standard Error of Means
SEA	Schistosome Egg Antigen
SSP	18 hr Soluble Schistosomule Protein
SWAP	Soluble Worm Antigen Protein
't' test	Two sample Student's 't' test
T cells	Thymus derived cells
TLC	Thin Layer Chromatography
TNF β	Tumor Necrosis Factor beta
µg	Microgram
µl	Microlitre
µm	micrometre
UoN	University of Nairobi
UV	Ultraviolet
v/v	Volume to volume
WHO	World Health Organization
Wk (s)	Week (s)

ABSTRACT

Plants may contain ingredients that have anti-parasitic activity against parasites of medical significance. *Chenopodium ambrosoides* (Wormseed) a wide spread herb in the Family Chenopodiacea was investigated for anti-schistosomal activity against the human trematode parasite, *Schistosoma mansoni*. The plant is well known for its vermifuge and anti-helminthetic properties. The root, stem, leaves and fruit of the plant were obtained and then the dried powder was extracted sequentially using *n*-hexane, dichloromethane, methanol and distilled water as solvents. After drying, the texture of the extracts ranged from semi-solid to solid. The texture of the extracts was probably due to the solvent used and the contents of part of the plant extracted. Mice were infected with *S. mansoni* and treated with extracts at week 4 postinfection. They were perfused at week 6 to recover worms. Gross pathology and histopathology were recorded. Aqueous (leaf) had 46% worms reduction, methanol (fruit) had 23% worms reduction and Praziquantel had 34% worms reduction. Aqueous (leaf) and methanol (fruit) extracts were significantly similar to Praziquantel ($p > 0.05$) and significantly different from infected controls ($p < 0.05$). This showed that aqueous (leaf) and methanol (fruit) extracts were efficacious against *S. mansoni* in terms of worms reduction. Other extracts worms recovery were similar to infected controls, hence they were not protective. Aqueous (leaf) and methanol (fruit) had similar gross pathology to Praziquantel while all the other extracts had similar gross pathology to infected controls. This means that aqueous (leaf) and methanol (fruit) extracts reduced gross pathology as a protective measure to *S. mansoni* while other extracts did not protect against *S. mansoni* infection. Both aqueous (leaf) and methanol (fruit) had mean glanuloma sizes significantly similar to Praziquantel ($p > 0.05$) meaning that they were able to protect by reducing granuloma sizes. The two extracts aqueous (leaf) and methanol (fruit) found to be efficacious were used in *in vitro* test against *S. mansoni* worms (5 males and 5 females). Aqueous (leaf) extract killed all worms at concentration of 0.05 mg/ml and in 5^{th} min. Its killing was dependent on ionizing effect of water. Methanol (fruit) extract killed all worms at concentration of 0.3 mg/ml in 15^{th} min. The killing was dependent on concentration of the extract. The

mortality effect aqueous (leaf) and methanol extracts were statistically similar to Praziquantel ($p > 0.05$). Schistosome antigens (SWAP and SSP) IgG responses of serum obtained from mice infected with *S. mansoni* and treated with aqueous (leaf) extract, methanol extract, Praziquantel and infected controls were assayed using ELISA. The aqueous (leaf) extract, methanol (fruit) extract and the Praziquantel had similar IgG responses to infected controls ($p > 0.05$) inspite of the fact that the first 3 had lower worms recovery than infected controls. This shows that they were killing worms directly and also via the immune response. This characteristic has been reported for Praziquantel. Aqueous (leaf) extract was better than methanol (fruit) extract. Isolation of crude extracts was carried out using Thin Layer Chromatography (TLC). Results of finger profiles mobile of *C. ambrosoides* extracts showed aqueous (leaf) extract had more R_f spots than methanol (fruit) extract but they were significantly similar ($p > 0.05$) suggesting that both aqueous (leaf) and methanol (fruit) extracts had similar compounds possessing anti- schistosomal activity. The results of this study suggest that *Chenopodium ambrosoides* aqueous (leaf) and methanol (fruit) extracts have remarkable anti-schistosomal properties which are similar to Praziquantel. Both aqueous (leaf) and methanol (fruit) extracts should be investigated to determine their toxicity and their active ingredients characterised. They should also be tested against other parasites as a source of anti-parasitic compounds for novel drug development.

CHAPTER ONE

INTRODUCTION

1.1 Schistosomes and Schistosomiasis

Schistosomes (or blood flukes) are digenetic trematode which utilize freshwater or amphibious snails as intermediate hosts and cause schistosomiasis in humans and other susceptible vertebrate animals which serve as definitive hosts (Ross *et al.,* 2002; Scholte, *et al*., 2012*).* The adult parasites live in the blood vascular systems of the definitive hosts, where they mate and produce eggs.

Human schistosomiasis is a major public health problem in tropical and sub-tropical regions of the world where an estimated 207 million people are actively infected and close to a billion people are at risk of contracting the disease (WHO, 2010). Endemic regions include parts of South America, the Caribbean area, Africa and the neighbouring regions, China and South East Asia (Ross *et al.,* 2002*).*

In Africa, both *S. mansoni* (causal agent of human intestinal schistosomiasis) and *S. haematobium* (causal agent of urinary schistosomiasis) are endemic and, snail species belonging to the Family Planorbidae serve as intermediate hosts of the parasites (Ross *et al.,* 2002; Scholte *et al*., 2012*).* Planorbid snails of the genus *Biomphalaria* are hosts of *S. mansoni* whereas those in the genus *Bulinus* are the hosts of *S. haematobium*. With respect to *S. mansoni*, although several *Biomphalaria* species may be involved in the transmission of the parasite in Africa, B. *pfeifferi* is probably the most widespread and probably the most important snail host on the continent (Ross *et al.,* 2002; Scholte *et al*., 2012). In addition to infecting humans, *S. mansoni* also naturally infects non-human primates and rodents (Nelson *et al.,* 1962), and these hosts can maintain and transmit the infection in the wild (Guillauine *et al*., 2012; Nyindo & Farah, 1999). It is for this reason that both baboons and mice provide excellent experimental models for *S. mansoni* producing pathology and diseases that closely resembles that observed in humans (Manivannan *et al*., 2010; Smithers & Doenhoff, 1982).

Schistosomiasis tends to run a chronic course and the eggs produced by the adult worms are largely responsible for the pathology associated with the disease (Manivannan *et al*., 2010; Ross *et al*., 2002). Schistosomes are carried passively from the skin to the lungs where they undergo a sequence of development lasting several days. This adapts them for onward migration through the vascular beds (Wilson & Coulson, 1985).

Schistosomiasis is a threat to economic development, as it causes suffering to men and women during their most productive years (Nawal, 2010; Savioli *et al.*, 2002). The key pathological events in this disease are the formation of granulomas around schistosome eggs in the liver and intestines (Manivannan *et al*., 2010). The presence of granulomas in the liver is associated with fibrosis and venous hypertension, which is the main cause of morbidity and mortality in both human and rodent schistosomiasis (Manivannan *et al*., 2010; Smithers & Doenhoff, 1982).

1.2 The Biology of Schistosoma mansoni

Schistosomes have a complex life cycle involving a snail intermediate host and a vertebrate definitive host (Scholte, *et al*., 2012). The adult worms live in the blood stream of the vertebrate host (usually birds and mammals). In the vertebrate host, the male and female adult worms live paired in the blood stream where they mate and reproduce (Scholte *et al*., 2012). Fig.1 provides a diagramatic representation of the life cycle of human schistosomes.

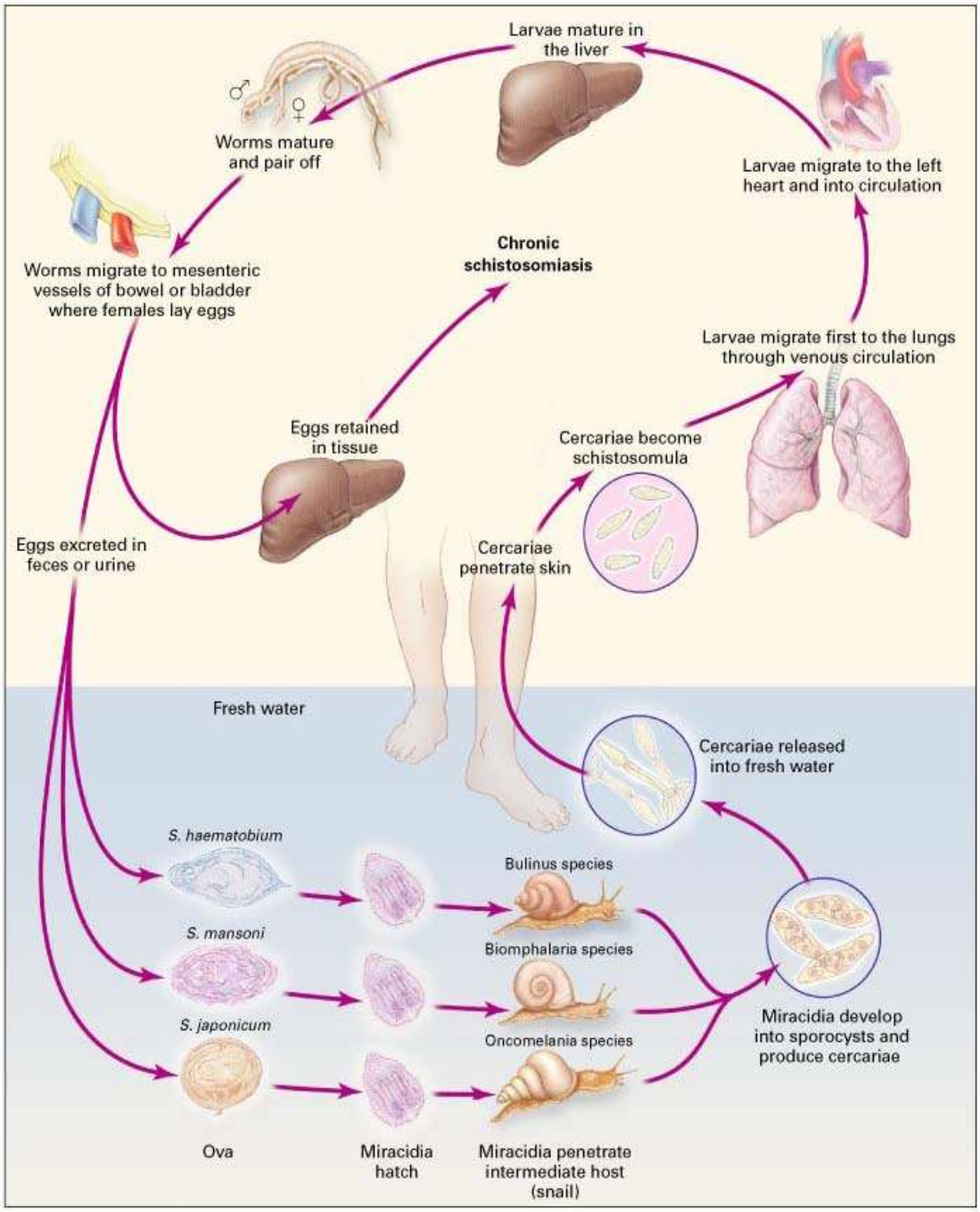

Figure 1.1: Diagrammatic life cycles of human Schistosomes

(htt:/www.Standford/ed/group/ParaSites 2008).

A detailed review of the reproductive biology of *S. mansoni* is provided by Ross *et al*. (2002). Planorbid snails in the genus *Biomphalaria* are involved in the transmission of *S. mansoni*. Globally, at least 32 *Biomphalaric* species exist (Franden, 1997) and while several species are susceptible to infection, only four; namely *B. pfeifferi, B. glabrata, B. sudanica, and B. alexandrina* are considered to be the most important in the transmission of *S. mansoni* (Franden, 1997).

In Africa and the surrounding regions, all species of *Biomphalaria* tested have proved compatible with *S. mansoni* to a greater or lesser degree (Frandsen, 1997). Eleven *Biomphalaria* species exist (Ross *et al*., 2002). Majority are known to be susceptible to infection with *S. mansoni*, and most likely play a role in schistosomiasis transmission. *B. pfeifferi* is the most widespread and probably the most important transmittor of *S. mansoni* in the Sub-Saharan Africa (Scholte *et al*., 2012).

The adult *S. mansoni* live in the mesenteric veins of the definitive host usually humans, non-humans primates (primarily baboons) and rodents (Tchuem and Techuente 1996), where male and female worms live paired, and mate and produce the characteristic laterally spined eggs which work their way into the lumen of small intestines where they are passed out into the environment via faeces (Tchuem &Techuente, 1996).

When the eggs pass into the environment, they hatch on coming into contact with fresh water, into miracidia (free-swimming ciliated larval forms), which are infective to snails. In the snail host, the miracidium transforms into a mother sporocyst then into daughter sporocysts. Daughter sporocysts undergo multiplication and produce cercariae (the free swimming larval forms that infect the definitive host). An individual miracidium will give rise to single sex cercariae and adult worms (May & Woodhouse, 1993). Cercariae are then released into the aquatic environment within 4 – 5 weeks after miracidia penetration and initiate the life cycle all over again once they come into contact with definitive host. The schistosome cercariae penetrate intact skin of the definitive host and transform into schistosomule(s) [juvenile

schistosome(s)]. The schistosomules then migrate in the vascular system through the heart, lungs and end up in the liver of the vertebrate host where they mature, and finally move to the mesenteric veins, their final destination (Tchuem & Techuente 1996).

1.3 Schistosomiasis Transmission

Schistosomiasis transmission depends absolutely on the presence of a snail intermediate host in a water body contaminated with human excreta from schistosome infected individuals (Guillauine *et al*., 2012). In general, *schistosomiasis* transmission is influenced by biological, economic and socio-cultural factors (Chitsulo *et al.,* 2000). Some of the factors involved include distribution, biology and population dynamics of the intermediate hosts, human water contact activities, knowledge, attitude and practices of the communities in the endemic areas (Brown, 1994; Vercruysse *et al.*, 2001). Water development projects, particularly construction of dams, artificial lakes and irrigation canals often associated with population settlement, have become a major factor in the spread of the disease (Hunter *et al.,* 1993; Chitsulo *et al.,* 2000).

Climate and environmental variables influence the distribution of schistosomiasis (Brooker, 2002; Brown, 1994). Malone *et al.* (2001) showed using GIS and remote sensing technology that, in East Africa, temperature may be important in influencing the broad-scale distribution of schistosomiasis. Children are the most vulnerable to schistosomiasis and high intensities occur in children aged 10 – 14 years (Vercruysse *et al.,* 2001). Children play a significant role in environment contamination and transmission of schistosomiasis (Vercruysse *et al.,* 2001).

1.4 Immunity and Morbidity in Schistosomiasis

Immunoglobulin IgG is an anti-body which is a protein complex consisting of four peptide chains. Two identical chains are heavy while the other two identical chains are light. These chains are arranged in a Y- shape characteristic of anti-body

monomers. The IgG has two binding sites and is the most common type of antibody found in blood circulation (Junqueira, *et al*., 2003).

Antibodies are important constituents of humoral immunity. IgG is the main anti-body found in the blood and extracellular fluid that control infection of body tissues. IgG protects the body by encapsulating pathogens such as bacteria, viruses and fungi thus protecting the body from infection. The IgG uses several mechanisms to protect body tissues from infection. IgG-mediated binding of pathogens causes their immobilization and binding together through agglutination. The IgG coats pathogen surfaces by a process called opsonisation. This allows the phagocytic immune cells to recognise and digest the pathogens. IgG also activates the complement system, a cascade of immune proteins production that assist in elimination of pathogens ((Mallery *et al.,* 2010).

IgG anti-bodies (IgGs) resulting from *Schistosoma mansoni* infection are directed to glycans as reduced IgGs binding are observed on periodate-treated schistosome antigens compared to untreated antigens (Kariuki *et al.,* 2008). However, it is not clear if the dominant anti-glycan IgG responses in schistosome infection are linked to protective immunity (Kariuki *et al.,* 2008).

Several studies have provided evidence for the development of an age-acquired immunity in human schistosomiasis in endemic areas (Zhang *et al.,* 1997 a). Immune protection is thought to be immunologically mediated and related to increased production in the serum of the adult worm-specific IgG (Nyindo & Farah, 1999). It has been shown that infectivity of *S. mansoni* is higher in primates than in rodents. Increase in infectivity induces the presence of more worms in a host and could increase the pathogenicity due to presence of eggs (Boissier & Mone, 2001). Studies done in mice infected with *S. mansoni* suggested that the reduction in granuloma size in K-12 treated mice was associated with a shift in Th2 cytokines response to one dominated by production of IFN-γ (Wynn, 1999). It has been shown that in mice, immunity arising from exposure to irradiated cercariae is species-specific and is

dependent on functional population of T and B cells (Coulson, 1997; Nyindo & Farah, 1999).

Invading schistosomes larvae are usually invisible to the host immune response while maturing adult worm stimulate both the humoral and cellular arms of the host immune response. Adult worms induce a Th1 type cytokine profile with elevated levels of IFN- γ, IL-12 and TNFß with the onset of parasite egg laying between 5 and 6 weeks post infection. This predominantly Th1-like response is modulated by Th2 cytokines such as IL-10 and IL-4 (McKerrow, 1997). Th1 induction is based on the observation that the altered parasites, irradiated cercariae, depend on macrophage effector cells' IFN- γ. At least there is a positive correlation between activation of these cells and protective immunity in mice (James, 1992). Another Th1 mechanism is based on the susceptibility of schistosomes to nitric oxide produced by cytokines-activated macrophages (James & Naney, 1993). However, Wynn and Hoffman (2000) downgraded the importance of Th1 and Th2 dichotomy for protection against schistosome infection. This inflammatory disease is also characterized by the release of several biologically active compounds including cytokines such as interleukin - 2 (IL-2), IL-5 and tumour necrosis factor ß TNFß (Amiri, 1992; Aloe & Fiore, 1998). This latter cytokine appears to be functionally relevant because injections of anti-TNFß antibody reduce the development and the formation of granulomas (Amiri, 1992).

1.5 Cellular and Humoral Interactions

Molecular and cellular components form the immune system including their function and interaction. The immune system is divided into innate and acquired or adaptive immune system which contains humoral and cellular components. Humoral immunity is so named because it involves substances found in humours or body fluids. The anti-body-dependent cell- mediated cytotoxicity (ADCC) is a process of cell- mediated immune defence whereby an effector cell of immune system lyses a target cell. The membrane surface of antigen is surrounded by specific antibodies. In humans, antibody-dependent cell- mediated cytotoxicity is mediated by IgG as part

of humoral immune response mechanism that binds and control infection (Jean-Luc Teillaud, 2012). Antibody-dependent cell-mediated cytotoxicity is mediated by natural killer (NK) cells. This involves activation of natural killer cells by antibodies. A natural cell expresses CD16 which is an Fc receptor. The receptor recognizes and binds to the Fc portion of an antibody such as IgG bound to the surface of an antigen-infected target cell. Once the Fc receptor binds to the region of IgG, the natural killer cells release cytokines such as IFN- γ (Jean-Luc Teillaud, 2012).

