EVALUATION OF MOLLUSCICIDAL AND ANTISCHISTOSOMAL ACTIVITIES OF SELECTED MEDICINAL PLANTS TRADITIONALLY USED IN MAKUENI COUNTY-KENYA

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Award of the Degree of Masters of Science (Biotechnology) of Kenyatta University

November, 2014
DECLARATION

I, Kimeu Benjamin Mwonga, declare that this thesis is my original work and has not been presented for a degree in any other university or for any other award

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DEDICATION

This thesis is hereby dedicated to my wife and children.
ACKNOWLEDGEMENT

This research work would not have been possible were it not for the immense contribution of the following people to whom I am very grateful and hereby acknowledge; I recognize with deep appreciation, my supervisors, Prof Eliud NM Njagi, and Dr George O Orinda for their support and tremendous guidance from the start of this work to its conclusive end. They were all available to me for consultations and encouragement.

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Sincere thanks go to my wife Juliana Mbithe. This is one lady whose goodness I need more time to figure out. Together with our children, Faith Mwende and Mark Mwendwa they were always by my side throughout the research work. My parents, Mwaitu and Tata, you packed for me the plant materials in secure packs and escorted me to the bus stop on my way to Nairobi; Mbaïtı̀ wìà ùù nì wenyù.
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PZQ</td>
<td>Praziquantel</td>
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<td>SEA</td>
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<td>Schistosome Worm Antigen Preparation</td>
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<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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ABSTRACT

Schistosomiasis (also known as Bilharzia) is a disease caused by species of parasitic worms or helminths of the genus *Schistosoma*. It continues to be a serious world wide public health problem. The pathological changes in schistosome infestations are caused mainly by the deposition of the eggs into various tissues and organs where granulomas or pseudo tubercles are formed around them. Schistosomes and their intermediate snail hosts are integral parts of the freshwater aquatic environments in which they are found. *Biomphalaria* and *Bulinus* are the two primary genera of snails capable of harbouring infections with *Schistosoma mansoni* and *Schistosoma haematobium*. Some of the methods of controlling of schistosomiasis include: control of snails, public health education, sanitation, and community-based chemotherapy employing praziquantel. No single method, regardless of location, has been shown to work because of the large number of environmental variables involved in the parasitic transmission. Some of the control programmes include curbing transmission. The objective of this study was to bioscreen aqueous extracts of five medicinal plants identified using ethnobotanical and pharmacological information gathered from traditional healers for molluscicidal and antischistosomal activity. Molluscicidal activity was assessed by determining the ability of various concentrations of the aqueous plant extracts to kill adult *Biomphalaria pfeifferi*, the intermediate host of *S. mansoni*. The antischistosomal effect was determined by oral administration of various concentrations of the aqueous plant extracts to *S.mansoni* infected BALB/c mice, and observing the effects of these extracts on the worm burden, histological sections of the liver and serum IgG levels of the infected mice. *Amaranthus hybridus* caused the greatest mortality to *Biomphalaria pfeifferi* of 44.0±2.3. This value was considered statistically significant compared to the reference molluscide Niclosamide which caused mortality of 48.67±0.7. *Azadirachta indica* exhibited the most encouraging antischistosomal effects. This was determined using the following parameters: least worm recovery of 6.8±3.1 compared to praziquantel which exhibited 4.8±3.1; least mean granuloma diameter of 7.6±3.4 µM compared to praziquantel which showed 5.6±1.1 µM, and greater ability to boost immunological response. Based on these findings it was concluded in this study that *Azadirachta indica* and *Amaranthus hybridus* have antischistosomal and molluscicidal activity respectively, and recommends that toxicity studies be conducted to establish their safety in bilharzia control.
CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Schistosomiasis (also known as Bilharzia) is a debilitating disease caused by species of parasitic worms or helminths of the genus *Schistosoma*, which are also known as blood flukes. It is the second most prevalent tropical disease in Africa after malaria. Bilharzia is of great public health and social economic importance in the developing world. World Health Organization (WHO) reports estimate that 500-600 million people in 74 tropical and sub-tropical countries are at risk of schistosomiasis. Over 200 million people in these countries are infected, 85% of whom live in sub-Saharan Africa where *S. haematobium*, *S. intercalatum* and *S. mansoni* are endemic (Chitsulo et al., 2000).