Humoral immunity is mediated by macromolecules (as opposed to cells). Humoral immunity refers to antibody production and the accessory processes that accompany it and include: Th2 activation and cytokines production. It also refers to the effector functions of antibodies which include pathogens and toxins neutralization, complement activation and opsonin promotion of phagocytosis and pathogens elimination (Janeway, Jr, 2001).

The immune system involve recognition of infectious pathogens and is able to remove pathogens without harming the host. Immune responses to pathogens are initiated by innate immune system, which reacts quickly to generic pathogen-associated molecules and primes the adaptive immune systems, which is slower to react but has exquite specificity and memory (Leonard *et al.*, 2010).

Protective responses are mediated by IgG. Naus *et al.* (1999) compared antibody responses of an immunologically naïve population which had recently inhabited an area in Kenya endemic for *S. mansoni*, with those of chronically infected resident inhabitants of the same region. He found that adult worms increased in older infected individuals and was associated with resistance to re-infection. However, the level of immunity may instead depend on the outcome of a balance of blocking and protecting immune responses as suggested by Caldas *et al.* (2000). Protective studies related to *S. mansoni* infection implicated both humoral and cell-medicated mechanism. For instance, resistance to re-infection in drug-cured patients has been correlated with the presence of anti-parasite IgG antibodies. *In vitro* experiments

have implicated esonophils as effector cells of IgG antibody dependent cell-mediated cytotoxicity (Berquist *et al.*, 2002).

1.6 Pathology caused by Infection of *S. mansoni*

The key pathological events in this disease are the formation of granulomas around schistosome eggs in the liver and intestine (Aloe & Fiore, 1998; Manivannan *et al*., 2010). Schistosomiasis causes granulomatous inflammation due to immunopathological reaction mediated by MHC class 11 – restricted CD4 T cells sensitized to egg antigens (Hermandez *et al.*, 2002). Eggs deposited by adult worms can become trapped in the liver and induce the development of granulomatous lesions which ultimately lead to liver fibrosis and portal hypertension (La Flamme *et al.*, 2001).

Granulomas, which are a particular form of inflammation are caused mainly by the host's reaction to the parasite infection and are characterized by an aggregation of macrophages, lymphocytes and eosinophils around the schistosome eggs (Aloe & Fiore, 1998). The presence of granulomas in the liver is associated with local venous hypertension, which seems to be the main cause of morbidity and mortality in human and rodent's schistosomiasis (Smithers & Doenhoff, 1982).

Using both T-cell enrichment experiment and flow cytometry analysis, Metwali *et al*. (2002) were able to show that granuloma $CD4^+$ T cells are the major source of cytokine in mice. NK T cells are also the source of Th1 and Th2 cytokines. The differentiation and regulation of Th1 and Th2 cells and control of TNF–γ within these granulomas are complex processes (Metwali *et al*., 2002). Schistosome eggs can also reach the central nervous system (CNS) inducing local granulomas leading to what has been defined as neuroschistosomiasis (Aloe and Fiore, 1998). Studies carried out demonstrated that brain granulomas are formed in mice infected with schistosomes for 15-18 weeks (Aloe, 1996).

1.7 Problem Statement

Schistosomiasis is a serious poverty-related health problem and more than 207 million people are affected. It is estimated that more than 800 million people live in Schistosomiasis–endemic areas (Engels *et al.,* 2002; Steinmann *et al.,* 2006). The only drug of choice for effective treatment of schistosomiasis is Praziquantel (PZQ). There has been evidence of resistance **(**Ross *et al*., 2002). In addition, Praziquantel has a short half – life (1 – 1.5 hours), it is expensive and it is also not effective against the juvenile stages of schistosome worms (Ross *et al*., 2002).

1.8 Rationale and Justification

The plant *Chenopodium ambrosoides* has been identified as an effective anti-helmintic, and as source of anti-parasitic agent. It makes sense to examine this plant for anti-schistosomal properties. *Chenopodium ambrosoides* is widespread, world wide and also occurs in the Schistosomiasis endemic areas (Agnew *et al*., 1994).

1.9 Hypotheses

1.9.1Null Hypothesis

There is no significant difference between mice treated with extracts of *Chenopodium ambrosoides* plants and those mice which have not been treated.

1.9.2 Alternative Hypothesis

There is a significance difference between mice treated with extracts of *Chenopodium ambrosoides* plants and those mice which have not been treated.

1.10 Objectives

1.10.1 General Objective

To investigate anti-schistosomal activity of *Chenopodium ambrosoidies* in mice infected with *S. mansoni.*

1.10.2 Specific Objectives

1. To determine effect of *Chenopodium ambrosoidies extracts in S. mansoni* worms reduction *in vivo.*
2. To assess effect of *Chenopodium ambrosoidies* on pathology caused by *S. mansoni* worms in mice.
3. To determine effect of *Chenopodium ambrosoidies* extracts on *S. mansoni* worms *in vitro.*
4. To determine whether *Chenopodium ambrosoidies* extracts elicits immunomodulatory responses in mice.
5. To determine anti-schistosomal finger profiles using thin layer chromatography (TLC).

CHAPTER TWO

LITERATURE REVIEW

2.1 *Schistosomiasis* Control

Several strategies are used in the control of Schistosomiasis and include sanitation and piped water supply, health education, community involvement and chemotherapy (Hagan *et al.,* 2004). Chemotherapy however, is probably the most effective strategy as it results in reduction in morbidity, reduction in environment contamination with parasite eggs, and directly benefits the infected individual (Hagan *et al.,* 2004). Widespread use of chemotherapy is however, often hindered by several factors such as poor infrastructure for drug distribution and the prohibitive cost of the drug of choice, Praziquantel (WHO, 2002). Furthermore, Praziquantel has a short half – life (1 – 1.5 hours), and is also not effective against the juvenile stages of schistosomes (Ross *et al.,* 2002). However, according to the Science Alerts Social Network (2013), derivatives of Praziquantel were synthesized and tested for anti-schistosomal activity against juvenile and adult stages of *S. mansoni* in infected mice. It was reported that a Praziquantel ketone derivative had the best activity against juveniles and adults, but it had no effect on the motility of adult *S. mansoni* in *in vitro* culture Network (2013). Of particular concern, is the fact that Praziquantel is the only anti-schistosomal drug currently on the market (Shuhua *et al.,* 2000), and even more worrying, Praziquantel resistance may slowly be emerging after nearly 30 years of intensive use in some endemic areas (Fallon *et al.,* 1994; Bridley & Sher, 2002; Raso *et al.,* 2004). Given the problems relating to anti-schitosomal chemotherapy, there is need to search for new drugs, preferably drugs with novel modes of action and probably involving different classes of compounds from existing drugs. Plants are frequently discussed as possible sources of novel drugs and in years, they have been investigated as possible sources of novel anti-schistosomal agents (Hagan *et al.,* 2004; Sher, 2001). In this study, the plant *Chenopodium ambrosoides* was investigated for anti-schistosomal properties using *S. mansoni* as the target parasite.

Conceptually, the schistosomiasis control can be interrupted at four distinct points:

Preventing excreta from reaching fresh water (sanitation), preventing the interaction of free-swimming larval stages of schistosomes; miracidia, with the intermediate host snail (broadly snail control), preventing exposure of susceptible humans to the infective free-swimming Cercariae (reduction of water contact) and chemotherapeutic attack on the parasite population living within human host (mass or targeted chemotherapy).

The main objective of schistosomiasis control is to achieve reduction of disease due to schistosomiasis. Historically, snails are considered the most vulnerable link in the life cycle of schistosomes. Decades of research and control efforts have tested an astounding array of chemical, biological and physical weapons with which to reduce snail populations below the level, which would permit transmission (Tchuem, 1996). With rare exceptions, these efforts have failed (Savioli *et al.,* 1997). Molluscicides such as N–tritylmorpholine and sodium pentachlorophenate (NaPCP) have been used to control snail's populations in dams, drains, rice paddies and canals (Appleton, 1985). However, the use of inorganic molluscicides is not suitable for application into water bodies where aquatic snail hosts are abundant and on which people's daily life depend. The application of molluscicides must be carefully planned to take advantage of focal and seasonal pattern of transmission; better strategies and delivery systems are needed to improve the cost effectiveness of applying molluscicides (McCullough, 1980).

Sanitation and piped water supply, health education, community involvement and improved chemotherapeutic treatment of the infection are the major tools needed for the control of the infection. Chemotherapy is probably the most effective strategy as it results in the reduction of environment contamination with parasite eggs and directly benefits infected individual. School–age children have the highest intensity of worm infection of any age group and chronic infection negatively affects all aspects of children health, nutrition, cognitive development, learning and educational access and achievement (World Bank, 2003).

2.2 Chemotherapy of *Schistosomiasis*

2.2.1 History and Advances

Since the discovery of Praziquantel in 1970's, it has replaced other anti-schistosomal drugs to become the only drug of choice for the treatment of human schistosomiasis (Wang *et al.,* 2012).

Anti-monials were the first drugs to be used for the treatment of schistosomiasis. However, they were abandoned as a result of side effects associated with their administration. These included skin ulceration, vomiting and failure of liver functions. Antimony compounds were introduced for clinical use (Cioli *et al.,* 1995; Parise-Filho & Silveira, 2001). The clinical use of antimony compounds was suspended on account of their severe side and toxic effects and patients' low tolerance them (Cioli *et al*., 1995; Cioli, 1998; Novaes *et al*., 1999). Niridazole (Ambilhar) was introduced into market for the treatment of schistosomiasis in 1964 (Lambert & Stauffer, 1964). However, Niridazole showed low activity against *S. japonicum*, moderate activity against *S. mansoni* and very high activity against *S. haematobium* (Berge *et al*., 2011). *The* drug had some issues, such as the number of doses and various severe side effects (high toxicity in the central nervous system, kidneys and liver, in addition to mutagenic, carcinogenic and teratogenic effects (Berge *et al*., 2011).

2.2.2 Oxamniquine and Metrifonate

With the introduction of oxamniquine as a treatment of schistosomiasis, studies have indicated some disadvantages in the use of oxamniquine.

Oxamniquine the only alternative to Praziquantel but only effective against *S. mansoni* infection is not readily available on market especially in Sub-Saharan Africa. It is now reserved for treatment of acute, chronic and complicated cases of *S. mansoni* infections, with uniformly good results (Ross *et al*., 2002). Oxamniquine mechanism may be associated with inihibition of nucleic acid metabolism of the

parasite (Abdulla *et al.,* 2007). Although associated with severe side effects including dizziness, drowsiness and headaches, Oxamniquine is well tolerated (Ross *et al*., 2002). It has limited supply because it is expensive (Ross *et al*., 2002). There are some disadvantages in the use of oxamniquine. The drug has no cell specificity, which results in undesired peripheral toxicity; there are also some side effects on the central nervous system (Davis; 1993; Soyez *et al*., 1996). It was also observed that oxamniquine showed low activity in the period between the 3rd and the 9th week of infection, which coincided with the egg production stage (Frézard & Melo, 1997; Silmara *et al*., 2012). Furthermore, there were reports of patients infected with *S. mansoni* who were resistant to the treatment (Cioli *et al.*, 1992). Oxamniquine is as effective as Praziquantel in eliminating *S. mansoni* infection but not effective against *S. haematobium* (Ross *et al*., 2002).

Metrifonate (Bilarcil) on the other hand is as effective as Praziquantel in eliminating *S. haematobium* infection but not effective against *S. mansoni* infections. Metrifonate has to be administered in three doses; this is its main drawback (Berge *et al*., 2011).

2.2.3 Praziquantel

Praziquantel, a *pyrazino isoquinoline* derivative, (whose structure is shown in Fig. 2.1) is the main drug for treatment of schistosomiasis and is the drug of choice.

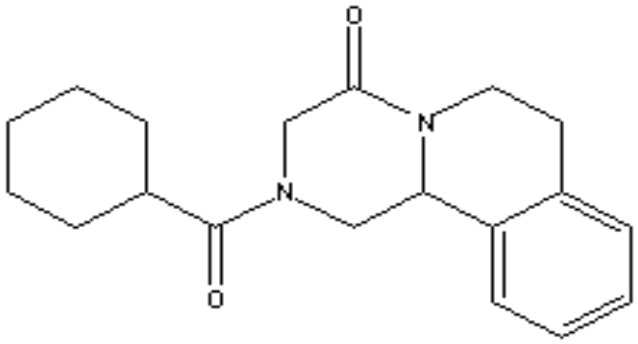

Figure 2.1: Molecular structure of Praziquantel. Molecular Formular = C19 H24 N2 O2 MW 312.41

The anti-parasitic activity of the *pyrazino isoquinoline* ring system (the con-structure of Praziquantel) was discovered in 1970's by the Bayer Laboratory in Germany (Andrews *et al.,* 1983; Cioli & Pica, 2003). The drug is considered relatively safe and effective for the treatment of schistosomiasis and this has led to its widespread use (Dawo-Appiah *et al*., 2013; Ross *et al.*, 2002). Although its mode of action is not fully understood (Day *et al*., 1992; Cioli, 2000), Praziquantel is known to cause titanic contractions and tegumental vacuoles which cause the worms to detach from the walls of the veins and die (Ross *et al*., 2002). The schistosomes exposed to the drug either *in vitro* or *in vivo* show paralysis of the worm musculature (Cioli & Pica, 2003). This contraction is accompanied and probably caused by a rapid calcium ions influx inside the schistosomes (Cioli & Pica, 2003; Pax *et al*., 1978). It has been suggested that schistosome calcium channels could be possible molecular target of Praziquantel (Khon *et al.,* 2001). Of particular interest is the fact that the anti-

schistosomal activity of Praziquantel, seems to be dependent on the host's immune **r**esponses (Cioli & Pica, 2003).

Praziquantel is effective against *S. mansoni, S. haematobium, S. japonicum, S. intercalatum, and S. mattheei* (Cioli, 2000; Hagan *et al*., 2004). In animal models, the presence of host's antibodies is shown to be critical for its efficacy (Brindley and Sher, 1987). In humans, Praziquantel produces cure rate of 60 – 90% and substantially decreases the worm burden and parasite egg production in those patients who are not completely cured (Ross *et al.,* 2002). Nevertheless, treatment failures have been reported with Praziquantel in some regions of Sub – Saharan Africa (Ross *et al.,* 2002).

Immature worms are not susceptible to the effect of Praziquantel (Hagan *et al.,* 2004). Although the cost of Praziquantel has decreased substantially with a recent focus on control of schistosomiasis and other neglected parasitic diseases, logistical problems are often encountered in respect of drug distribution in Sub –Saharan Africa (Hagan *et al.,* 2004). Praziquantel has a very low aqueous solubility and its dissolution rate is considered the limiting factor for its bio-availability (Naglaa *et al.,* 2012). Praziquantel is currently the drug of choice for the treatment of schistosomiasis. The drug has no toxic effects against vital organs systems and their functions (WHO, 2010). The modern rational approach to drug design is focused upon the structure and function of biochemical and molecular targets. The requisite pharmacological properties for new anti-parasite drugs should not be ignored in this process (Croft, 1997). The current thrust of the most effective control strategies is towards preventing the development of chronic, severe pathology (Guyatt, 1998).

2.2.4 Artemether

Artemether has been explored as anti-schistosomal drug, which appears to be effective against juvenile shistosomes (Shuhua *et al*., 2000; Lu *et al.,* 2010). In combination with Praziquantel, it appears to produce a synergetic killing of adult worms (Shuhua *et al*., 2000). When treatments with Praziquantel fail, both

artemether and artesunate are potential prophylactic drugs for schistosomiasis (Lu *et al*., 2010). Myrrh, *Commiphora molmol* (Family: Burseraceae) have also been used experimentally as anti-schistosomal agent against various schistosome species including *S. mansoni, S. haematobium, S. japonicum* (Barakat *et al.,* 2005).

2.3 Plants with *Anti-Schistosomal* Activity

2.3.1 Plants as possible therapeutic agents

Development of plant–derived anti-schistosomal agents for use or in combination with synthetic drugs might be a promising approach. Combination of chemotherapy, in particular, is seen as a novel, more versatile approach for schistosomiasis control (Barakat *et al.,* 2005) and because of potential problems with Praziquantel resistance/tolerance research effort have been initiated to develop anti-schistosomal drugs that target more than one point in a biochemical pathway (Barakat *et al.,* 2005). Interest in exploiting plant-derived products such as artemether is also intensifying (Sher, 2001), and reports from China suggest that research in this area is worthwhile, and future is bright (Xiao *et al*., 2000).

Medicinal plants and natural products can provide an alternative to conventional chemotherapy for a variety of diseases (Archana *et al.,* 2011). Plants have been used for medicinal purpose since ancient Egyptian civilization (Hamed, 2009). Oregano (*Origanum vulgare*) Family: Lamiceace, a perennial herb native to Europe, Asia and Mediterranean regions, has anti-microbial properties (Ningthoujam, 2011).

Plants are frequently discussed as possible sources of novel drugs. They have been investigated as potential sources of antiparasitic agents including novel antischistosomal agents (Sher, 2001; Hagan *et al.,* 2004). Medicinal plants extracts and isolated metabolites can inhibit protozoan parasites and intestinal worms (Wink, 2012). Bioactive natural products isolated from higher plants e.g mangrove plants, have been produced and selected as compounds with anti-microbial, anti-parasitic, cytotoxic as well as neuroprotective activities (Amal *et al.,* 2010). Non-alkaloidal natural compounds from plants have been isolated as products with anti-plasmodial

and anti-malarial properties (Ronan *et al.,* 2009). Artemisinin derivatives, now seen as important anti-malarials, have been shown to have an effect on schistosomes. In China, artemether has been used successfully for acute *S. japonicum* infections in times of flood (Xiao *et al*., 2000). Another natural product, an extract of *Commphora molmol* (myrrh) has been licensed and marketed as Mirazid for clinical use against *Fasciola* and schistosome infections in Egypt (Sher, 2001).

Plants have been used as traditional medicine for anti - parasites. It has been shown that extracts of two plants, the dotted dalea (*Psorothamnus polydenius)* and the majave dalea (*Psorothamnus arborescens)* were capable of killing haemoflagellates, protozoas, Leishmania and trypanosomes (Salem *et al.,* 2006). Essential oil, isolated by hydrodistillation from *Chenopodium botrys* (Chenopodiacea) aerial parts, expressed significant bacterialcidal and fungalcidal activity against selected strains of micro organisms (Maksimovic *et al.,* 2005).

Ascaridole, a terpene isolated from C*henopodium ambrosoides* was studied to determine its antihelmintic activity on the *in vitro* development of *Plasmodium falciparum*. Ascaridole was found to be a potent inhibitor of plasmodial growth (Pollack *et al*., 1990). The following plants *Ambrosia hispida* (Asterraceae), *Aristolochia trilobata* (Aristochiaceae), *Chenopodium ambrosoides* (Chenopodiaceae), *Portulaca oleracea (*Portulacaceae) and *Artemisia absinthium* (Asteraceae) were studied and showed that they had biochemical properties which provided efficacious treatment for intestinal parasite loads (Quinlan *et al*., 2002). *In vivo* tests of compounds in the pith of *Venonia amygdalina* (Compositae) showed anti-helmintic activity against schistosomes (Gasquet *et al*., 1985).

In view of the fact that schistosomiasis control relies on Praziquantel as drug of choice, and given the concern about Praziquantel resistance (Raso *et al.,*2004) , there is need to search for new anti-schistosomal agents preferably with novel modes of action or the development of new treatment strategies.

Some drugs in the modern pharmacopoeia and other naturally occurring products e.g quinine from the bark of cinchona tree and artemisinin from the herb *Artemisia annua* are known for the treatment of malaria (Tagbito & Townson, 2001) and against monogenetic parasites of water fish *Sarotherodon melanotheron* (Obemeata *et al*., 2012). The essential oil from leaves and stalks of *Lippia myrltifolia* (Verbenaceae) was tested for anti-malarial activity on *in vitro* cultures of *Plasmodium falciparum*. It was found out that the oil inhibited growth at the trophozoite – schizont stage, indicating a potential effect on the first nuclear division of the parasite (Valentine *et al.*, 1995). The essential oil and its main component ascaridole of *C. ambrosoides* is reported to be a potent inhibitor of plasmodial growth in lower *c*oncentrations and kill malarial parasites in higher concentrations (Okuyama *et al*., 1993). *C. ambrosoides* contains saponin, which is located in the roots; the aglycone fraction of the saponin is echinocystic acid. The saponin is reported to have anti-fungal activity (Kishore *et al.*, 1993). *C. ambrosoides* extracts were shown to inhibit egg development of parasites and maturation of larva but these tests have not been confirmed in *in vivo* studies (Sagrero & Bartley, 1995; Ketzi & Brown, 1998). Ascaridole the active principle of essential oil paralyses but does not kill the intestinal worms, eg. *Ancylostoma duodenale, Ascaris lumbricoides, Necator americanus and Trichuris trichiura;* they must be expelled by a laxative (Ketzis & Brown, 1998). The study evaluating *Chenopodium ambrosoides* (paico) leaves as an anti-parasitic agent was conducted in patients (children and adults) with *Ancylostoma, Trichuris and Ascaris* infections in a village near Tarapoto, San Martin. With respect to the parasites tested for, the efficacy was 100% for *Ancylostoma duodenale, Trichuris trichiura and Ascaris lumbricoides* 50% (Nakazawa, 1996).

2.3.2 Immunomodulatory Properties of Some Medicinal Plants

Aloe vera has been shown to modulate immune response and to play an essential role as the first line of defence against invading pathogens especially *Candida albicans*, a communal and opportunistic pathogen in humans (Farahnejad, *et al.,* 2011). Studies carried out show that both methanolic and ethanolic extracts of *Withania somnifera*

(Solanaceae), have significant anti-inflammatory, activity on carrageenan induced paw oedema in Swiss mice (Satish *et al*., 2012). The aqueous suspension of *Withania somnifera* root powder was also investigated for its *in vivo* and *in vitro* immunomudulatory properties using mice. The results of the root powder showed potent inhibitory activity towards the complement system, mitogen induced lymphocyte proliferation and delayed-type hypersensitivity reaction (Ashwani, 2012). On the other hand, Sathianarayanan and Rajasekaran, (2012) conducted studies using albino wistar mice on the effect of both methanolic and ethanolic extracts of the leaves of the plant *Wrightia tinctoria* (Apocynaceae). The study showed that the extracts act by stimulating both specific and non-specific arms of immuno system. Several Indian medicinal plants have been reported to possess anti-stress and immuno-modulatory activity. For example, *Withania somnifera* (Solanaceae), *Ocimum sanctum* (Labiatae) *and Emblica officialis* (Eupborbiaceae) have been demonstrated to have significant activity (Pande & Vijaykumar, 1994). Rao *et al.* (1996) successfully demonstrated the immuno-modulating activity of a combination of the extracts of these plants. They also demonstrated the immuno-modulating effect of Zeetress in commercial broilers by studying the broiler's anti-body titres. They observed that the antibody titres against Newcastle disease (ND) significantly improved in the group treated with Zeetress compared to the titres in the control group. Weight gain and feeding conversion were better in the treatment group with both groups being kept under the same feeding management and health coverage scheme. The results confirmed the stimulation of humoral immune response of Zeetress **(**Pande &Vijaykumar, 1994).

A study carried out in the treatment of mice with a herb extract of Anamu (*Retiveria alliacea*) showed that the plant stimulates the production of lymphocytes and interleukine (IL) -2, IL- 4 and interferon (Kubec *et al*., 2003). An immunomodulatory polysaccharide-rich substance (Noni–ppt) from the fruit juice of *Morinda citrifolia* has been found to possess both prophylactic and therapeutic potential against the immunomodulator sensitive sarcoma 280 tumor system. The anti- tumor activity of Noni–ppt produced a cure rate of 25% - 45% in allogenic

mice. Its activity was completely abolished by concomitant specific inhibitors of macrophages (2-chloroadenosine), T cells (Cylosporine) or Natural killer (NK) cells (anti-asialo GM1 antibody). Noni-ppt showed synergistic or additive beneficial effects when combined with a broad spectrum of chemotherapeutic drugs such as adriamycin, mitomycin-c and comptothecin. Noni-ppt also showed beneficial effect when combined with the Th1 cytokines, interferon gamma, but activity was abolished when combined with Th2 cytokines, interleukin 4 or interleukin 10, thereby suggesting that Noni-ppt induced a Th1 dominant immune status *in vivo* (Furusawa *et al.,* 2003).

The regulation of T helper (Th)1 and Th2 type cytokine patterns is important in the final outcome of Leishmaniasis in human and murine models (Ghazanfari *et al*., 2000). Studies carried out in the treatment of BALB/c mice with garlic (*Alliun sativum*) extract, showed that the mice developed Th1- type cytokines responses. In contrast, glucantime therapy led to a Th2-type response in the control group with lower levels of IL- 2. However, a combination of garlic and glucantime treatment was found to be more effective than either treatment alone, and resulted in a Th1-type response similar to that which developed with garlic treatment. These results suggest that garlic extract in combination with antimonial drug may provide effective therapy against *Leishmania major*. The modulatory properties of garlic were elucidated in terms of shifting the cytokine response to a Th1- type pattern and therefore causing the protective response (Ghazanfari *et al*., 2000). Studies carried out to evaluate the anti–inflammatory properties of ethanolic extract of *Capparis deciduas* (Capparaceae) and the aqueous extract of *Capparis spinosa* (Capparaceae) showed significant anti–inflammatory activity against carragreenan induced oedema in rats (Ageel *et al.,* 1986).

2.3.3 Anti–Pyretic Properties of Plants

The ethanolic leaf extracts of *Gongrenema latifolium* were tested against rodents. Neha and Rekha (2010). The leaves of the plant *Haldinia cordifolia* were found to possess anti- pyretic properties (Yatagiri *et al.,* 2012). Neha and Rekha (2010)

carried out a study using crude methanolic extracts of *Madhuca indica* (Sapotaceae) in male wistar mice. The study confirmed that methanolic extracts of *Madhuca indica* possess anti-pyretic properties due to the presence of the phytoconstituents of the plant. According to Zankara *et al.* (2007) the aqueous (leaf) extracts of the *Melastoma malabathricum, have* anti-conceptive, anti-inflammatory and anti-pyretic activities. Bum *et al*. (2011) evaluated four mdecianal plants from Cameroon: *Afromosia laxifora, Chenopodium ambrosoides, Macroglossa pyrifolia and Mimosa pudica* and confirmed that *C. Ambrosoides, M. pyrifolia and M. pudica* possess anxiolytic–like and antipyretic activity while *A. laxifora* possesses only laxiolytic like properties (Bum, 2011).

Chenopodium ambrosoides plant extract was studied using thermal (hot plate) test. The plant aqueous extract was found to be effective at a dose of 0.4 g/kg and 0.89 g/kg in elevating pain threshold test. *C. ambrosoides* was found to protect mice from the sensation of pain due to the thermal stimulus (Amole, 2002).

A study was carried out to evaluate the anti–pyretic potential of the methanol extract of the aerial part of *Inssiaea suffruticosa* Linn (MEJS) on normal body temperature. Yeast induced pyrexia in albino rats and showed significant reduction in body temperature. The anti- pyretic effect of MEJS was comparable to that of paracetamol (150 mg/kg body wt), a standard; anti–pyretic agent (Muragesan *et al.,* 2000). The methanol extract of the whole plant of *Vernonia cinerea* (Compositae) was evaluated for its anti-pyretic potential in rats. The anti-pyretic effect on the extract at a dose of 500 mg/kg was found to be identical to that of the standard drug paracetamol (Gupta *et al.,* 2003). The following plants were also tested for their anti–pyretic effect and analgesic activity. *Capparis deciduas* (Capparaceae) were also found to possess significant anti–pyretic effect. Both *Capparis deciduas* (Capparaceae) and *Vernonia cinerea* (Compositae) were found to be devoid of analgesic effect (Ageel *et al.,* 1986).

Experiment carried out on the fruit pulp of *Adansonia digitata* (Bombacaeae) exhibited significant anti-inflammatory and anti-pyretic activities at both 400 mg and

800 mg/kg. Oral administration of the extract to BALB/c mice at 800 mg/kg had significant analgesic effect and a shift analgesic effect at 400 mg/kg (Ramadan *et al*., 1994).

2.3.4 Plants with *Anti-Schistosomal* Properties

A chemical compound holothurim, extracted from an Egyptian sea cucumber was found to have anti-schistosomal activity (Mona *et al*., 2012). An amide isolated from plants of the genus piper (Piperaceae) revealed anti-schistosomal properties against *S. mansoni* worms (de Moraes *et al.,* 2012). Mostafa *et al.* (2011) demonstrated that the plant ginger (*Zingiber officinale*) possesses anti-schistosomal activity. Schistosomiasis was treated using myrrh, a gum extract from the stem of *Commiphora Myrrha (molmol- Somali)* of the family: Burserceae (Abebe, 2008). The extract had some anti-schistosomal properties which cause worm pairs to separate. The female worms then shifted to the liver, where they were killed (Sher, 2001). Tests carried on vernodalin, a highly toxic sesquiterpene lactose, isolated from *Venonia amygdalina* (Compositae) also showed significant activity against schistosomes as well as *Plasmodium* and *Leishmania* (Ohigashi *et al.,* 1994). Crushed seeds of the plant *Nigella sativa* (Rununculaceae) were found to have anti-schistosomal activity against different stages (cercariae and juvenile) of *S. mansoni* (Mohamed *et al*., 2005). The plant, *Ammi visnaga* (Synonyms - *Ammi majus)* belonging to the family Umbellifereae was found to decrease the worm burden and produce oogram change in mice infected with *S. mansoni* (Abdulla *et al*., 1978).

Studies carried out on plants extracts from *Berkheya speciosa* (Asteraceae), *Euclea natalensis* (Ebenaceae), and *Trchilia emetica* (Meliaceae) showed them to be lethal to the schistosomule of *S. haematobium* (Sparg *et al*., 2000). Rug and Ruppel (2000) compared the toxic activities of the aqueous extract with that of methanol extract from the plant *Jatropha curcas* L. (Euphorbiaceae) and found that methanol extract showed the highest toxicity against cercariae and miracidia of *S. mansoni*. Some quassinoides isolated as plant growth compounds from the leaves of *Eurycoma longifoha* Jack (Simaroubaceae) were subjected to *in vitro* tests on anti-tumor

promoting anti-schistosomal and plasmodicidal activities. The most active compound for inhibition of tumor promoter, induced Epstein–Barr virus activition (anti-tumor, promotion) was 14, 15β-dihydroxyklaineanone 5, (IC_{50} =5μM). Longilactose gave significant anti- schistosomsmal effect at a concentration of 200 μg/ml. The 15β-0-acetyl-14- hydroxyklaineanone showed potent plasmodicidal activity (IC_{50} = 2 μg/ml). Thus, it was suggesting that *E. longifolia* possess high medicinal value due to the occurrence of a variety of quassinoids (Murakami *et al*., 2003).

A list of medicinal plants with anti-schistosomal activity is shown in Table 1.

Table 1: Medicinal plants with anti-schistosomal activity

Scientific Name	**Used Part of the Plant**	**Extract/ Compound**	**Species**	**Observations**
Abrus precatorius	Stem and Roots	Aqueous Extract	*S. mansoni*	*In Vitro* Test - Schistosomulum
Ozoroa insignis	Leaves and Bark of the Roots	Aqueous Extract	*S. mansoni*	*In Vitro* Test - Schistosomulum
Elephantorrhiza Goetzei	Barks of the Stem	Aqueous Extract	*S .mansoni*	
Commiphora Molmol Myrrh	Stem	Myrrh	*S.mansoni and S.haemato-bium*	-
Nigella sativa Seeds,	Oil Aqueous, Ethanolic and Chloroform Extracts		*S. mansoni*	*In Vivo* Test with Egyptian strain (Oil), *In Vitro* Test with Miracidia, Cercariae and Adults (Extracts)
Allium sativum	Crushed Scale Leaves	Aqueous Extract, Dry Crude Extract	*S. mansoni*	Egyptian strain, *In Vivo* Test
Clerodendrum umbellatum	Leaves	Aqueous Extract	*S. mansoni*	Cameroonian strain, I*n Vivo* Test
Curcuma longa	-	Curcumin	*S.mansoni*	LE strain,
Zanthoxylum naranjillo	Leaves	Fraction of Ethyl Acetate	*S.mansoni*	*In Vitro* Test
Dryopteris sp.	Rhizome	Phloroglucinol	*S.manson*	LE strain
Piper tuberculatum	Inflorescence	Piplartine	*S.mansoni*	BH strain, *In Vitro* Test
Baccharis dracunculifolia	Leaves	Essential Oil	*S.mansoni*	Native of Brazil, LE strain, *In Vitro* Tests
Cratylia mollis	Seeds	Cramoll 1,4	*S.mansoni*	Native of Brazil,BH strain, *In Vivo* Test
Zingiber officinale	Rhizome	Aqueous Extract	*S.mansoni*	*In Vivo* Test
Ageratum conyzoides L	Leaves	Essential Oil	*S.mansoni*	LE strain, *In Vitro* Test
Allium cepa	Scale Leaves	Dry Crude Extract	*S.mansoni*	Egyptian strain, *In Vivo Test*

Source .Unicamp, (2011) http://intechopen.com

2.4 Anti-parasitic Properties of *Chenopodium ambrosoides* (Wormseed) and its Phytochemicals

Chenopodium ambrosoides is an erect herb that grows to a height of 40 cm, often branched and is widely distributed throughout the world (Wong, 2012). At least 250 species of *Chenopodium* are known to exist *(*Agnew and Agnew, 1994*)*. Several infraspecific taxa have also been described giving rise to at least 12 different varieties. Most important are var. *ambrosoides* and var. *anthelminticum* (L.) A. Gray (synonym. *C. anthelminticum* L.) which are commonly cultivated in warmer parts of the world (Wong, 2012)). The stem is angularly ribbed, glabrous or finely pubescent. The leaves are oval, up to 4 cm long and 1 cm wide and toothed. The leaves have glandular hairs. The flowers are small, green in color and arranged in clusters in the axil of bract-like leaves. The seeds are also very small and green in color when fresh and black when dry.

Chenopodium ambrosoides occurs abundantly along roadsides and in waste places, sometimes also in upland rice fields. In Java it occurs at 1600 – 2000 m altitude (Agnew & Agnew, 1994). In Kenya, the distribution and availability of *C. ambrosoides* is variable. It occurs mainly in the highlands of Aberdaes, Kitale, Machakos, Narok and Nairobi (Agnew & Agnew, 1994). In parts of South East Asia, e.g. in the Java and Philippines, the plant is cultivated for medicinal purposes. It exhibits considerable variation in chemical composition e.g var. *anthelminticum* has a high proportion of ascaridole which is considered the active ingredient against a variety of parasites (Wong, 2012). Methanol extracts of *Chenopodium ambrosoides* were bioassayed on germination and seedling growth inhibition test of *Hycopersicon asculentum* (Solanaceae) and *Betavulgaric* var. rapa (Chenopodiaceae) and two weeds; *Melilotus indicus* (Leguminosae) and *Sonchus oleraceus* (Compositae). The inhibitory effect of different treatment on both germination and seedling growth increased according to the following order: oil extract > methanol extract >water extract (Hegazy & Farvaq, 2007).

Using essential oils of *Chenopodium ambrosoides* for intraperitoneal treatment at dose 30 mg/kg in BALB/c mice infected with L*eishmania amazonesis*, the data

demonstrated that the essential oils had better anti-leishmanial effect than the reference drug (Amphotericin B at 1mg/kg) suggesting that the essential oils could be used as a drug (Monzote *et al*., 2011). Detailed classification of *Chenopodium ambrosoides* is shown on Table 2.2 below.

Table 2.2: Classification of *Chenopodium ambrosoides*

Classification	**Scientific Name**	**Common Name**
Kingdom	Plantae	Plants
Super division	Spermatophyta	Seed plants
Division	Magnoliophyta	Flowering plants
Class	Magnoliopsida	Dicotyledons
Subclass	Caryophyllidae	-
Order	Caryophyllaes	-
Family	Chenopodiaceae	Goosefoot family
Genus	*Chenopodium L.*	Goosefoot
Species	*Chenopodium ambrosoides L.*	Mexican tea

Chenopodium is used all over the world as a vermifuge (Wong, 2012). The crushed plant, the expressed juice, the flower spikes, the seeds and the seed oil of C. *ambrosoides* are well known for their use as anthelmentics (Sagrero-Nieves & Bartley, 1995). Additional uses were reported in Southern Africa, Mexico, and Brazil and include the application of an infusion against cold, stomachache and treatment of cutaneous Leishmaniasis (Okuyama *et al., 1993*). According to Arisaw*a* (1971) there are many compounds found in C*henopodium ambrosoides* such as *ascariodole* (60 - 80%), ascaridole, p-cymene, limoneae and x-terpinene.

Oil yield is highest in fruits, up to 25%. The level of these compounds varies depending on the part of the plant, the age of the plant and whether it is used dried or

fresh form (Cathy Wong, 2012). When fresh stem of *C. ambrosoides* was extracted using distillation it was found to yield 0.03 – 0.04%, a yellow ethereal oil [a^{20o}, 0.908; (np^{20o}, 1.491; acid vial, 0.6 and ester vial, 22.8] with a disagreeable odour containing 5% aldehyde, ketone and 1% phenol. All parts of the plant are rich in an essential oil called chenopod essence. The leaves and inflorescence contain 35%, the fruit 0.6 - 3% (Duke, 1985; Ayensu, 1982). The grandur hairs present on leaves and fruits exude oil. (Sagrero-Nieves and Bartley, 1995). The essential oil, which is generally considered as nervine and anti-rheumatic, is very effective against amoebae causing dysentery (Ketzis & Brown, 1998). The plant contains saponin and triterpenic hiterosides which are located in the roots and are reported to have anti-fungal activities (Jaramillo *et al.,* 2012; Okuyama *et al.,* 1993; Kishore *et al.,* 1989).