Schistosomiasis is caused by digenetic trematodes belonging to phylum Platyhelminthes, super family Schistosomatoida, and genus Schistosoma. It is usually attributed to three species, subdivided into intestinal *Schistosoma mansoni* and *Schistosoma japonicum* or urinary *Schistosoma haematobium* types, according to the site preferred by the adult worms. In Kenya, the two species of bilharziasis are *Schistosoma mansoni* and *S. haematobium* whose intermediate hosts are fresh water snails, *Biomphalaria pfeiferi*, and *Bulinus truncatus* respectively (WHO, 1985). In humans, these blood flukes reside in the mesenteric and vesicle venules. They have a life span of many years and produce large numbers of eggs daily, which must traverse the gut and bladder tissues on their way to the lumens of the excretory organs. Many of
the eggs remain in the host tissues, inducing immunologically mediated granulomatous inflammation and fibrosis. Heavy worm burdens may produce hepatosplenic disease in schistosomiasis *mansoni* and *japonica* and urinary tract disease in schistosomiasis haematobia. Since both the schistosomes and the eggs utilize host metabolites, and because the host responses to the parasite are affected by its nutritional status, malnutrition strongly affects both the parasite and the complex host-parasite relationship (Warren, 1982).

At least four approaches to controlling infection have been tried at the community level. These are control of snails, public health education, sanitation, and community-based chemotherapy employing praziquantel (Jolcelyn, 2004). Selective molluscicide treatment in snail-infested bodies of water at main human contact points is the preferred way to approach controlling snail populations (Jolcelyn, 2004). Three drugs have been used for schistosomiasis treatment. These are Praziquantel (effective in the treatment of all forms of schistosomiasis, with hardly any side effects), Oxamniquine (used to treat intestinal schistosomiasis), and Metrifonate (effective for the treatment of urinary schistosomiasis (WHO, 2004). Praziquantel has been found to be more effective in treating *S. haematobium* infections compared to metrifonate and more effective in treating *S. mansoni* when compared with oxamniquine because it is effective when treating advanced hepatosplenic schistosomiasis, with few side effects. Praziquantel is currently the drug of choice for treatment of any kind of schistosomiasis. The main limitation is the cost which restricts its use in many developing countries. Unfortunately, the long-term worldwide application of Praziquantel coupled with the
recent discovery of Praziquantel-tolerant schistosomes has generated concern over the development of drugs-resistant *Schistosoma* strains (Shengliang et al., 2001; Appiah & DeVlas, 2002). A large number of plant products, which possess molluscicidal activity, have been identified and used for treatment of schistosomes, (Hostettmann & Lea, 1987; Agarwal & Singh, 1988; Alard *et al.*, 1991; Ndamba *et al.*, 1994; Sparg *et al.*, 2000; Molgaard *et al.*, 2001).

Traditional medicine, being a significant element in the cultural patrimony, still remains the main resource for a large majority of people in Kenya, for treating various diseases and ailments. This research dealt with ethnobotanical study in Makueni District with the aim to identify antischistosomal and molluscicidal plants used in the control of various ailments.

**1.2 Statement of the Problem**

In order to achieve effective schistosomiasis control, we require intervention programs that are effective, reliable and sustainable. In addition, the control method(s) should have minimal environmental impact. Currently, Control of schistosomiasis relies heavily on diagnosis and mass chemotherapy principally using the drug, praziquantel (PZQ). However, the prospect of relying on a single drug for a disease affecting 200 million people is an alarming situation (Cioli, 2000). There is a lot of concern due to loss of Praziquantel efficacy (Charles *et al.*, 2000) and its inaccessibility (Anthony *et al.*, 1994) and high cost requiring hard currency (Kloos and McCollough, 1982) which set back helminthes control efforts. In addition, use of molluscicides in the control of
intermediate host snails is important in integrated schistosomiasis control. Despite the
great deal of attention directed to the control of aquatic molluscs using saponins-
containing materials, no investigation is known to previously have been made to use
these compounds to control *Biomphalaria sp.* This is so despite the significant problems
posed by the snails in schistosomiasis transmission. Synthetic molluscicides have
shown a number of drawbacks including high cost, altering of the structure of the
environment by acting as biocides and destroying flora and fauna of the ecosystem.