Ascaridole from *C. ambrosoides* is reported to be a potent inhibitor of plasmodial growth in lower concentrations, and to kill malarial parasites in higher concentrations. It is effective at about the same dose as chloroquine and artemisinin (Wong, 2012). Like artemisinin, the compound is one of the few naturally occurring terpenes bearing a peroxide group. This peroxide group must be essential for the anti-malarial activity of ascaridole, because 1, 8-cineol (which has an epoxide group instead of a peroxide group) at identical concentrations is inactive (Kliks, 1985). In Brazil, the plant was used in the treatment of cutaneous leishmaniasis (Okuyama *et al.,* 1993). *In vitro*, studies with oil of chenopodium and chenopodium extracts were shown to inhibits egg development of parasites and inhibits maturation of larva. However, these results have not been confirmed in *in vivo* studies. According to Salifou (2013), *Chenopodium ambrosoides* aqueous extract is effective against gastrointestinal parasitic nematodes. Wong (2012) has also indicated that *C. ambrosoides is* used as a herbal remedy in the tropics to treat roundworms, hookworms and tapeworms.

The chemical composition of the oil seems to differ considerably depending on the origin of the plants and botanical variety. The crushed plant, the expressed juice, the flower spikes, the seeds and the seed oil of *C. ambrosoides* are well known for their use as anthelmintics (Sagrero-Nieves & Bartley, 1995). *C. ambrosoides* is an

effective anthelmintic with a long history of use. It is used to treat ascarids (Ketzis & Brown, 1998). It is effective against hookworms in humans (*Ancylostoma duodenale and Necator americanus*), roundworms (*Ascaris ambricoides*) and whipworms (*Trichuris trichiura*) (Kliks, *1985;* Zulane *et al., 2012*). The extracts from the plant have been used to treat worm infection in humans. According to WHO (2003), a single dose of 20 g causes a fast expulsion of parasites, without apparent secondary effects (Anjaneyulu, 1977).

2.5 Reasons for Search of an Alternative Drug

Praziquantel has been found to be effective in the treatment of both *S. haematobium* and *S. mansoni* infection (Cioli & Pica-Mattoccia, 2002; Ross *et al*., 2002**)**. It is effective in treating advanced hepatosplenic schistosomiasis, with few side effects. Praziquantel is currently the drug of choice for treating all forms of schistosomiasis (Cioli & Pica-Mattoccia, 2002; Ross *et al*., 2002**)**. However, there are limitations. The cost of Praziquantel restricts its use in many developing countries (Ross *et al*., 2002). Chemotherapy using Praziquantel may be costly on the long term, and unsustainable, especially in the resource limited countries. There is also concern that the efforts to eliminate schistosomiasis will result in intensive use of Praziquantel for schistosomiasis control. In this scenario, Praziquantel resistance or reduced parasite susceptibility to Praziquantel is likely to occur. The problems of therapeutic and drug resistance have been reported from some developing countries (Chandiwana &Taylor, 1990). Even with substantial decrease in cost of Praziquantel, cost of drug distribution may still be a problem in many endemic countries. The main constraints of population based chemotherapy are drug procurement, transport and logistics of drug delivery (Chandiwana & Taylor, 1990). Under these circumstances, alternative novel *anti-schistosomal* drug should be sought.

CHAPTER THREE

MATERIALS AND METHODS

3.1 The Research Design

This study is based on Experimental design. Ethical approval was obtained from the Institute of Primate Research (IPR) and the Kenya Medical Research Institute (KEMRI), Kenya.

3.2 Experimental Plan

Scheme 3.1 shows the experimental plan for entire study was carried out as indicated in the flow chart here below.

Figure 3.1: The experimental plan for entire study

3.3 Determination of the Effect of Chenopodium ambrosoides Extracts in *S. mansoni* Worms Reduction *In vivo*

3.3.1 Pilot I: Determining the best Parameters for *Schistosomal* Activity

Before initiation of this study, pilot experiments were carried out to find the best parameters for determination of the presence of schistosomes in mice. Mice infected with *S. mansoni* were used. The following parameters were tested: (Methods used for pilot experiment will be fully described in the main experiment).

3.3.1a Collection of Faecal Samples from Treated BALB/c Mice

The purpose for this procedure was to determine eggs counts as a measure of anti-schistosomal activity of extracts from different parts of *C.ambrosoides*.

The mice were exposed to *S. mansoni* cercariae 8 wks after weaning them using the ring method described by Smithers and Terry (1965).

Faecal samples were collected from infected mice. The faeces were collected on a weekly basis; starting from week 0 to week 7. Each mouse was placed in a clear plastic bottle (8 x 5½ cm) containing Physiological saline (0.85% NaCl), with enough volume to cover the base of the container. Each mouse was then allowed to stand in the container for a period of 30 min. The cold saline was used to agitate the mouse and cause it to defecate. The other reason for using saline was to protect the eggs in the faeces from hatching.

The faeces from each mouse was collected using a spatula and placed in a small plastic bag and kept in a refrigerator (4^{o}C) until ready to be screened for eggs using the Kato technique described in section 3.1.1c below.

3.3.1b Weight Measurement of Treated BALB/c Mice

The purpose of this study was to determine changes in infected mice body weight.

Body weight from each male and female mouse was taken starting at week 0 (infection week) to week 7 (perfusion week) using a 3 digit scale balance (Kern CB12KIN. Kern & Shn-GmbH, German). Using a 't' test to compare total mean weights of both male and female mice, the results showed variation in body weight measurements throughout the infection period was not statically significant ($p < 0.05$). The highest total mean weight for male mice was 55.44 ± 2.73 and the lowest total mean weight was 52.22 ± 4.31 while the highest total mean weight for female mice was 62.66 ± 3.21 and the lowest total mean weight was 55.88 ± 3.90. Weight gain or weight loss may have varied from individual to individual mice due to inappetance, stress or completion of food and water intake. According to Heleen *et al*., (2002), competition of food and water may also affect variation in weight. In this study, weight was found not be a reliable parameter to determine the effect of *C. ambrosoides* in mice infected with *S. mansoni*.

3.3.1c Kato Technique for Screening Egg

Faeces from mice infected with *S. mansoni* obtained as described in 3.1.1a was screened for Schistosomes eggs using the Kato technique. Ten grams of faeces from each infected mouse was pressed through a mesh screen. A template was placed on a microscope slide and the sieved faeces was transferred to evenly fill the hole of the template. This represents 50 mg of faecal sample. The template was removed and the pellet of faeces was covered with a cellophane paper soaked in malachite green/glycerol mixture. The slide was turned upside down onto a blotting paper. The preparation was then pressed gently to spread the faeces between the slide and the cellophane paper. The slide was kept until the glycerol cleared the faecal particles and the malachite green stained all the particles leaving the ova to stand out. The number of eggs were counted using a x 10 objective. The number of eggs/gm of faeces were calculated using the following formula: Number of eggs counted per

slide x 20. (NB since only 50 mg of faeces was used, it was multiplied by 20 to give 1000 mg =1g).

The results showed that there was variation in egg counts per gram of faeces from infected mice. In previous studies, it was found that there is variation in egg production in mice (Kato *et al.*, 2000). This variation is supported by this study. Eggs counts was therefore found not to be a useful parameter in determining the effect of *S. mansoni* in mice.

3.3.1d Recovery of Worms from Infected BALB/c Mice

The mice infected with *S. mansoni* were perfused at week 6 using a modified method of Smithers and Terry (1965). (See 3.2.14 and 3.2.15). The mean worm recovery from male mice was 11.9 ± 3 while in female mice it was 12.2 ± 4. There was no statistical significance difference between male and female mice ($p < 0.05$). Worm counts was found to be a suitable parameter to determine presence of schistosomes.

Conclusion

Among the above parameters that were tested to evaluate the anti-schistosomal activity of *Chenopodium ambrosoides* in BALB/c mice infected with *S. mansoni*, worm counts was found to be reliable. The following parameters were found not useful in this study: Eggs counts and weight measurement.

3.3.2 Pilot II: Confirmation of Identity *Chenopodium ambrosoides*

The purpose of this experiment was to confirm the identity of *Chenopodium ambrosoides* plants before the actual work began.

Seeds of *Chenopodium ambrosoides* plants were obtained from the Gene Bank facility at the Kenya Agriculture Research Institute (KARI), Muguga, Kenya. These seeds were subsequently planted in a botanical garden at Chiromo Campus, University of Nairobi. The plants were allowed to grow until they produced fruits.

The purpose of planting these seeds was to compare what was to be collected in the field with the authentic plants grown from the seeds at Gene bank.

Figures. 3.2, 3.3 and 3.4 below show *Chenopodium ambrosoides* plants grown at the botanical garden at Chiromo.

Figure 3.2: *Chenopodium ambrosoides* plants growing in the garden.

Figure 3.3: *Chenopodium ambrosoides* plant showing fibrous roots

Figure 3.4: *Chenopodium ambrosoides* plant showing maturing seeds

After the Pilot studies the main experiments to determine the effect of *Chenopodium ambrosoides* in *S.mansoni* worms *in vivo* were carried out as summarized in the flow chart below:

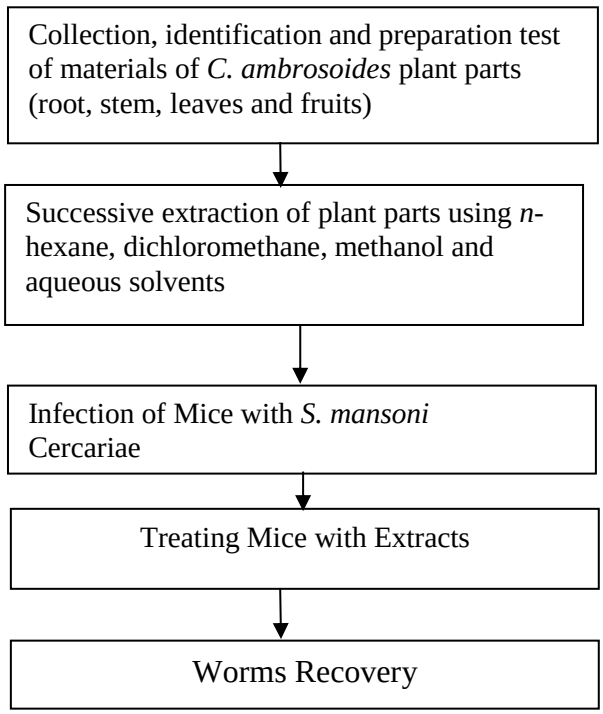

Figure 3.5: Summary of effect of *Chenopodium ambrosoides* in *S.mansoni* worms *in vivo*

3.3.3 Collection and Identification of *Chenopodium ambrosoides* (Wormseed)

The plants used in this study were collected from Westlands and Umoja Estates of the City of Nairobi, which have similar soil texture and climatic conditions (Agnew and Agnew, 1994). The plants were found growing on the banks of water trenches in Westlands and on dump site in the Umoja area. The identity was confirmed using the plant grown from seeds obtained at the Gene Bank facility of the Kenya Agriculture Research Institute (KARI), Muguga, Kenya. (See 3.2.2).

The Visual confirmation test of the plants was established by taxonomists from the National Museums of Kenya (Herbarium) and Department of Botany, University of Nairobi.

3.3.4 Preparation of Plant Materials

Plants collected from the field were transported back to the laboratory at the School of Biological Sciences, University of Nairobi in plastic bags. The plants specimens were examined for any unusual conditions and healthy plants were sorted out into the different parts: roots, stem, leaves, and fruits. The plants were dried in good air draft for a period of 6 weeks at room temperature (RT, approximately 25^{o}C), before being prepared for extraction. The respective dried plant parts were crushed into powder form using a Mekon Micromealers Single Phase grinder at the Chemistry Laboratory at Jomo Kenyatta University of Agriculture and Technology (JKUAT). The ground materials were standardized by sieving through a 0.5 mm mesh size sieves. The powdered materials were sealed in plastic bags and stored at room temperature, until ready for extraction.

3.3.5 Successive Extraction of the Plant Parts of *Chenopodium ambrosoides*

Mode of extraction depends on the texture and water content of the plant materials being extracted or the type of substances that are being isolated (Harborne, 1984). The procedure used for obtaining organic constituents from dried plant tissues involves continuous extraction of the powdered materials in a rotary evaporator (Buchi Rotavapor R-200. Helzbad HB, German) with a range of solvents starting with compounds which elute non-polar materials to more polar components (Harborne, 1984). The extract is then filtered through celite or a water pump, and concentrated *in vacuo*. This is carried out in a rotary evaporator which concentrated bulky solutions down to small volumes without bumping at temperature between 40^{o} – 45^{o}C. Each plant part was extracted separately.

3.3.5a *n* – Hexane Extraction:

The powdered root, stem, leaf and fruit powdered materials were individually first subjected to extraction by soaking them in *n* - hexane for 3 days (72 h) to elute the non-polar materials. The suspensions were then filtered through Whatman's Filter paper (No.1 medium crystalline). This process was repeated three times for each plant part in order to make sure that all non-polar materials were fully eluted. The filtrates were concentrated using Bochi-Rota Evaporator R 200 at 40^{o}C and the resulting products were labeled 'Extract **I'.**

3.3.5b Dichloromethane Extraction:

The residues obtained from *n* - Hexane extraction were then re-soaked in dichloromethane (DCM) to elute the medium polar materials. The process was repeated 3 times as for as for *n* - Hexane extraction above and the resulting products were labeled **'Extract II'**.

3.3.5c Methanol Extraction:

The residues from DCM extraction above were then re-soaked in methanol to elute the polar materials. The extraction process was repeated 3 times as for dichloromethane (DCM) and the resulting products were designated **'Extract III'.** All the 3 concentrated filtrates above, were dried in a fume cupboard at 25ºC for several weeks to remove most of the solvents.

3.3.5d Aqueous Extraction:

The residues from methanol extraction were finally soaked in distilled water for 3 days (72 h) in order to extract any remaining polar materials. The process was repeated 3 times as for as for methanol extraction. The resulting suspensions were filtered and subjected to freeze drying using Chemlabs Instrument Ltd (Edwards). The freeze dried materials constituted the aqueous **'Extract IV'.**

3.3.6 Snails Collection

B. pfeifferi snails, the intermediate host of the *S. mansoni*, were originally collected from Kakuyuni, in Kangundo area, Machakos County. The snails were collected with a scoop made of fine wire mesh from the stagnant water of Kakuyuni river. The healthy snails were carefully selected using a blunt forceps. The snails were placed in plastic basins containing water from the river during collection. They were then placed in plastic containers with holes to allow aeration. The containers were lined with wet cotton to keep the snails cool. Once the snails were placed on the first layer of cotton, they were covered with another layer of cotton. The process of layering the snails continued until the container was full. It was then covered with a final layer of cotton. The snails were transported to the snail laboratory at the Institute of Primate Research (IPR). The process of collection of the snails is shown in Figure. 3.6 below.

Figure 3.6: Collection of snails from Kakuyuni, in Kangundo area, Machakos County.

The snails were maintained in a temperature controlled snail room, under temperature ranging from 25 – 28ºC and a 12h light/12h darkness cycle. The snails were maintained in aquatic aquarium tanks measuring 42 cm long by 25 cm wide and 12 cm deep. The tanks were layered with heat sterilized sand (150 ºC for 12hrs) and gravel from Kkuyuni as a substratum. Water from IPR well which is chlorine free was used to maintain the snails. Water level in each aquarium was maintained at ¾ - level. The aquaria were also provided with micro crustaceans, Daphnia species, to provide water movement and aeration.

3.3.7 Parasite and Mouse Host

Schistosoma mansoni strain originally from Kibwezi, Machackos and maintained in baboons at the Institute of Primate Research (IPR) was used. BALB/c mice used in the study were purchased from Kenya Medical Research Institute (KEMRI). These were bred and maintained in the Animal House at the School of Biological Sciences, University of Nairobi, using the laboratory animal breed's procedures. The mice were fed on commercially compressed mice cubes purchased from the Unga Feeds Company Nairobi and were given water *ad libitum.* The mice diet was supplemented with cabbages and carrots. The mice were exposed to *S. mansoni* cercariae 8 weeks after weaning them.

3.3.8 Hatching of Miracidia

24 h faecal samples from chronically infected baboons were collected and a faecal suspension prepared in physiological saline using a wooden spatular in a 250 ml beaker. The suspension was poured through two sieves mesh size 600 and 250 μm into collecting trays. The sieves were washed thoroughly with saline from a wash bottle to ensure that all the eggs were in the collecting tray. The suspension was then transferred into urine jars and the urine jars filled with more saline. The urine jars with suspension were placed in the dark. After standing in the dark for 30 min, the supernatant was carefully poured off without disturbing the deposit, and the egg containing deposit suspended in more saline. The jars were allowed to stand for

another 30 min, in the dark. The process was repeated until the supernatant was clear. The clear supernatant was then decanted and the deposit transferred into a glass Petri dish. Using a wash bottle, the urine jar was thoroughly sprayed with water in order to ensure that all of the deposit was transferred on the Petri dish. Water was gently added to cover the deposit in the Petri dish. The Petri dish was left under artificial light for 30 min. The emergence of miracidia was observed using a dissecting microscope x 10 – 40 magnifications.

3.3.9 Infecting *Biomphilaria pfeifferi* Snails with Miracidia

B. pfeifferi snails were placed individually in 24-well culture plates. 3 - 5 miracidia were picked from the Petri dish using a drawn out pipette mounted with a rubber bulb under a dissecting microscope and dispensed in each well with a snail. The culture plate was covered to prevent snails from crawling out and the plate left for 30 min on top of the bench to allow for miracidia penetration. The snails were then transferred into newly prepared tanks. At 4 weeks post- infection, the snails tanks were covered with a dark cloth to prevent trickle shedding of cercariae.

3.3.10 Shedding Snails to obtain Cercariae

Snails with a patent infection of *S. mansoni* (5 wks post- infection) were artificially stimulated to shed cercariae using the procedure described by Yole *et al*., (1996). The snails were shed en masse for 3h in a 100 ml beaker containing 20ml of snail water, under a light bulb (100 Watts), shielded with glass to safeguard the snails from the direct heat. After shedding, cercarial suspension was transferred to a clean beaker and snails were returned to their tanks. The cercarial suspension was well mixed using a cross – style of mixing. This helps to distribute cercariae evenly. Three 50 µl aliquots of the suspension were taken and observed under a dissecting microscope (x 40 objective). Three aliquots of 50 µl each of the cercarial suspension were placed separately as droplets in a clear Petri dish. Iodine was added to immobilize and stain the cercariae. Cercariae from each of the 3 aliquots were counted under dissecting microscpe. Average of the cercariae was calculated. This

gave number of cercariae in 50 µl. The volume containing 200 cercariae was worked out based on the above.