The near exclusive use of praziquantel (PZQ) for treatment of human schistosomiasis
has raised concerns about the possible emergence of drug-resistant schistosomes.
Further, some molluscicides like copper sulphate and sodium pentachlorophenate are
general biocides and have been shown' to destroy most aquatic organisms. They have
high mammalian toxicity, and the durability of copper may result in a build-up of
copper salts in the water after prolonged treatment. Also, Sodium pentachlorophenate is
highly irritant and inhalation of the powder produces acute symptoms. Sprayers
continually using this substance suffer from lacrimation and chronic coughs. This
research aimed at evaluating native plants for potential active substances against
schistosomes and schistosome snail hosts.

1.3 Justification of the Study

Schistosomiasis is a debilitating disease with 500-600 million people in 74 tropical and
subtropical countries being at risk. Development of antischistosomal resistance in
helminthes reported in a number of countries (WHO, 2004) gives a clear indication that
control programs based exclusively on their use are not sustainable. The molluscicidal and antischistosomal agents used conventionally to control this disease have many setbacks besides being very expensive. Many plants have been investigated for molluscicidal and antischistosomal activities and have been found to exhibit some activity. Herbal medicines are cheap, more readily available to people and are less toxic, although a lot still needs to be done to confirm this.

Continuous research for new drugs with high activity and reduced adverse effects is very important, especially considering that in Kenya parasitic diseases constitute a serious public health problem. Screening the biodiversity of the Kenyan plants can reveal new phytotherapeutic drugs. The biodiversity existing in the Kenyan flora is a potential source of many new bioactive molecules

1.4 Research Hypothesis

Aqueous leaf extracts of *Aloe secundiflora* (AS), roots extracts of *Balanites aegyptiaca* (BA), leaf and flower extracts of *Aspilia pluriseta* (AP) and *Amaranthus hybridus* (AH), and root and leaf extracts of *Psidium guajava* (PG) and *Azadirachta indica* (AI), respectively, have both molluscicidal and antischistosomal activities.

1.5 General Objective

To identify and evaluate antischistosomal and molluscicidal activities of aqueous extracts of *Aloe secundiflora* (AS), *Balanites aegyptiaca* (BA), *Aspilia pluriseta* (AP),
Amaranthus hybridus (AH), Psidium guajava (PG) and Azadirachta indica (AI) used traditionally in Ukambani area for control of various diseases.

### 1.5.1 Specific Objectives

i. To evaluate the molluscicidal activity of aqueous extracts of *Aloe secundiflora* (AS), roots of *Balanites aegyptiaca* (BA), leaves and flowers of *Aspilia pluriseta* (AP) and *Amaranthus hybridus* (AH), and roots and leaves of *Psidium guajava* (PG) and *Azadirachta indica* (AI) on *Biomphalaria pfeifferi*.

ii. To evaluate the antischistosomal activity of aqueous extracts of the plants in (i) above.
CHAPTER TWO

LITERATURE REVIEW

2.1 Schistosomiasis

2.1.1 Milestones in the study of schistosomiasis

Schistosomiasis has been recognized since the time of the Egyptian Pharaohs. Egyptians have had a long history of symptoms caused by schistosomiasis, notably haematuria, which appeared classically in young boys and was once deemed to be a sign of puberty. It was in Egypt in 1851 that Theodore Bilharz (a young German pathologist, from whom the disease took its original name, Bilharziasis) discovered, in autopsy material that the causative agent of haematuria was Schistosoma (El-Khoby et al., 1998). In 1903, Manson observed lateral spined eggs in the faeces of a patient who had no hematuria. He suggested that more than one species of the worm was involved in the vesical and intestinal forms of the disease on grounds of dissimilar geographical distribution of both types of infestation. In 1907, Sambon verified Manson’s suggestion and named the worm that produced lateral spined eggs and caused intestinal infestation as Schistosoma mansoni.