3.3.11 Infection of Mice with *S. mansoni* Cercariae

Mice were exposed to *S. mansoni* cercariae using modified ring method described by Smithers and Terry (1965). Mice were anaesthetized with a Ketamine/xylazine mixture (ratio 3: 1). A volume of 0.02 ml per 30 g mouse body weight was inoculated intraperitoneally with a 1- ml syringe fitted with a 25G needle. Mice were shaved on the abdomen. The shaved abdominal area was wiped with damp cotton wool to facilitate cercariae penetration. The mice were placed with abdomen up on a specially designed wooden rack; a 1cm diameter metal ring was placed on the shaven area. A suspension containing 200 cercariae was dispensed dispensed in the metal ring using 1000 µl micropipette per mouse. The cercariae were allowed 30 min to penetrate after which that the mice were transferred into labelled cages.

3.3.12 Preparation of Extracts for Dosing Mice

Aqueous, dichloromethane, methanol extracts and Praziquantel were prepared as suspension in distilled water. *n* -Hexane, was prepared as a suspension in 10% Tween 80 solution. The *n* - hexane, dichloromethane, methanol and aqueous extracts, were each administered at 150 mg/body wt. Praziquantel at (450 mg/body wt) was used as a reference drug and was used to treat another group of infected mice. The dosage volume for each mouse was 200 µl /30g body weight. In order to avoid wastage, dosing volume was made up for 10 mice (ie 50 µl x10 =500 µ ~ 2 ml). Therefore 0.15 g of aaqueous, dichloromethane, methanol extracts was dissolved each in 0.2 ml distilled water while *n*- hexane extract was dissolved in 10% Tween 80 solution. Each suspension was prepared in a 1.5 ml Eppendoff tube and thoroughly mixed on a Votex apparatus.

3.3.13 Treating Mice with Extracts

Mice with 4 weeks infection of *S. mansoni* were used to test extracts from different parts of *C. ambrosoides*. 6 groups of mice consisting of 6 mice (3 males and 3 females) were set up for each plant part extract. The following groups of mice groups of mice were set up:

- *n*- Hexane (root), *n*- hexane (stem), *n*- hexane (leaf) and *n*- hexane (fruit).
- Dichloromethane (root), dichloromethane (stem), dichloromethane (leaf) and dichloromethane (fruit).
- Methanol (root), methanol (stem), methanol (leaf) and methanol (fruit).
- Aqueous (root), aqueous (stem), aqueous (leaf) and aqueous (fruit).
- Praziquantel
- Distilled water

Each mouse per group was administered 200 µl suspension at 30g body wt. using a 200 µl micropipette. The mice groups were marked on the skin with picric acid for easy of identification and each group was placed in separate cages. Each experiment for each extract was repeated once.

3.3.14 Perfusing Mice

Perfusion of mice was done using a modified method of Smithers and Terry (1965) and Yole *et al.* (1996). On wk 6 (post-infection), mice from each group i.e the experimental and the controls, were perfused with a perfusion pump (Monostat Variastat. New York, USA. Model A Series) to recover the adult worms. A transverse mid-ventral nick was made on the skin of the abdomen with a pair of scissors and skinned upwards and downwards. The abdominal wall was opened without cutting the viscera. The thoracic cavity was opened by cutting through the diaphragm along the edge of the rib cage. Ribs were increased on either side of the sternum taking care not to puncture the heart or lungs. The hepatic portal vein was severed. The Perfusion fluid contained 0.85% Sodium chloride and 1.5% Sodium

citrate. Perfusion needle, 21G, was inserted in the left ventricle of the heart and perfusion carried out until the liver and mesenteries were clear. The perfusate was collected in glass Petri dish (20 cm diameter)

3.3.15 Worms Recovery

The perfusate was transferred to a urine jar and left to settle. The supernatant was sucked out and discarded. The settling method was repeated three times. After the third settling procedure, excess supernatant was discarded. Worms's suspension was poured into ruled Petri dish (10 cm diameter). Male, female and stunted worms were counted separately. The total, mean and standard error (S.E) was worked out for worms from each group.

3.4 Assessment of the Effect of *Chenopodium ambrosoides* on Pathology caused by *S. mansoni* in mice.

3.4.1 Gross Pathology from Infected and Treated Mice

Before perfusion of each mouse, gross pathology of the liver was observed for gramulomas which if present would be distributed over the surface of the liver lobes. Gramulomas were subjectively grouped either into: no gramuloma (0 granulomas per lobe), mild (1- 3 granulomas per lobe), moderate (4 -10 granulomas per lobe) and severe (more than 10 granulomas per lobe). In addition, the livers were checked for inflammation. The inflammation was recorded as either non-inflammed or inflamed. Adhesions on the livers were also checked which indicated livers' infection. The adhesions were recorded as being absent or present.

3.4.2 Histopathology from Infected and Treated Mice

After perfusion, livers were fixed in 10% saline-buffered formalin for 2 wks. Dehydration was done on a 2 mm sample using ethanol in water, with gradually increasing percentages of alcohol changing through solutions at 30%, 50%, 70%, 80%, 95% and absolute alcohol. Clearing was done using xylene before embedding the tissues in paraffin wax. Seven micrometer (7 µm) thick sections were cut using a

Sledge microtome (Leitz Westlar, German). The sections were stained using Haematoxylin/Eosin and observed under a microscope for granulommas. Granuloma sizes were measured under a microscope using an ocular micrometer which was calibrated with a stage micrometer. The vertical and horizontal diameter of granulomas with a visible centrally placed eggs were taken. The average of the horizontal and vertical diameter was taken to be the diameter of the granuloma. A total of 10 granulomas were measured for each mouse. The photographs were taken using an inverted Nikon compound microscope (S.N. 24408) mounted with a Digital Camera.

3.5 Determination of the Effect of *Chenopodium ambrosoides* Extracts on *S. mansoni* Worms using Methanol (fruit) an Aqueous (fruit) Extracts *In vitro*

Efficacy of all the extracts was determined using worm counts as described in 3.2 and gross and histopathology as described in 3.3. Two extracts, methanol (fruit) and aqueous (leaves) were found to be efficacious. The two were used in this experiment. 100 mice were infected with *S. mansoni* cercariae as described in 3.2.11. They were perfused at wk 6 as described in 3.2.14 and the worms were collected as described in 3.2.15.

Eight Groups of 5 males and 5 female adult worms were collected and placed into separate Petri dishes (5cm diameter). Methanol (fruit) and aqueous (fruit) extracts were each weighed, and diluted into 3 different test concentrations: 0.05 mg/ml, 0.015 mg/ml and 0.3 mg/ml using distilled water. Each of the 6 concentration was dispensed into a separate Petri dish containing worms. Two controls were set up. PBS as a negative control and Praziquantel (450mg/ml) as positive control. Observations were made under a dissecting microscope after 5 min, 10 min, 15 min 20 min, and 30 min for each group. The number of dead worms from each concentration together with the controls were noted and recorded. The procedure was repeated and average of dead or live worms taken.

3.6 Determination of Immunomodulatory Effect of *Chenopodium ambrosoides* Extracts in Mice infected with *S. mansoni*

3.6.1 Infection and Treatment of Mice

The plants extracts, aqueous (leaf) and methanol (fruit) which had been found to be efficacious were used in this experiment. Mice were infected with *S. mansoni* and treated as described in 3.2.11, 3.2.12 and 3.2.13. They were divided into 5 groups of 6 mice. At wk 4 post-infection, 2 groups were treated separately with aqueous (leaf) and methanol (fruit) extracts. The other 2 groups were used as positive control (treated with Praziquantel and negative controls (infected but not treated). The naives group was not infected or treated.

3.6.2 Preparation of Serum

At wk 6 post-infection blood was drawn from the anaesthetized mice by cardiac puncture using a 25G needle and 1 ml syringe. The thoracic cavity was opened as described in 3.2.14. The left ventricle of the heart was pierced using the needle with the bevel of the needle facing upward. Blood was drawn by small jerks. About 1ml of blood was collected. The whole blood was placed into clean epperndoff tubes. The tubes were allowed to stand at room temperature for 3 h to form a clot. The tubes were then transferred into a refrigerator (4°C) overnight. The tubes were spun in microcentrifuge at 7000 rpm for 10 min. The clear supernatant, (the serum), was collected using sterile Pastuer pipette and transferred into clean nunc tubes. The tubes containing the serum were stored in a freezer (- 20ºC) until use.

3.6.3 *Schistosome* Egg Antigens (SEA)

Schistosome Egg Antigen was required as antigens for Immunoglobulin G Enzyme Linked Immunoabsorbent Assay (IgG ELISA).test to determine antibody levels. Schistosome eggs recovered from the livers of infected mice were suspended in phosphate – buffered Saline (1x PBS, pH 7.4) and spun twice at 450 g at 4°C for 10 min, each time decanting the supernatant. The eggs were sonicated 10 times for 10 sec. each time. The eggs homogenate was then centrifuged at 100,000 g at 4°C for 1

h. The clear supernatant (SEA) was recovered and the protein concentration determined using Biorad technique (1976). The procedure was carried as follows:

This method utilizes bovine serum albumin, BSA, (Bio- Rad Co.) as a standard protein. The optical densities at 595 nm, 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 spectrophotometry (Cecil Instruments CE 6600R, England). The protein was aliquoted and sterilized by exposure to UV light (10 min, 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.

3.6.4 *Schistosomule* Soluble Proteins (SSP)

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 discontinous percoll gradient and washed two times in complete media. Two concentrations of Percoll (Sigma Co. Sweden were prepared; one which was 70% Percoll 21ml 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 discontinous gradient. Cercariae suspension was chilled for one hour at 4°C. The cercariae settled at the bottom of the beaker. Excess water from the chilled cercariae suspension was sucked out using a Pasteur pipette and the cercariae re-suspended. The cercariae suspension was placed in chilled glass tubes containing 0.5 ml of 5% glucose (in distilled water). The suspension was shaken on vortex for 90 seconds to separate the heads from the tails of chilled cercariae. The separated heads and tails of the cercariae were dispensed gently on the Percoll gradient using a Pasteur pipette and centrifuged at 450 g 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°C, in the presence of 5% carbon dioxide for 3 h to release proteins. After 3 h of incubation, the schistosomules (head) suspension was centrifuged (10 min at 450 g, at 37°C). The supernatant was discarded. The schistosomules placed in PBS were sonicated, centrifuged and protein assay determined as for SEA above (3.5.4). This was shistosomule soluble proteins (SSP).

3.6.5 Schistosome Specific IgG ELISA (Enzyme - Linked Immunoabsorbent Assay)

Fifty microliters of 10 mg/ml of SSP or SEA in coating buffer (1% PBS w/v 0.05% Tween 20 pH 9.6 Fisher Chemicals, USA) was dispensed in specified wells in ELISA plate (ELISA (NUNC IMMUNO F96 CERT.MAXI-SOP) and incubated at 4ºC overnight. The plate was washed x 6 with washing buffer (PBS/0.05% Tween 20) to remove free antigens. In the plate 100 µl per well of 3% BSA in PBS/0.05% Tween 20 was added and incubated for 1 h at 37^0C to block any non-specific binding sites. The plate was washed x 6. A dilution 1:50 of mouse sera in 1% PBS was prepared. Fifty microliters of test sera and standards (positive from infected controls and negative from naives mice sera) were dispensed into the specified wells. The plate was incubated for 2 h at 37ºC. Excess sera was washed x 6. Goat- mouse IgG peroxidase conjugated to horseradish peroxidase was added and incubated at 37ºC for 1 h. The plates were then washed x 6 to remove excess conjugate. Fifty microliters of peroxidase substrate (TMB microwell peroxide substrate, Kirkgaard and Perry labs, USA) per well was added. Absorbance at 630 nm was determined using a Maxi Kinetic Microplate Reader (Molecular Devices, Palo, CA).

3.7 Determination of Anti-schistosomal Finger Profiles Using Thin Layer Chromatography

3.7.1 Thin Layer Chomatography (TLC) of the Plants Crude Extracts

Chromatograpy is a versatile technique for separating chemical compounds in a sample. Thin Layer Chromatograpy is primarily used for identifying chemical components in sample and for preparative purposes.

The crude extracts of the efficacious plant, methanol (fruit) and aqueous (leaf) were subjected to thin layer chromatography (pre-coated silica gel 60 F_{254}, Merck, German). TLC was carried out by ascent, in a tank which was paper – lined so that the atmosphere inside was saturated with dichloromethane as mobile phase. Detection of compounds on TLC plates was carried out by spraying with Vanillin reagent which was heated for 10 min at 110 ºC. The plates were observed under the 254 and 366 nm UV wavelengths (Min. uvis DESAGA-SARSTEDT.GRUPPE). Spots were outlined and R_f values of the separated fractions were recorded and reported.

3.7.2 Thin Layer Chromatography of extracts with some anti-schistosomal activity

This process separates the solution into minute spots with each spot representing the compound of the original solution. The number and the size of the minute spots indicate the number of components and the amounts in the original solution.

3.7.3 Methanol (fruit) extract

One gram methanol (fruit) crude extract which showed some anti-schistosomal activity was chromatographed on silica gel plates (Silica gel 60 F_2S_4 Merck, German) and developed using a mixture of dichloromethane and methanol (6: 4) as mobile phase. Vanillin reagent was used for identification of compounds.

3.7.4 Aqueous (leaf) extract

One gram aqueous (leaf) crude extract was also chromatographed on silica gel plates and developed using a mixture of methanol and distilled Water (1: 1) as a mobile phase. Vanillin reagent was used for identification of spots.

3.8 Data Management and Analysis

Data collected were analyzed using Holm-Sidak (1979) statistical package software. p - values < 0.05, as determined by the Student's 't' test and one ANOVA were considered significant.

CHAPTER FOUR

RESULTS

4.1 Introduction

Three experiments were carried for all the work in the mouse. The experiment reported is a representative of the three.

4.2 Determination of the Effect of *Chenopodium ambrosoidies* Crude Extracts on *S. mansoni* Worms Recovery *In vivo*

4.3 Preparation of Test Powder from *Chenopodium ambrosoides* and Yields of Plant Parts

Chenopodium ambrosoides dry materials and the powder from the plant parts (fruits, leaves, stem and roots) were each weighed as shown in Table 4.1.

The results shows stem yielded the highest amount of powder 1240 g, the fruits yielded 811 g of powder, the root yielded 441 g and the leaves yielded the lowest amount of powder 308 g. Table 4.1: Dry Materials Weights and the Yields of Powder from each of the Plant Part.

Table 4.1: Dry Materials Weights and the Yields of Powder from each of the Plant Part.

Plant Parts	Dry Weights (g)	Yields of Plant Parts in (g)	% Yields
Roots	452	441	97
Stem	1502	1240	82
Leaves	568	308	54
Fruits	919	811	88

4. 4 Aqueous and Solvents Extracts

The characteristics and the yields of each plant part of *Chenopodium ambrosoides* after extraction are as shown in Table 4.2. Methanol (stem) produced a semi-solid material with a black/green colour and yielded the highest amount of extract (10.3 g) followed by methanol (root) which produced a sticky solid, brown/yelowishin colour with a yield of 9.4 g. *n*- hexane (root) produced a Sticky Semi-solid, and yellowish brown in colour with yield of 0.85g. *n*– hexane (stem) produced tar-like solid with a dark brown colour with a yield of 0.98 g.

Table 4.2: Characteristics of the extracts from each of the Chenopodium ambrosoides plant parts studied.

Solvent System	Plant Part	Texture of Extracts	Colours of Extracts	Semi - Solid Yields (g)
n - Hexane	Root	Sticky Semi-solid	Yellowish brown	0.85
	Stem	Tar-like	Dark brown	0.98
	Leaf	Tar-like	Brown	4.52
	Fruit	Semi-solid	Blackish green	1.92
Dichloromethane (DCM)	Root	Semi-solid	Brown	2.18
	Stem	Semi-solid	Dark brown	2.46
	Leaf	Semi-solid	Blackish green	5.26
	Fruit	Semi-solid	Blackish green	5.78
Methanol (MeOH)	Root	Sticky solid	Brown/yellow	9.40
	Stem	Semi-solid	Blackish green	10.30
	Leaf	Semi-solid	Blackish green	4.49
	Fruit	Sticky semi-solid	Dark brown	5.10
Aqueous	Root	Sticky Semi-solid	Brown	3.20
	Stem	Sticky Solid	Brown	2.39
	Leaf	Sticky powder	Brown	2.32
	Fruit	Tar-like	Brown	2.1

4.5 Worms Recovery

Worms recovery results are shown in Tables 4.3, 4.4 and Fig 4.1. Aqueous (leaf) extract had the lowest mean worm counts of 16.75 ± 2.87 followed by methanol (leaf) extract which had a mean worm counts of 23.60 ± 2.04. Methanol (fruit) extract had a mean worm counts of 24.00 ± 1.31 and aqueous (fruit) extract had a mean worm count of 24.50 ± 3.07. Praziquantel had a mean worm counts of 20.50 ± 1.71 and the infected untreated control had a mean worm counts of 31.20 ± 3.23. Aqueous (leaf) extract reduced worms more effectively than the rest of the extracts.

Percentage worms reduction showed that aqueous (leaf) extract had the highest worms reduction of 46.3%, methanol(leaf) extract was second with 24.3% worms reduction and methanol (fruit) extract was third with 23.% worms reduction and aqueous(leaf) extract was fourth with 21.4% worms reduction and Praziquantel had 34.2% worms reduction. Aqueous (leaf) extract had the best worms reduction. There was statistical difference when Praziquantel was compared with infected control ($p < 0.05$). There was no statistical difference when aqueous (leaf), methanol (leaf) and methanol (fruit) were compared with Praziquantel ($p > 0 .05$). There was a statistical difference when other extracts were compared with Praziquantel ($p < 0.05$).

When aqueous (leaf), methanol (leaf); methanol (fruit) and aqueous (fruit) were compared with infected control, there was a significance difference ($p < 0.05$). However, there was no significance ($p > 0.05$) when all the other extracts were compared with infected controls.