In 1915, Leiper discovered the two genera of snails in Egypt (Bulinus and Biomphalaria) that transmitted the two species S. haematobium and S. mansoni, respectively. In 1937, Scott reported on the prevalence of schistosomiasis in 100 Egyptian Villages. At that time, S. haematobium infestations were common, while S. mansoni infestations were rare in the Nile delta. Since 1977 this pattern of
schistosomiasis in Egypt changed as the prevalence of *S. mansoni* infestation increased and of *S. haematobium* decreased (Abdel-Wahab *et al.*, 1980). This change has important public health implications, because the hepatosplenic schistosomiasis caused by *S. mansoni* is more difficult to trace and is associated with greater morbidity and mortality than the urinary schistosomiasis caused by *S. haematobium*.

### 2.1.2 Geographical distribution

Schistosomiasis (also known as bilharzia, bilharziasis or snail fever) is a parasitic disease caused by several species of fluke of the genus *Schistosoma*. It is most commonly found in areas with water that is infested with freshwater snails, which may carry the parasite (El Alamy and Cline 1977). The disease is found in tropical countries in Africa, Caribbean, eastern South America, east Asia and in the Middle East. *Schistosoma mansoni* is found in parts of South America and the Caribbean, Africa, and the Middle East; *S. Haematobium* is found in Africa and the Middle East; while *S. japonicum* in the Far East (Mason, 1903). *S. mekongi* and *S. intercalatum* are found focally in Southeast Asia and central West Africa, respectively. Although it has a low mortality rate, schistosomiasis is an often chronic illness that can cause liver and intestinal damage and can be very debilitating (Elliott, 1996).

A few countries have eradicated the disease, and many more are working towards it. WHO is promoting efforts working towards this goal. In some cases, urbanization, pollution, and/or consequent destruction of snail habitat has reduced exposure, with a subsequent decrease in new infections. The most common way of getting schistosomiasis in developing countries is by wading or swimming in lakes, ponds and
other water bodies which are infested with the snails (usually of the *Bulinus*, or *Oncomelania* genus) that are the natural reservoirs of the *Schistosoma* pathogen (Boros, 1989).

2.1.3 Snail intermediate host

Schistosomes and their intermediate snail hosts are integral parts of the freshwater aquatic environments in which they are found. *Biomphalaria* and *Bulinus* are the two primary genera of snails capable of harbouring infections with *Schistosoma mansoni* and *S. haematobium*. Both are pulmonate molluscs obtaining oxygen directly from the atmosphere. A small percentage of its oxygen demand is satisfied by diffusion across the epithelium of exposed tissues (Barbosa and Costa, 1981).

Pulmonates are hermaphroditic, but the male reproductive system may be incompletely developed. They produce viable eggs via self-fertilization or cross-fertilization. Pulmonates can produce up to thousands of eggs in their lifetime. They do not usually lay eggs in temperatures below 18°C. Egg laying behaviour increases proportionally with temperatures up to 30-35°C. Above this level, snail and egg mortality rise dramatically. Eggs are laid in clusters within transparent, yellow, gelatinous masses. *Biomphalaria* eggs are circular or oval, and attached to flat surfaces, while *Bulinus* eggs are more elongated and are often wrapped around the curved surfaces of plant stems. Eggs mass and size are proportional to the size of the parent snail. The mass may exceed 1 cm in diameter or length and contain 30 or more eggs. Snail hatching and development is directly linked to temperature. On average, eggs hatch within 5-10 days,
and the hatchlings measure 0.5-1.0 mm in length. It takes about 4-12 weeks to reach sexual maturity. Most often this occurs when snails have reached 5 mm in diameter or height. In the laboratory, *Biomphalaria* species native to Africa live less than one year, reaching a maximum diameter of 15-20 mm. In contrast, those found throughout South America and the Caribbean basin live for two years or more, reaching a maximum diameter of 30mm. In the field, many species of *Biomphalaria* outlive their laboratory-reared counter-parts.

Figure 2.1 *Biomphalaria pfeifferi*; snail intermediate host of *Schistosoma mansoni* (Boros, 1989)
2.2 Schistosomes

2.2.1 Species

There are four species of schistosome which are infective to humans: They include:

- **Schistosoma mansoni**, found in Africa, Brazil, Venezuela, Suriname, the lesser Antilles and Puerto Rico. It is also known as Manson’s blood fluke or swamp fever. *Biomphalaria pfeifferi* (Figure. 2.1) a freshwater snail is an important host for this trematode.