Table 4.3: *Anti-schistosomal* activity of *C.ambrosoides* extracts (root, stem, leaf and fruit) using *S. mansoni* as target parasite in BALB/c mice

Solvent	Root	Stem	Leaf	Fruit
n-Hexane	32.86 ± 2.27	35.14 ± 3.56	33.40 ± 4.35	32.83 ± 2.27
Dichloromethane	36.25 ± 5.02	34.40 ± 2.79	37.00 ± 8.51	37.00 ± 3.89
Methanol	32.50 ± 1.61	40.33 ± 4.09	23.60 ± 2.04	24.00 ± 1.31
Aqueous	37.17 ± 2.18	39.60 ± 5.14	16.75 ± 2.87	24.50 ± 3.07
PZQ	20.50 ± 1.71			
Infected control	31.20 ± 3.23			

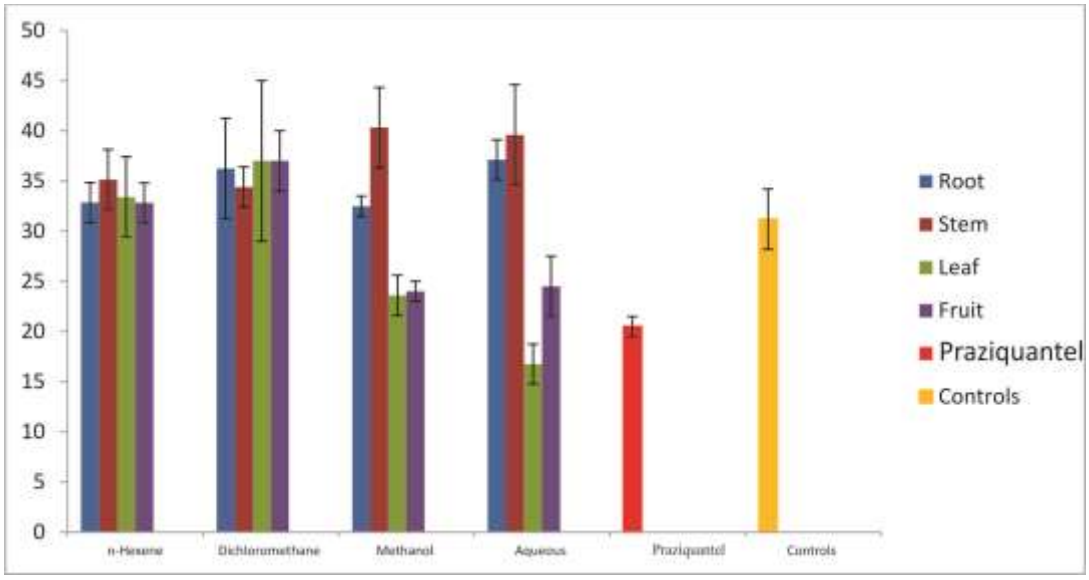

Figure 4.1: Anti-schistosomal activity of C.ambrosoides extracts (root, stem, leaf and fruit) using S. mansoni as target parasite in BALB/c mice

Key: PZQ = Praziquantel
Infected Controls consisted of *S. mansoni* infected mice but not treated
Naïve = Consisted of mice that were not infected or treated

Table 4.4: Mean worm counts and percentage worms reduction of biologically active extracts

Extract	Mean ± S.E	% Worms Reduction
Aqueous (leaf)	16.75 ± 2.87	46.3
Methanol(leaf)	23.60 ± 2.04	24.35
Methanol (fruit)	24.00 ± 1.31	23.07
Aqueous(fruit)	24.50 ± 3.07	21.47
PZQ	20.50 ± 1.71	34.29
Infected control	31.20 ± 3.23	

4. 6 Assessement of the Effect of Chenopodium ambrosoides Crude Extracts on Pathology caused by S. mansoni in Mice

4.6.1 Gross Pathology

The results of gross pathology in BALB/c mice are shown in Table 4.5.

The livers of all the mice groups infected with *S. mansoni*, whether treated or not, were inflamed. Livers of all groups had adhesions. Adhesion are either present (100%) or absent (0%) therefore aqueous (leaf) and aqueous (fruit) had adhesions (100%).

Among the livers of mice treated with Praziquantel, 67% had none granulomas while 33% had few granulomas. The livers of infected control, had 50% moderate granulomas and 50% severe granulomas.

The extracts which exhibited similar results to Praziquantel were aqueous (leaf) with 83% no granulomas and 17% few granulomas. Aqueous (fruit) extract had 50% none and 33% few and 17% moderate granulomas. Methanol (fruit) had 50% no granulomas and 33% few granulomas and 17% moderate granulomas. *n*-hexane had 60% no granulomas, 23% few granulomas and 17% moderate granulomas.

Table 4.5: Gross liver pathology readings in percentage from BALB/c mice

		G r a n u l o m a s							
Solvent/Extract	**No of mice**	**None**	**Few**	**Moderate**	**Severe**	**Adhesions**		**Inflammation**	
						AP	**AA**	**I**	**NI**
n–hexane (root)	8	40	60	-	-	100	-	100	-
Stem	8	25	75	-	-	100	-	100	-
Leaf	3	60	23	17	-	100	-	100	-
Fruit	3	33	67	-	-	100	-	100	-
DCM (root)	6	-	-	100	-	100	-	100	-
Stem	5	60	20	20	-	100	-	100	-
Leaf	6	-	67	33	-	100	-	100	-
Fruit	6	-	33	67	-	100	-	100	-
MeOH(root)	6	-	67	33	-	100	-	100	-
Stem	6	-	66	17	17	100	-	100	-
Leaf	7	43	28	28	-	100	-	100	-
Fruit	6	50	33	17	-	100	-	100	-
Aqueous (root)	5	20	60	20	-	100	-	100	-
Stem	7	43	43	14		100	-	100	-
Leaf	6	83	17	-	-	100		100	-
Fruit	6	50	33	17	-	100		100	-
PZQ	6	67	33	-	-	100	-	100	-
Infected Controls	8	-	-	50	50	100	-	100	-

Key: AA = Adhesions Absent, AP = Adhesions Present, I = Inflamed and NI = None Inflamed

4.6.2 Histopathology

Granuloma sizes are shown in Table 4.6 and Fig 4.2. Methanol (fruit) extract had the lowest mean granuloma size of 242.88 ± 22.32 µm and the aqueous (leaf) extract had the second lowest mean granuloma size of 256.52 ± 29.27 µm. Praziquantel had a mean granuloma size of 403.63 ± 20.65 µm and the Infected controls had a mean granuloma size of 971.52 ± 80.65 µm.

Aqueous (leaf) and methanol (fruit) were significantly similar to Praziquantel ($p > 0.05$) and significantly different from infected controls. All the other were significantly different from Praziquantel ($p < 0.05$) but similar to infected control ($p > 0.05$).

When all the extracts were compared with Praziquantel, it was only aqueous (leaf) and methanol (fruit) which had their worm reduction, gross pathology and histopathology similar to Praziquantel.

Table 4.6: The effect of extracts treatment on mean liver granuloma sizes (µm) in BALB/c mice

Solvents	**Root**	**Stem**	**Leaf**	**Fruit**
n –Hexane	347.31 ± 16.92	790.83 ± 124.85	403.92 ± 56.08	575.52 ± 72.48
Dichloromethane	624.21 ± 86.01	540.67 ± 67.10	608.26 ± 45.82	673.46 ± 82.22
Methanol	528.26 ± 5.85	1157.57 ± 31.52	455.26 ± 70.68	242.88 ± 22.32
Aqueous	823.68 ± 43.17	783.55 ± 74.27	256.52 ± 29.27	499.84 ± 22.26
Praziquantel	403.63 ± 20.66			
Controls	971.52 ± 80.65			

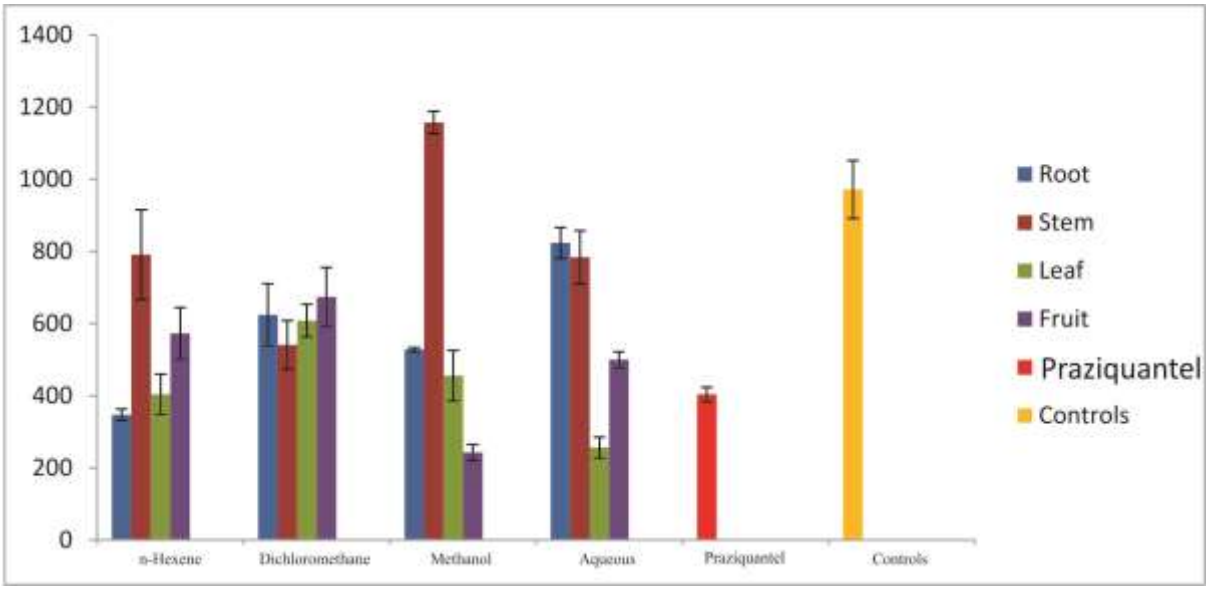

Figure. 4.2: The effect of extracts treatment on mean liver granuloma sizes (µm) in BALB/c mice

Fig.4.1: shows granuloma with high infiltration of immune cells around *S. mansoni* egg as seen in the photomicrograph. Fig. 4.2 shows hyperplasia of the bile ducts (ie. Splitting of the bile duct as result of schistosome infection) as seen in the photomicrograph. Fig. 4.3 shows normal liver with liver cells, kupffer cells and a venulo (thin vein wall).

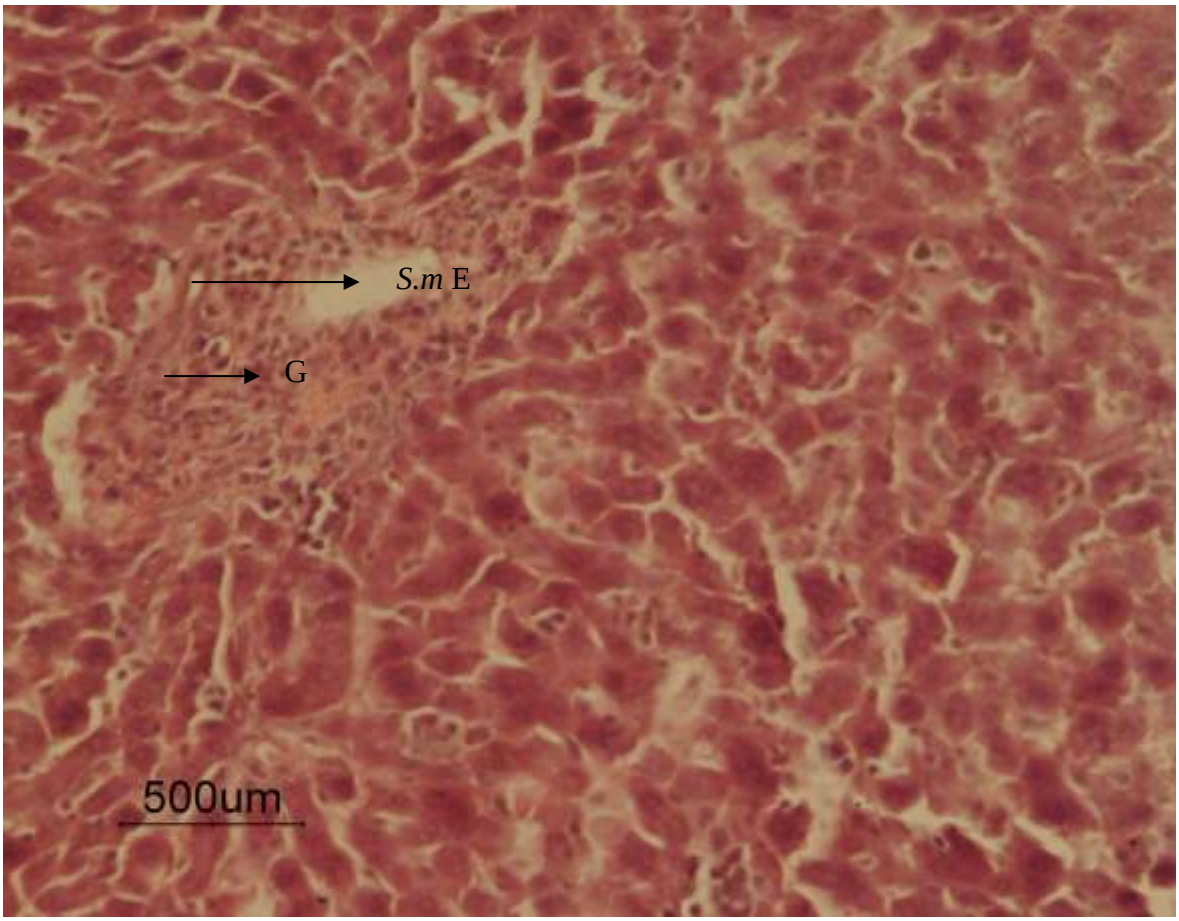

Figure.4.3: Photomicrograph of liver section from BALB/c mice treated with *C.ambrosoides* methanol (fruit) extract

Key: *S.m* E= *Schistosoma masoni* Egg.

G=Granulom

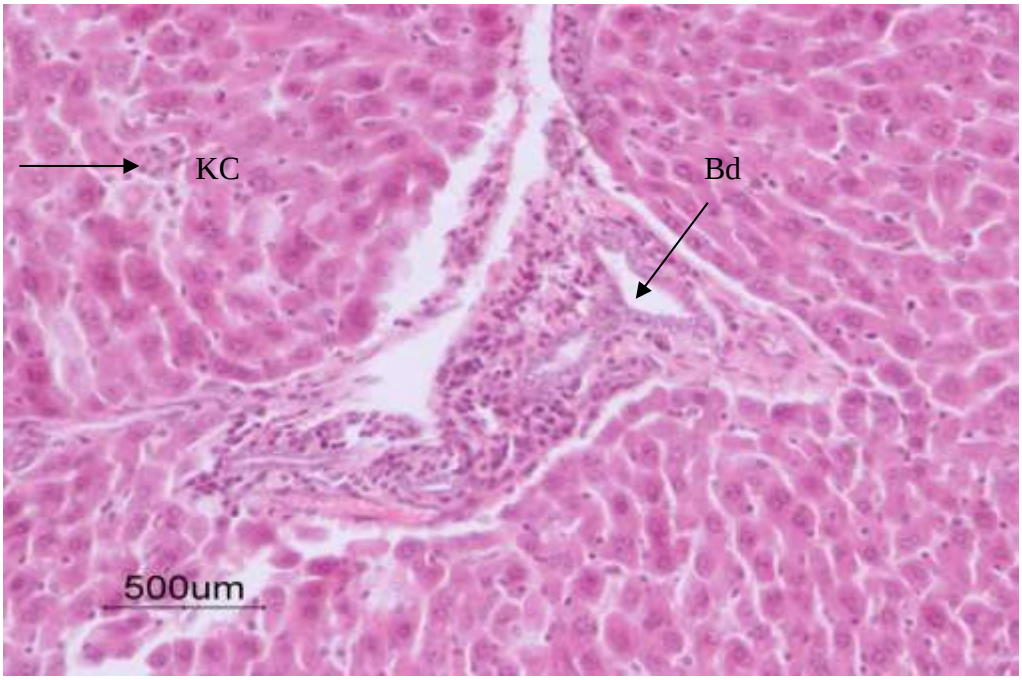

Figure.4.4: Photomicrograph of liver section with Hyperplsia of bile duct as a result of S. mansoni infection

Key: B d = Bile duct

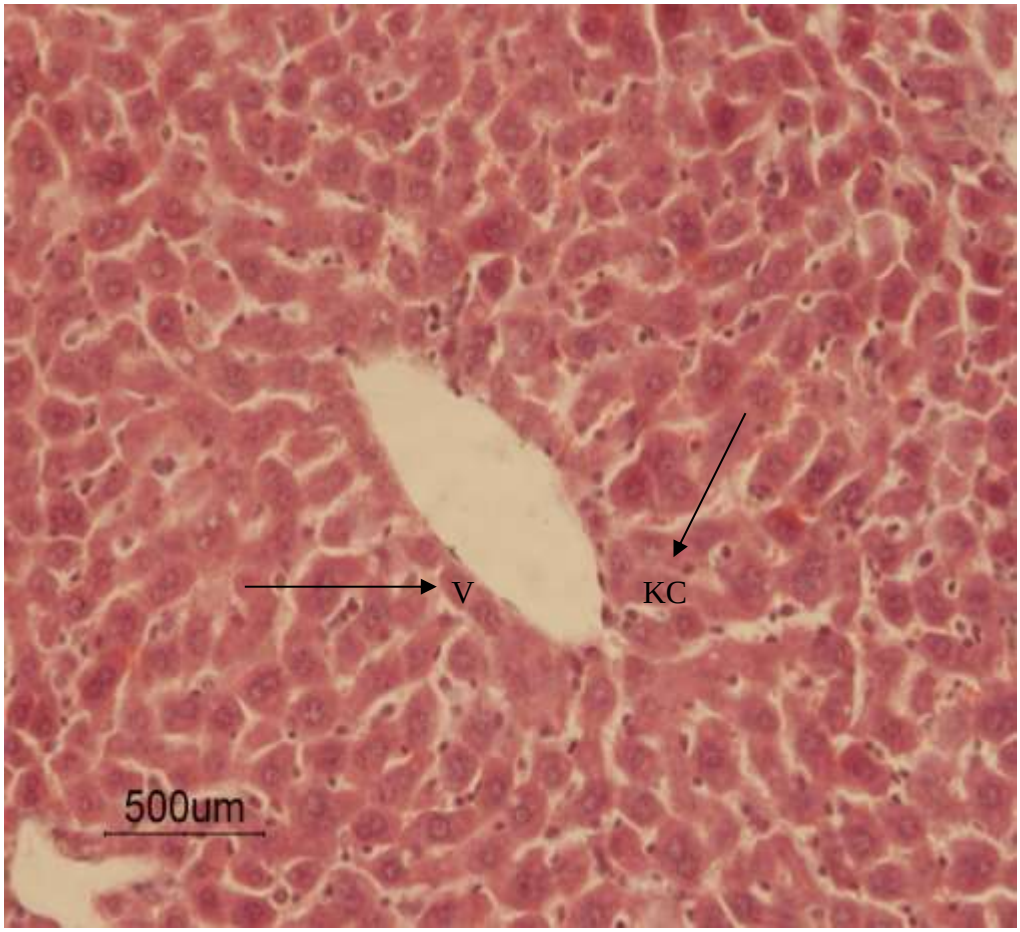

Figure. 4.5: Photomicrograph of normal liver section from BALB/c mice

Key: KC = Kupffeer Cells

V = Venulo (vein with thin wall)

4.7 Determination of the Effect of *Chenopodium ambrosoidies* Extracts on *S. mansoni* Worms *In Vitro*

The two extracts which were most efficacious in terms of worms reduction and pathology were used in this assay i.e aqueous (leaf) and methanol (fruit). When aqueous (leaf) extract was used to treat adult worms, 100% adult worms died at 5^{th} min in dose 0.05 mg/ml. Total mortality of 100% adult worms was observed in dose of 0.15 mg/ml at 5 min. 20% adult worms died at 5^{th} in higher dose of 0.3 mg/ml and this did not increase with time.