- **S. japonicum** whose common name is simply blood fluke is found widely spread in Eastern Asia and the south-western Pacific region. In Taiwan this species only affects animals, not humans. Freshwater snails of the *Oncomelania* genus are an important host for *S. japonicum*.

- **S. mekongi** is related to **S. japonicum** and affects both superior and inferior mesenteric veins. *S. mekongi* differs from *S. japonicum* in that it has smaller eggs, a different intermediate host, and longer prepatent period in the mammalian host.

- **S. haematobium**, commonly referred to as the bladder fluke, originally found in Africa, the Near East, and the Mediterranean basin was introduced into India during World War II. Freshwater snails of the *Bulinus* genus are an important host for this parasite.

2.2.3 Morphology

Schistosomes, unlike other trematodes, are long and slim worms. The male *S. mansoni* is approximately 1 cm long (0.6 to 1.4 cm) and is 0.11 cm wide. It is white, and it has a
funnel-shaped oral sucker at its anterior end followed by a second pediculated sucker. The external part of the worm is composed of seven layers and is continually renewed as the outer layer is continually shedding. The tegument bears a large number of small tubercules. The suckers have small thorns in their inner part as well as in the buttons around them. The male genital apparatus is composed of 6 to 9 testicular masses, situated dorsally. There is one deferent canal beginning at each testicle which is connected to a single deferent that dilates into a reservatory, the seminal vesicle, located at the beginning of the gynaecophoric canal. The copula happens through the coaptation of the male and female genital orifices (Boros. 1989).

The female has a cylindrical body, longer and thinner than the male (1.2 to 1.6 cm long by 0.016 cm wide). The female parasite is darker, and it looks grey. The darker colour is due to the presence of a pigment (haemozoin) in its digestive tube. This pigment is derived from the digestion of blood. The ovary is elongated and slightly lobulated and is located on the anterior half of the body. A short oviduct conducts to the ootype which continues with the uterine tube. In this tube it is possible to find 1 to 2 eggs (rarely 3 to 4) but only 1 egg is observed in the ootype at any one time. The genital pore opens ventrally. The posterior two-thirds of the body contain the vittelogenic glands. The winding canal unites with the oviduct a little before it reaches the ootype. The digestive tube begins at the anterior extremity of the worm, at the bottom of the oral sucker. The digestive tube is composed of an oesophagus which divides in two branches (right and left) and that reunite in a single caecum. There is no anus.
Figure 2.2 Schistosomes encopul. (Sambon, 1907)
2.2.4 Reproduction
Unlike other trematodes, the schistosomes are dioecious – that is, the sexes are separate.

The two sexes display a strong degree of sexual dimorphism, and the male is considerably larger than the female. The male surrounds the female and encloses her within his *gynaecophoric canal* (Figure. 2.2) for the entire adult lives of the worms, where they reproduce sexually (Jones *et al.*, 2008).

2.2.5 Life Cycle

The three species of schistosomes that commonly affect human (*S. haematobium*, *S. mansoni* and *S. japonicum*) have similar life cycles and develop by a succession of stages: the egg, miracidium, first stage sporocyst, second stage sporocyst, cercariae, schistosomule, lung stage, liver stage and adult. All the species of schistosomes are contracted in the same way: by direct contact with infested surface water containing free-living forms of the parasite known as cercariae (Basch, 1981).