When methanol (fruit) extract was used to treat adult worms, 80% adult worms died at 5^{th} min in dose 0.05 mg/ml at 15^{th} min in 0.15 mg/ml. Total mortality of 100% adult worms was observed in the highest dose of 0.3 mg/ml at 15^{th} min. When worms were placed in Praziquantel (450mg/ml) there was sluggish movement of the adult worms within 5^{th} min and at 10^{th} min. There was 100% mortality at 15^{th} min. in dose 0.05 mg/ml. In the 0.15mg/ml. and 0.03mg/ml concentrations, 100% mortality occurred at the 5^{th} min. Physiological buffered saline was used as a control; there was no mortality between 5^{th} and 30^{th} min in all the doses.

Table 4.7: Percentage adult worms mortality using different time and concentrations of aqueous (leaf) and methanol (fruit) extracts (n = 10)

Concentration	Time	Aqueous (leaf)	Methanol (fruit)	PZQ	PBS
0.05 mg/ml	5 min.	100	80	0*	0**
	10 min	100	80	90	-
	15 min	100	80	100	-
	20 min	100	80	-	-
	30 min	100	80	-	-
0.15 mg/ml	5 min	100	50	100	-
	10 min	100	70		-
	15 min	100	80		-
	20 min	100	80		-
	30 min	100	80		-
0.3 mg/ml	5 min	20	-	100	-
	10 min	20	-		-
	15 min	20	100		-
	20 min	20	100		-
	30 min	20	100		-

4.8 Determination of the Effect of *Chenopodium ambrosoides* Extracts Eliting Immunomodulatory Responses in Mice

Two *C. ambrosoides* crude extracts, aqueous (fruit) and methanol (fruit) which were found to be efficacious on the basis of worms recovery and pathology were used in this experiments.

4.8.1 Schistosomule Soluble Protein (SSP) Specific IgG Responses

Responses of SSP are shown in Table 4.7 and Fig. 4.6. Aqueous (fruit) extract had an optical density of 0.236 ± 0.012, methanol (fruit) extract had an optical density of 0.248 ± 0.013, Praziquantel had an optical density of 0.258 ± 0.021, infected control had an optical density of 0.279 ± 0.017, and naïve had an optical density of 0.049 ± 0.001.

The result showed there was no statistical difference in IgG responses among the four infected groups i.e Praziquantel, infected control, aqueous (leaf) and methanol (fruit) ($p > 0.05$). However, there was statistical difference between the naives and the infected groups ($p < 0.05$).

Table 4.8: Schistosomula soluble protein mean optical densities of aqueous (fruit), methanol (fruit), controls and the naives

Group	**Mean (O.D) ± S. E**
Aqueous /leaf	0.236 ± 0.012
Methanol/ fruit	0.248 ± 0.013
PZQ	0.258 ± 0.021
Controls	0.279 ± 0.017
Naïve	0.049 ± 0.001

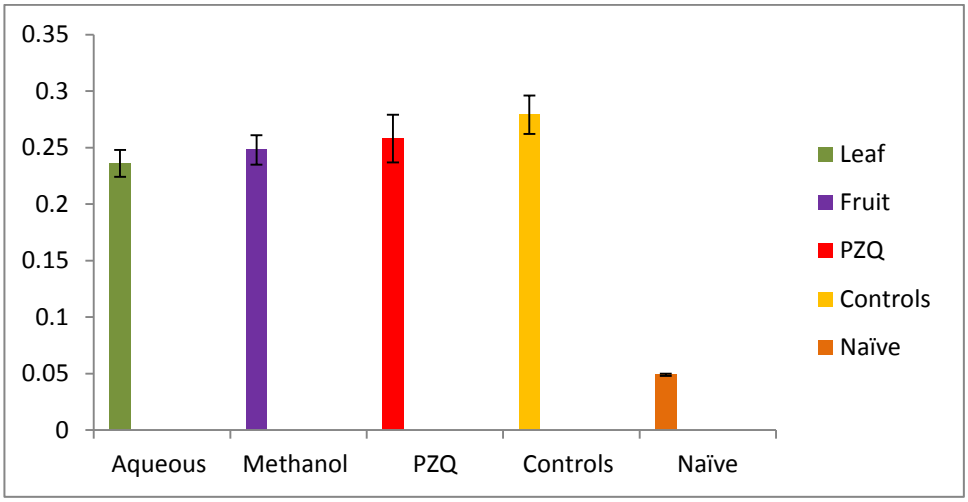

Figure 4.6: Schistosomula soluble protein mean optical densities of aqueous (leaf), methanol (fruit), controls and the naives

4.8.2 Soluble Egg Antigen (SEA) Specific IgG Respose

The results of SEA are shown in Table 4.8 and Fig 4.7. Aqueous (fruit) extract had an optical density of 0.401 ± 0.052, methanol (fruit) extract had an optical density of 0.433 ± 0.163, Praziquantel had an optical density of 0.420 ± 0.037, infected control had an optical density of 0.472 ± 0.012, and the naïves had an optical density of 0.060 ± 0.010.

The result showed there was no statistical difference among the four infected groups i.e Praziquantel, infected control, aqueous (leaf) and methanol (fruit) ($p > 0.05$) but all were statistically different from naives ($p < 0.05$). The results of soluble egg antigen are double those of schistosomula soluble protein.

Table 4.9: Soluble egg antigen mean optical density of aqueous (leaf) extracts, methanol (fruit), controls and the naives.

Extract treatment	Mean (O.D) ± S. E
Aqueous /leaf	0.401 ± 0.052
Methanol/ fruit	0.433 ± 0.163
Infected untreated controls	0.472 ± 0.012
PZQ	0.420 ± 0.037
Naïve	0.060 ± 0.010

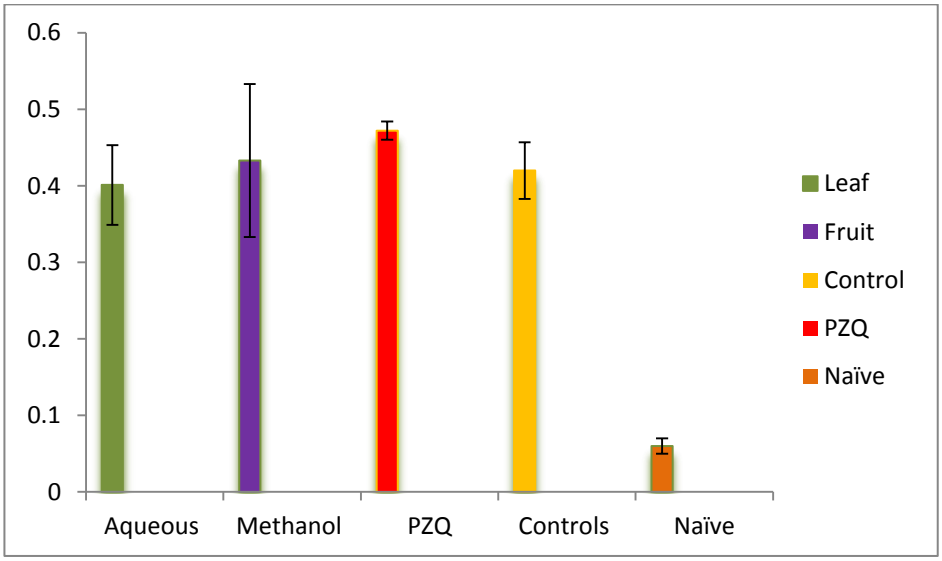

Figure 4.7: Soluble egg antigen mean optical density of aqueous (leaf) extract, methanol (fruit) extract, infected controls and the naives.

4.9 Determination of Anti-schistosomal Finger Profiles using Thin Layer Chromatography (TLC)

4.9.1 Methanol (fruit) Crude Extracts

The results are shown in Table 4.9; Fig.4.6 and Fig.4.7. Methanol (fruit) extract showed that a dilution of dichloromethane : methanol (9 :1) eluted two sports with R_f values of 0.362, 0.525; a dilution of dichloromethane : methanol (7 : 3) eluted two sports with R_f values of 0.731, 0.902; a dilution of dichloromethane : methanol (3 : 7) eluted two sports with R_f values of 0.700, 0.925 and a dilution of dichloromethane : methanol (1:9) eluted two sports with R_f values of 0.236, 0.796 and a dilution of dichloromethane : methanol (3 : 2) eluted four sports with R_f values of 0.171, 0.526, 0.723 and 0.907.

A repeat fractionation of methanol (fruit) extract was done to confirm the number of sports eluted using a dilution of dichloromethane: methanol (3 : 2). Methanol (fruit) extract showed that only three spots with R_f values of 0.562, 0.725, 0.850 were eluted. The fractionation of methanol (fruit) extract using a dilution (MeOH: Dist.H_2O (1: 1) was done and no spots were eluted.

4.9.2 Aqueous (leaf) Crude Extracts

The aqueous (leaf) extracts was subjected to fractionation using dichloromethane: methanol dilutions. Aqueous (leaf) extracts showed that using dichloromethane: methanol (9: 1) no spots were eluted. Using a dilution of dichloromethane: methanol (7: 3) two sports with R_f values of 0.562 and 0.605 were eluted. Using a dilution of dichloromethane: methanol (3: 7), one sport with R_f values of 0.787 was eluted. Using a dilution of dichloromethane: methanol (1: 9), two sports with R_f values of 0.162 and 0.637 were eluted and using a dilution of dichloromethane: methanol (3 ; 2), two sports with R_f values of 0.320, 0.705 were eluted. A repeat fractionation of aqueous (fruit) extract was done to confirm the number of sports eluted using a dilution of dichloromethane: methanol (3: 2). The repeat aqueous (fruit) extracts showed that three spots with R_f values of 0.212, 0.562, and 0.687 were eluted. The

fractionation of aqueous (leaf) extract using a dilution (MeOH: Dist.H_2O (1: 1) was done. Two spots with R_f values of 0.087 and 0.85 were eluted.

This experiment showed that the dilution of dichloromethane: methanol (3: 2) eluted more compounds in both the methanol (fruit) and aqueous (leaf) fractions than other solvents. The mobile phases of methanol (fruit) and aqueous (leaf) extracts showed that methanol (fruit) had more spots than aqueous (leaf) against *S. mansoni* parasites.

Table 4.10: TLC (Rf) spots from Chenopodium ambrosoides aqueous (leaf) and methanol (fruit) fraction crude extracts

Dilution ratio of solvents	**No. of spots from Methanol (fruit) crude extract (R_f)**	**No. of spots from aqueous (leaf) crude extract (R_f)**
DCM : MeOH (9 : 1)	0.362, 0.525	-
DCM : MeOH (7 : 3)	0.731, 0.902	0.562, 0.605
DCM : MeOH (3 : 7)	0.700, 0.925	0.787
DCM : MeOH (1 : 9)	0.236, 0.796	0.162, 0.637
DCM : MeOH (3 : 2)	0.171, 0.526, 0.723, 0.90	0.320, 0.705
Repeat DCM: MeOH (3:1)	0.562, 0.725, 0.850	0.212,0.562,0.687
MeOH : Dist.H_2O (1 : 1)	-	0.087, 0.85

Figure 4.8: TLC profile of Chenopodium ambrosoides Methanol (fruit) Crude Extract using DCM: MeoH (3: 2) solvent system

A solvent of DCM: MeOH (3: 2) was used as mobile phase. Two spots were identified with R_f values at 0.320 and 0.705.

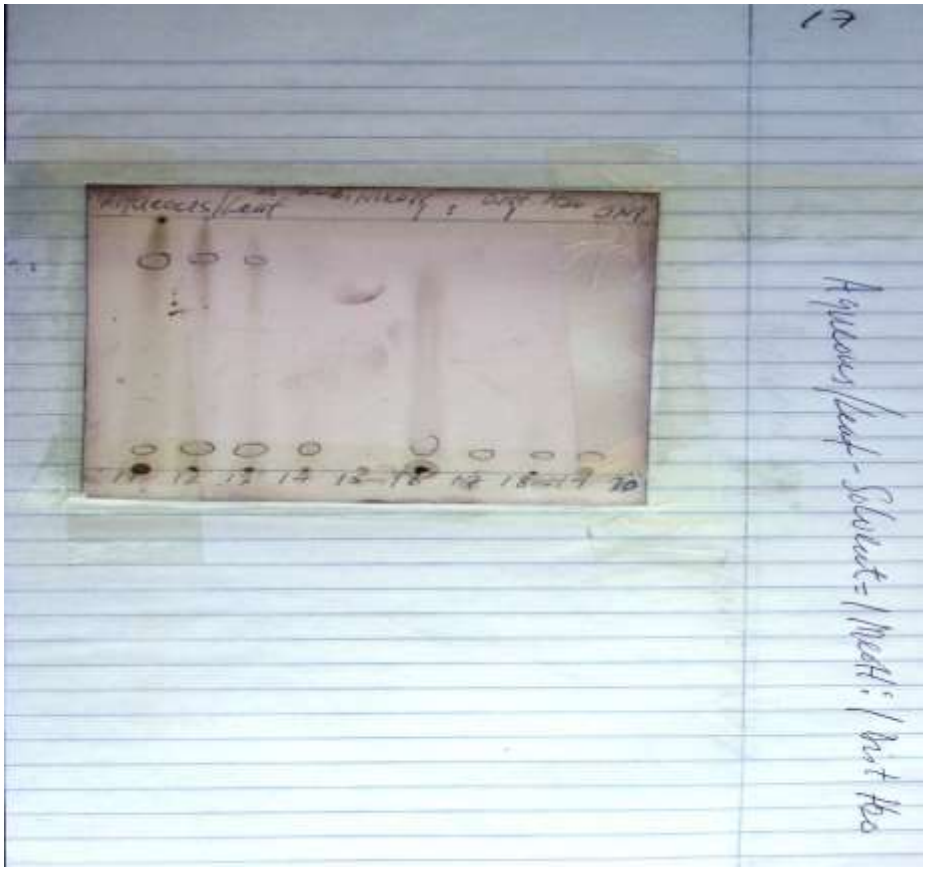

Figure 4.9: TLC Profile of Chenopodium ambrosoides Aqueous (leaf) Crude Extract using Methanol : Distilled water (1 : 1) Solvent System

A solvent of methanol: distilled Water (1: 1) was used as mobile phase. Two spots were identified with R_f values at 0.087 and 0.85.

CHAPTER FIVE

RESULT AND DISCUSSION

5.1 Characteristics and Yields of the Extracts from Each of the *Chenopodium ambrosoides* Plant Part

Chenopodium ambrosoides (Chenopodiacea L.) which is distributed all over the World has been used as a vermifuge Wong, (2012). In Kenya, the plant is found in waste places and in the landfills. In this study, the plant was chosen on the basis of its activity in the treatment of intestinal parasites and the fact that it was being investigated here in Kenya for the first time.

Plants were collected from Westlands and Umoja Estates of the City of Nairobi, which have similar soil texture and climatic conditions (Agnew & Agnew, 1994). The identity of the plants was confirmed by taxonomists from the National Museums of Kenya (Herbarium) and Department of Botany, University of Nairobi. Plants were transported back to the laboratory where they were dried and crushed into powder form using a Mekon Micromealers Single Phase grinder.

Successive extraction of plant parts was done using *n*-hexane, dichloromethane, methanol and aqueous solvents. This method is similar to what was described by Harborne (1984) and Heftmann (1983) and Bobbit (1963).

Organic constituents were obtained from dried plant materials (stem, root, leaves and dried seeds) by continuous extraction with a range of solvents i.e *n* - hexane for non-polar, dichloromethane for medium polar, ethanol and water for polar compounds. The aqueous (leaf) extract resulted in a powder form after extraction was carried out progressively using *n*-hexane, dichloromethane, methanol and finally distilled water. The rest of the extracts from other solvents ranged from semi-solid to solid extracts. The texture of the extracts was probably due to the solvent used and the contents of part of the plant extracted (Harborne, 1984). The leaves texture was oily probably due to ascaridole, a chenopod essence found in leaves of Chenopodium species

(Ayensu, 1982). Different colours of the extracts were also exhibited due to the type of the substance being isolated (Harborne, 1984).

Chenopodium ambrosoides have been shown to contain active ingredients e.g ascaridole 60 -80%, p-cymene, limonese and x- terpene (Arisawa *et al.,* 1971; *Pollack et al.,* 1990). Saponin which is associated with antihelmintic is located in roots of the plant *Neuclea latifolia* (Bishnu & Zeev, 2005.)

5.2 Determination of the Effect of *Chenopodium ambrosoidies* Extracts in *S. mansoni* Worms Reduction *In vivo*

Plants have been used as traditional medicine for anti- parasites. *Chenopodium ambrosoidies* have been used as anti- parasitic agent against *Ancylostoma, Trichuris and Ascaris* infections (Nakazawa, 1996).

BALB/c mice were each infected with 200 cercariae of *S. mansoni.* Specified groups of mice were treated with extracts of *Chenopodium ambrosoidies* at week 4 post infection as follows:

n – Hexane (root, stem, leaves and fruits), DCM (root, stem, leaves and fruits), Methanol (root, stem, leaves and fruits), Aqueous (root, stem, leaves and fruits). The mice were perfused at week 6 to recover worms and percentage worms reduction calculated. The infected controls had the highest mean worm counts compared to the rest of the extracts. There was significant difference between the infected controls with Praziquantel ($p < 0.05$).

Percentage worms reduction showed that aqueous (leaf) extract had 46%, methanol (leaf) had 24% and methanol (fruit) extract had 23%. These were statistically similar to Praziquantel ($p > 0.05$) and statistically different from infected controls ($p < 0.05$). Among all the 16 extracts, tested for anti-schistosomal activity, only the 3 extracts had results similar to Praziquantel in terms of worms reduction showing that the extracts were able to invoke protection of the mice against *S. mansoni* infection. Praziquantel was used as a positive control since it is effective against *S. mansoni*

(Cioli, 2000; Hagan *et al*., 2004). Praziquantel is the drug of choice for the treatment of Schistosomiasis (WHO, 2010). It is known to cause titanic contractions and tegumental vacuoles which cause the worms to detach from the walls of the veins and die (Ross *et al*., 2002).