2.2.5.1 Life Cycle of *S. mansoni*

After the eggs of the human dwelling parasite are emitted in the faeces and into the water, the ripe miracidium hatches out of the egg. The hatching happens in response to temperature, light and dilution of faeces with water. The miracidium searches for a suitable freshwater snail (*Biomphalaria glabrata*, *B. pfeiferi*, *B. straminea* or *B. tenagophila*) to act as an intermediate host and penetrates it. Following this, the parasite develops via a so-called mother-sporocyst and daughter-sporocyst generations to the cercaria. The purpose of the growth in the snail is the numerical multiplication of the
parasite. From a single miracidium result a few thousand cercariae, every one of which is capable of infecting man. The cercariae emerge from the snail during daylight and they propel themselves in water with the aid of their bifurcated tail, actively seeking out their final host. When they recognize human skin, they penetrate it within a very short time. This occurs in three stages, an initial attachment to the skin, followed by the cercaria creeping over the skin searching for a suitable penetration site, often a hair follicle, and finally penetration of the skin into the epidermis using proteolytic secretions from the cercarial post-acetabular, then pre-acetabular glands. On penetration, the head of the cercaria transforms into an endoparasitic larva, the schistosomule. Each schistosomule spends a few days in the skin and then enters the circulation starting at the dermal lymphatics and venules. The schistosomule migrates to the lungs (5-7 days post-penetration) and then moves via circulation through the left side of the heart to the hepatoporal circulation (>15 days) where it feeds on blood. If it meets a partner of the opposite sex, it develops into a sexually mature adult and the pair migrates to the mesenteric veins (Boros, 1989). Male schistosomes undergo normal maturation and morphological development in the presence or absence of a female, although behavioural, physiological and antigenic differences between males from single-sex, as opposed to bisex, infections have been reported. On the other hand, female schistosomes do not mature without a male. Female schistosomes from single-sex infections are underdeveloped and exhibit an immature reproductive system (Clemens et al., 1989).
Although the maturation of the female worm seems to be dependent on the presence of the mature male, the stimuli for female growth and for reproductive development seem to be independent from each other. The adult female worm resides within the adult male worm's gynaecophoric canal, which is a modification of the ventral surface of the male forming a groove. The paired worms move against the flow of blood to their final niche in the mesenteric circulation where they begin egg production (> 32 days). The *S. mansoni* parasites are found predominantly in the small inferior mesenteric blood vessels surrounding the large intestine and caecal region of the host. Each female lays approximately 300 eggs a day (one egg every 4.8 minutes), which are deposited on the endothelial lining of the venous capillary walls (Boros, 1989). Most of the body mass of female schistosomes is devoted to the reproductive system. The female converts the equivalent of almost her own body dry weight into eggs each day. The eggs move into the lumen of the host's intestines and are released into the environment with the faeces.
Figure 2.3 Life cycle of the three schistosome species
2.3 Culture of schistosomes

*In vitro* cultivation methods can provide useful insights into the biology, nutrition and immunology of schistosomes. Among the key issues in parasite cultivation is the degree to which cultured organisms resemble their counterparts reared in normal hosts (Basch, 1981; Clemens and Basch, 1989). Trials to cultivate *S. mansoni* from cercariae have led to production of nonviable eggs by worm pairs grown entirely *in vitro* (Basch, 1981). Tiba et al. (1974) showed that artificially prepared schistosomules can develop to maturity when injected into mice shortly after preparation. Basch et al. (1981) demonstrated that 2 hour and 13 day old schistosomules grown *in vitro* from *S. mansoni* cercariae can complete normal development successfully after implantation into mouse mesenteric veins. An alternative approach, the study of egg production by pairs of mature worms maintained *in vitro*, was not successful (Davis, 1993). In general, egg laying has been observed for only a few days after adult worms were transferred from the host to cultures (Warren, 1982).

Wu and Wu (1986) demonstrated that the portal serum from various mammalian sources have components that stimulated *S. mansoni* oviposition *in vitro*. Hobbs et al. (1993) established protocols for the initiation and maintenance of cultures from juvenile worms of *S. mansoni*. These cultures exhibited properties characteristic of the organism from which they originated and could be maintained for as long as 6 month *in vitro*. Taylor et al. (1969) and Taylor (1971) showed that in single-sex infestations, schistosomes migrated to the portal-mesenteric venous system, indicating that each sex is capable of locating the preferred site independent of the other sex. Blood draining to the portal vein is derived from the gastrointestinal tract. Therefore, it is different from the peripheral
blood in many respects (Ishikawa, 1976). The site preference of *S. mansoni* could depend on a constituent of portal blood that is not present in the periphery. This might take the form of a substance that the parasite recognizes or requires to develop. It has been shown that egg production can be stimulated by portal serum components added to culture media, but not by serum from peripheral blood (Wu *et al*., 1985). This occurred regardless of whether the host is natural or not. Immature schistosomes in culture have been shown to have enhanced cellular proliferation when portal serum was added to the medium. This effect could not be reproduced by serum obtained from the vena cava (Butterworth, 1994). Furthermore, when the serum was fractionated, the size of the stimulatory substances was estimated to be larger than would be expected for simple nutrients absorbed from the gut (Shaker *et al*., 1998). In experimental animals, granuloma formation has been shown to be induced and elicited by soluble egg antigens (SEA) secreted by the miracidia within eggs (Hang *et al*., 1974).

Over the years, several laboratories have isolated antigenic fractions from crude egg homogenates. A number of partially purified glycoproteins have been shown to possess serological, dermal, lymphocyte-stimulating, hepatotoxic, and granuloma inductive properties (Harn *et al*., 1989). However, the relative importance of the various fractions as granulomatogenic agents remains unexplored. The differential responsiveness of acute-versus chronic-infestation murine lymphocytes to a panel of SEA-derived fractions was demonstrated. A 38-kDa fraction was found to be egg stage specific, to elicit strong lymphokine production *in vitro*, and to induce granuloma formation *in vivo* during the acute stage of murine schistosomiasis (Lukacs and Boros, 1991).
2.4 Pathogenesis and pathology

The pathological changes in schistosome infestations are caused mainly by the deposition of the eggs into various tissues and organs where granulomas or pseudo tubercles are formed around them. In primary infestations, the granuloma is composed of aggregations of mononuclear phagocytes, neutrophils, lymphocytes, plasma cells and fibroblasts. Onset of egg laying in humans is sometimes associated with an onset of fever (Katayama fever). This "Acute Schistosomiasis" is not however as important as the chronic forms of the disease (Kojima, 1998).

*S. mansoni* and *S. japonicum* are "Intestinal" and "Hepatic Schistosomiasis", associated with formation of granulomas around trapped eggs lodged in the intestinal wall or in the liver, respectively (Lukacs and Boros, 1991). The hepatic form of the disease is the most important, granulomas in this site giving rise to fibrosis of the liver and hepatosplenomegaly in severe cases (Harn *et al.*, 1989). Symptoms and signs depend on the number and location of eggs trapped in the tissues. Initially, the inflammatory reaction is readily reversible. In the latter stages of the disease, the pathology is associated with collagen deposition and fibrosis resulting in organ damage that may only be partially reversible (Hang *et al.*, 1974). Giant cells are also frequently observed in the granulomas. Granulomas may vary in size and cellular components with the immune status of the host in experimental infestations in immunized animals (Blum *et al.*, 1992). A dominant cellular infiltration of eosinophils and lymphocytes is observed around the eggs, (Kojima, 1998). Granuloma formation around schistosome eggs has been considered to be the result of delayed type hypersensitivity reactions mediated
through a T cell mediated immune response to soluble egg antigens (Warren, 1967). However, studies have demonstrated that there exist at least 2 subsets of T helper cells with a CD4 phenotype, termed Th1 and Th2 cells, which can be distinguished from each other by their cytokine production. The cytokines derived from Th1 cells, such as interleukin (IL)-2, interferon or tumor necrosis factor (TNF), may be responsible for activation of macrophages and cell-mediated immunity, whereas IL-4 or IL-5, the cytokine produced by Th2 cells, stimulates IgE production or eosinophilia, respectively (Weinstock, 1992).

Figure 2.4 S. mansoni egg with the characteristic lateral spine (Waine & McManus 1997)
2.5 The immune response in schistosomiasis

The immunology of schistosomiasis is largely dependent on the biological characteristics of the parasite itself (Capron et al., 1980). After skin penetration, schistosomula undergo a complex migratory life cycle in the vertebrate host before they settle, in the case of *S. mansoni*, in the blood vessels of the portal and mesenteric system. In this intravascular situation, the adult worms release a large amount of excretory and/or secretory material, which elicits a strong antibody response. Antigens may be found in the serum and various body fluids in the form of free antigens, and more generally as immune complexes (Waine and McManus 1997). This continuous release of soluble antigens has important implications in the regulation of the immune response, both in terms of antigenic competition and as direct factors of immunosuppression or tolerance.