The other extracts had similar worms recovery to infected controls ($p > 0.05$) and were significantly different from Praziquantel ($p < 0.05$). They were not protective. In this study, it can therefore be concluded that aqueous (leaf), methanol (leaf) and methanol (fruit) extracts of *Chenopodium ambrosoide* had anti-schistosomal active ingredients against *S. manoni* infection in BALB/c mice. The worms reduction of aqueous (leaf) was 46% which met the WHO criteria of 40% protection (Bergquist *et al.,* 2002).

Plants have been investigated as potential source of anti- schistosomal agents (Sher *et al*., 2001). *Chenopodium ambrosoides* plant has been shown to contain biochemiacal properties that control intestinal worm loads (Quinlan *et al*., 2002). *Chenopodium ambrosoides* has been used to treat a variety of intestinal worms (Kliks, 1985). Different parts of *C. ambrosoides* plant eg. the roots have biochemical products which have been used treat hookworms and ascarids in humans (Kishore *et al.,* 1989).

5.3 Assessment of the Effect of *Chenopodium ambrosoides* on Pathology caused by *S. mansoni* in Mice

5.3.1 Gross Pathology from Infected and Treated Mice

The formation of granulomas is usually in response to antigenic stimulation and is orchestrated through cytokines, immune cells and host genetics (Almadi *et al*., 2011).

The gross pathology of the liver was observed for granulomas which were subjectively grouped either into no granuloma (0 granulomas per lobe), mild (1-3 granulomas per lobe), moderate (4 – 10 granulomas per lobe) and severe (more than 10 granulomas per lobe). The infected controls showed presence of severe

granulomas suggesting heavy worms load producing high numbers of eggs while aqueous (leaf), methanol (fruit) extracts and Praziquantel exhibited reduced pathology suggesting few worms producing few eggs compared to the infected controls. Other extracts showed severe granulomas like the infected controls suggesting that they had heavy worms load producing many worms.

Aqueous (leaf) extract, methanol (fruit), aqueous (fruit) and *n*-hexane (leaf) extracts were similar to Praziquantel and different from infected controls. Other extracts were significantly similar to the in infected controls. Reduced pathology means that aqueous (leaf), methanol (fruit), aquous (fruit) and *n*-hexane (leaf) extracts were protective against *S. mansoni* infection in mice at a level similar to Praziquantel.

Livers from all groups of mice treated with the test extracts and the infected controls, exhibited presence of adhesion. Adhesions are as a result of *S. mansoni* infection and also due to the total number of *S. mansoni* eggs that end up in the liver of the mice (Cheever *et al*., 1994). The presence of adhesions indicates that the body was fighting infection.

All livers from all infected groups were inflamed indicating cell mediated immunity reaction. Egg antigens stimulate cellular immunity leading to inflammation reaction. Hepatic inflammation is one of the key pathological lesions of patent *S. mansoni* infection (Farah *et al.*, 1997). Granulomas are a form of inflammation caused mainly by host's reaction after schistosome eggs are trapped in the liver (La Flamme, 2001). According to Ibironke and Ajboye (2007), methanol extract of dried leaves of *Chenopodium ambrosoides* was investigated for anti-inflammatory and analgesic activities. The results showed that the extract dose dependently reduced granuloma tissue formation in rats. This is in agreement with findings of this study.

5.3.2 Histopathology from Infected and Treated in Mice

After the perfusion, livers were fixed in 10% buffered saline for 2 weeks. The liver tissues were dehydrated using ethanol in water, cleared using xylene and tissues embedded in paraffin. The 7 µm sections were stained using Haematoxylin/Eosin

and observed under a microscope for granulomas and granuloma sizes were measured. The average of the horizontal and vertical diameter of a granuloma with an egg at the centre was taken to be the diameter of the granuloma. A total of 10 granulomas were measured for each mouse.

Praziquantel had a mean granuloma size of 403.63 ± 20.65 µm and the infected controls had a mean granuloma size of 971.52 ± 80.65 µm. They were significantly different ($p < 0.05$)

Methanol (fruit) extract had mean granuloma size of 242.88 ± 22.32 µm. Aqueous (leaf) extract had mean granuloma size of 256.52 ± 29.27 µm. Although they had smaller granulomas than Praziquantel, aqueous (leaf) and methanol (fruit) extracts mean granuloma sizes were significantly similar to Praziquantel ($p > 0.05$) and significantly different from infected controls ($p < 0.05$). Granulom size reduction means reduced liver pathology hence less severe disease (Farah *et al*., 1997).

Worms reduction, reduced gross pathology and reduced histopathology for aqueous (leaf) and methanol (fruit) extracts were similar to Praziquantel. Therefore, these two *Chenopodium ambrosoides* extracts were protective to *S. mansoni* infection in mice.

5.4 Determination of the Effect of *Chenopodium ambrosoidies* Extracts on *S. mansoni* Worms using Aqueous (leaf) and Methanol (fruit) Extracts *In vitro*

The two extracts aqueous (leaf) and methanol (fruit) found to be efficacious in terms worms reduction, reduced gross pathology and histopathology were used in this experiment. Ten adult *S. mansoni* worms (5 males and 5 females) were tested against the following concentrations of aqueous (leaf) and methanol (fruit) extracts: 0.05 mg/ml, 0.15 mg/ml and 0.3 mg/ml.

Aqueous (leaf) extract killed 100% of the worms at 0.05 mg/ml in 5^{th} min and methanol fruit) extract killed 100% of the worms at 0.3 mg/ml in 15^{th} min and Praziquantel killed all the worms at 0.05 mg/ml in 15^{th} min. The killing effect of

methanol (fruit), and aqueous (leaf) extracts were statistically similar to Praziquantel ($p > 0.05$).

Aqueous (leaf) extract killed more worms at lower concentration than the higher concentrations. Killing effect seemed to be dependent on ionization of the extract; the higher the dilution (i.e lower concentration) the higher the ionization and the higher the wormicidal effect.

Methanol (fruit) extract potentency depended on concentration. The higher the concentration, the faster the killing. Aqueous (leaf) took the shortest time to kill (5min) compared to methanol (fruit) 15min.

Rug and Ruppel, (2000) compared the killing activities of the aqueous extract with that of methanol extract from the plant *Jatropha curcas* L. (Euphorbiaceae) and found that methanol extract showed the highest killing against cercariae and miracidia of *S. mansoni*. This is in contrast with the findings in this study.

5.5 Determination of Immunomodulatory Effect of *Chenopodium ambrosoidies* Extracts in Mice infected with *S. mansoni*

Aqueous (leaf) and methanol (fruit) extracts found to be efficacious in terms of worms reduction, gross pathology and histopathology were used in this test.

Mice were infected at week 0 with *S. mansoni*, then treated at week 4 with aqueous (leaf) and methanol (fruit) extracts. At week 6, when worms are mature and presence of eggs can be detected in the liver, the mice were bled and sera prepared. The sera was used in Enzyme imbed immunoabsorbent Assay (ELISA) to give IgG response to both schistosomule soluble protein (SSP) and soluble egg antigen (SEA) *S. mansoni* antigens.

The results of aqueous (leaf), methanol (fruit) Praziquantel and infected controls were significantly similar ($p > 0.05$). However optical density of naives was significantly different from the rest ($p < 0.05$).

Similar results were obtained with SEA although responses were double those of SSP. The higher response of SEA at week 6, can be attributed to the presence of eggs at this time. The response given by schistosomule soluble protein is due to schistosome shared antigens between shistosomules and egg stages, since at week 6 there are no schistosomules.

Praziquantel, which had very few worm had significantly similar response to infected controls. This strongly suggests that, in addition to Praziquantel destroying worms directly, it also stimulated the immune response to destroy the worms too. Both aqueous (leaf) and methanol (fruit), had similar response to Praziquantel. Praziquantel and the extracts showed high levels of IgG despite their lower number of worms compared with infected controls, indicating they killed the worms directly but also elicited immune responses against *S. mansoni* resulting in higher deaths. Studies in mice have shown that efficacy of Praziquantel against *S. mansoni* is probably linked to the host immune response (Kahama *et al.*, 1999).

This seems to be true for aqueous (leaf) and methanol (fruit). Therefore, the efficacy of aqueous (leaf) and methanol (fruit) extracts was based on worms reduction, gross pathology and histopathology reductions and modulation of worms number by the immune system.

5.6 Determination of Anti-schistosomal Finger Profiles Using Thin Layer Chromatography (TLC)

Isolation of the compounds from the crude extracts was carried out using chromatographic methods. Thin layer chromatography (TLC) technique was used in the purification process. The polar fractions of the methanol extract was eluted on gradual increase 50% to 90% methanol/ water and gradual decrease to 50% and was maintained at this level before the next injection. The process was repeated for each of the extracts. The flow rate was maintained and elution of the compounds was monitored at 254 or 366 nm µv wavelength (Min uvis. DESAGA-SARSTEDT.GRUPPE.).

When comparing two different compounds run under the identical chromatographic conditions, the compound with larger R_f value is assumed to be less polar because it interacts less strongly with the polar adsorbent on TLC plate. The relationship between the distance travelled by the solvent front and the substance is usually expressed as the R_f value. R_f is accurate to identify a compound because each compound is unique for a particular solvent. R_f values are strongly dependent upon the nature of the adsorbent and solvent. The R_f values can provide corroborative evidence as to the identity of a compound. The compounds from both methanol (fruit) and aqueous (leaf) extracts were identified by comparing the R_f values; this was done by monitoring the progress of a reaction and by comparing the intensity of the spots. If two compounds have the same R_f values, they are likely (but not necessarily) the same compounds.

The TLC finger profiles of *Chenopodium ambrosoides* methanol (fruit) and aqueous (fruit) extracts which had indicated anti-schistosomal activity were subjected to fractionation using dichloromethane : methanol (9:1), dichloromethane : methanol (7: 3), dichloromethane : methanol (3:2), dichloromethane : methanol (1: 9) and methanol : distilled Water (1: 1). In the present study, the result showed that methanol (fruit) fraction extract exhibited more spots than aqueous (fruit) extract suggesting that it had better anti-schistosomal activity against *S. mansoni* parasites.

Some medicinal plants with anthelmintic activity have secondary metabolites like alkaloids and flavonoids (Klicks, 1985). These metabolites are considered the sources of chemicals responsible for wide therapeutic activities of some medicinal plants (Klicks, 1985). Both ethanolic and aqueous crude extracts have been reported to have anti-parasitic inhibition (Oguntoye *et al.,* 2008). Although distinct chemical profiles of plants extracts are not known, in general, hydro-alcoholic extracts of plants may contain some non – polar organic chemicals with wide rage of polarity than aqueous extracts (Nisha *et al.;* 2006).

The TLC carried out on the aerial parts of *Chenopodium ambrosoides*, among the compounds isolated included hydroperoxids which was found to be against

epimastigotes of Trypanosoma cruzi (Kiuchi *et al.,* 2002). Powder and essential oil obtained from the leaves of *Chenopodium ambrosoides* were found to protect grains from damage of insect pests such as Sitophilus zeamais ambrosoides (Tapondjou *et al.,* 2002).

Ascoridol, a derivative of *Chenopodium ambrosoides* demonstrates that natural products can provide anti-parasitic agents (Klicks, 1985). MacDonald *et al.,* (2004) argues that, nematocidal activity of *Chenopodium ambrosoides* infusions are due to hydrophilic components from ascoridole.

Methanolic extracts of some medicinal plants (eg.*Centratherum anthelminticum*,) have been shown to have complete inhibition of worm motility and subsequent motality (Nisha *et al.;* 2006). Eloff, (1998) revealed that the aqueous extracts are better than ethanolic extracts in *in vitro* anthelmintic activity. Ellof (1998) argue that the active principle that reduces anthelmintic activity might be due to different classes of chemicals in the plant parts. These natural products offer an efficient approach to discovering and optimizing new pharmaceutical agents for disease control Eloff (1998).

In order to ensure the authenticity, quality and efficacy of the extracts used above, it is necessary to process to final products which were not done during this stage of the study.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

- Among *Chenopodium ambrosoides* extracts used in the treatment of *S. mansoni* infection in BALB/c mice, both methanol (fruit) and aqueous (leaf) had similar efficacy to Praziquantel, in terms of worm reduction. However, aqueous (leaf) extact was the best extract meeting 40% of WHO criteria for protection while methanol (fruit) extract was the second best.
- Both aqueous (leaf) and methanol (fruit) extracts had similar efficacy to Praziquantel in terms of reduced gross pathology. However, aqueous (fruit) extract was the best and methanol (fruit) extract second best in terms reducing granulomas.
- Both aqueous (leaf) and methanol (fruit) extracts had similar efficacy to Praziquantel in terms of histopathology in mice. However, methanol (fruit) extract had smaller granulomas than aqueous (leaf) extract though the difference is not significant.
- Both aqueous (leaf) and methanol (fruit) killed the worms. All needed ionization, the methanol (fruit) increased concentration. Aqueous (leaf) extract was better than methanol (fruit) extract, in terms of time taken to kill the worms.
- IgG responses for Praziquantel, aqueous (leaf) and methanol (fruit) were similar to infected controls, although the three had very fewer worm counts than infected controls. This shows that the three Praziquantel, aqueous (leaf) and methanol (fruit) kill worms directly and also induce immune responses.
- The two efficacious extracts were subjected to fractionation using Thin Layer Chromatography (TLC). Methanol (fruit) extract exhibited more spots than aqueous (fruit) extract.

- Overall, aqueous (leaf) extract was better than methanol (fruit) extract in terms of worms reduction both *in vivo* and *in vitro*.

6. 2 Recommendations

1. Investigation of toxicity of aqueous (leaf) and methanol (fruit) extracts.
2. Characterization of the active ingredients.
3. The *Chenopodium ambrosoides* (aqueous (leal) and methanol (fruit) extracts to be tested against other parasites.

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APPENDICES

Appedix1: Determination of Protein Concentration using the Bio-Rad Method 1976

The Bio–Rad protein assay, based on the method of Bradford 1976, is a simple and accurate procedure for determining concentration of solublized protein. It involves the addition of an acid dye protein solution and subsequent measurement at 595 nm with a spectrophotometer or microplate reader. Comparison to standard curve provides a measurement of protein concentration. The Bio – Rad protein assay is a dye binding assay in which a differential colour change of a dye occurs in a response to various concentration of protein. The absorbent maximum for an acidic solution of Coomusie brilliant blue G – 250 shifts from 465 nm to 595 nm when binding protein occurs. The Coomusie blue dye binds to primarily basic and romantic amino acid residues, especially arginine. Thus, Beers law may be applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration.

The procedure was carried as follows:

Five test tubes were arranged in the rack and labelled 1 to 5 for the standard for the sample of the antigen prepared. For test tube 1 for the standard, neat 100 µl of PBS was added to the tube no. 2 and mixed thereby and serial dilution carried out. The same was done for test samples. The dye reagent was prepared by diluting 1 part dye reagent concentration with 4 parts of distilled in deionized water and filtered through Watman paper no. 1 to remove the particulates. 5.0 mls of diluted dye reagent was added to each tube and voltexed. The tubes were incubated at room temperature for 5

min. The standards were read and a curve printed and also the sample read on spectrophotometer and concentration compared from the curve.

Appendix II: Anti-Schistosomal Activity of *Chenopodium ambrosoides* Extracts in Adult Worms *In Vivo* and *In Vitro*

APPENDIX III: Determination of Anti- Schistosomal Finger Profiles of *Chenopodium ambrosoides* Crude Extracts in BALB/c Mice Using Thin Layer

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Determination of Anti-schistosomal Finger Profiles of *Chenopodium ambrosoide* Crude Extracts *in* BALB/c Mice Using Thin Layer Chromatography (TLC)

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Abstract
Plants may contain ingredients that have anti-parasitic activity against parasites of medical siginificance. *Chenopodium ambrosoides* (Wormseed) a wide spread herb in the Family: Chenopodiacea was investigated for anti-schistosomal activity using, the human trematode parasite, *Schistosoma mansoni*, as the target. The plant is well known for its vermifuge and anti-helminthetic properties. The root, stem, leaves and fruit of the plant were extracted sequentially using *n*-hexane, dichloromethane, methanol and distilled water as solvents and tested for anti-schistosomal activity. TLC finger profiles mobile of *C. ambrosoides* extracts showed aqueous (leaf) extract had more R_f spots than methanol (fruit) extract but they were not significantly different ($P > 0.05$). The results of this study suggest that *Chenopodium ambrosoides* aqueous (leaf) and methanol (fruit) extracts has remarkable anti-schistosomal properties, and should be investigated to determine their toxicity and also tested against other parasites as a source novel anti-parasitic compounds.
Keywords: R_f - Mobility Relative to front TLC - Thin Layer Chromatography

1. INTRODUCTION
Chromatograpy is a versatile technique for separating chemical compounds in a sample. Thin Layer Chromatograpy is primarily used for identifying chemical components in sample and for preparative purposes. The sample is applied to the layer of absorbent near the edge as a small spot of a solution. The solvent which is at the bottom of the container creeps up the layer of adsorbent, passes over the spot, and, as it continues up affects separation of the material in the spot. TLC is carried out by ascent, in a tank which is paper – lined so that the atmosphere inside is saturated with solvent as mobile phase. Detection of compounds on TLC plates is normally carried out by spraying with Vanillin reagent which is heated for 10 min at 110°C. The plates are observed under 254 and 366 nm uv wavelength. Both methanol (fruit) and aqueous (leaf) crude extracts of *Chenopodium ambrosoide* which had indicated some anti-schistosomal activity in BALB/C mice were subjected to this techniques to determine which of the two extracts was better in reduction of schistosome infection.

2. MATERIALS AND METHODS
2.1 Collection of Plants Materials
The plants used for this study were collected from Westlands and Umoja areas of Nairobi city, Kenya which have similar soil texture and climatic conditions. The identity of the plants was established by a recognized taxonomist from the National Museums of Kenya (Herbarium) and Department of Botany, University of Nairobi.

The plants were freshly picked, stored in plastic bags and transported to the laboratory. The plant parts: roots, stem, leaves and fruits were then sorted out and checked for any unfamiliar conditions before separating them into different parts. The plant parts: roots, stem, leaves, and fruits were separated and dried in good air draft for a period of 6 weeks at room temperature (approx. 25°C) until ready for solvent extraction. The respective dried plant parts were crushed into powdery form using Mekon Micromealers Single Phase located at GoK Chemistry laboratory at Jomo Kenyatta University of Agriculture and Technology (JKUAT).

2.2 Extraction of Crude Extracts from Powdered Materials
The ground materials were standardized by passing them through 0.5 mm sieves. The powdered materials from roots, stems, leaves, and fruits were first subjected to extraction by soaking them in *n* – hexane, dichloromethane, methanol and aqueous solvents each for 3 days (72 hr) to elute the non-polar materials. The solutions were then filtered using Whatman's Filter paper (No.1 medium crystalline). The process was repeated three times for each plant part in order to make sure that all non-polar materials were fully eluted. The filtrates were concentrated using Bochi-Rota Evaporator R200 at 40°C. The product was labeled **Extract I.**