The major role of antibodies in protective immunity is to induce cytotoxic destruction of schistosomula targets, and antibody-cell mediated cytotoxicity appears to be the main mechanism for destruction of parasites both in rat and human schistosomiasis (Capron *et al.* 1980). The persistence of the trematodes in an immunologically hostile environment has been attributed to their ability to acquire or synthesize, during their maturation, surface antigenic determinants (host antigens) to which the animal is unresponsive (Boyer *et al.*, 1977). The worm tegument, which undergoes a continuous and rapid turnover, acquires numerous host molecules ranging from various serum proteins or glycolipid to major histocompatibility antigens. This phenomenon has been
considered as an essential escape mechanism (Torpier et al., 1979; Damian et al., 1973; Dean, 1977).

It has been assumed from epidemiological studies in endemic areas that age-dependent immunity may develop against infestation or against re-infestation after treatment, with *S. mansoni* (Butterworth, 1994) or *S. haematobium* infestation (Hagan et al., 1991). Using a mathematical model, it has also been shown that predicted patterns of variation in age-related changes in the intensity and prevalence of *S. haematobium* infestation are consistent with the epidemiological effects of acquired immunity (Woolhouse et al., 1991). At present, however, there is no effective vaccine against schistosomiasis or any other human parasitic disease. In order to develop such vaccines, it is important to elucidate mechanisms involved in protective immunity at the cellular and molecular levels because of the complex life cycle and stages of parasites which occur in the human body.

### 2.6 Clinical manifestations

Clinical manifestations reflect developmental stages of the parasites and host responses to toxic or antigenic substances derived from the parasite and eggs. During the early stage of infestation, the patient may present with signs caused by cercarial penetration of the skin (cercarial dermatitis), followed by broncho-pulmonary manifestations attributed to the passage of schistosomules through the lungs. Approximately five weeks after infestation, more dramatic symptoms, often known as Katayama Disease
consist of malaise, weight loss, gastrointestinal symptoms, eosinophilia and fever. They are caused by the initial deposition of eggs by female worms (Warren, 1987).

In the case of *S. mansoni*, female worms lay eggs in the mesenteric branches of the portal vein along the intestinal wall and although a relatively large part of the eggs are carried into the liver and other organs by the blood flow, the remainder of them may stay in the small venules until the embryo they contain develop into miracidia within 10 days. Antigenic substances excreted from miracidia diffuse out through submicroscopic pores in the egg shell, and elicit an acute inflammation in the surrounding tissues, resulting in the rupture of the vascular wall and escape of the eggs from the venules through the intestinal sub-mucosa and mucosa into the intestinal lumen. The inflammation causes recurrent daily fever, abdominal pain and enlarged tender liver and spleen, and discharge of eggs into the intestinal canal is accompanied by dysentery or diarrhoea (Kojima, 1998). Blood chemistry may reveal a transient elevation of glutamic pyruvic transaminase, glutamic oxaloacetic transaminase and alkaline phosphatase five to six weeks after infestation. Eosinophilia may be observed in most of the patients with or without increase of leukocyte counts. Serum level of IgE may increase as observed in other helminth infestations (Kojima *et al.*, 1972).

Chronic schistosomiasis is characterized by a series of chronic inflammatory lesions produced in and around blood vessels and tissues by the eggs or their product (Nash, *et al.*, 1982). The chronic manifestation in *S. mansoni* infestations is characterized by hepatosplenomegaly, although development of polyps or mucosal proliferation of the
intestine may also be observed in most cases. Egg granulomas are replaced by fibrotic tissues, which are prominent in the periportal areas and lead to the development of pipestem fibrosis (Von Lichtenberg et al., 1971). Hepatosplenic schistosomiasis refers to the major complication of chronic infestation with *S. mansoni*. It is usually, but not necessarily associated with enlargement of the liver and spleen (DeCock, 1986).

The liver is inevitably involved in intestinal schistosomiasis, but the extent of such involvement depends on many factors such as intensity and duration of infestation which are mainly responsible for the changes produced. The liver gradually decreases in size, but increases in hardness as fibrosis is gradually extended into the parenchyma, resulting eventually in liver cirrhosis in severe cases. The enlarged spleen may reach the level of the umbilicus or even at times expand to fill most of the abdomen (Kojima, 1998). End stage hepatosplenic schistosomiasis may be complicated by features of hepato-cellular failure, ascites often being the most obvious clinical sign. While this may all result from severe schistosomiasis, the possibility of other coexistent liver disease must be considered. The portal hypertension of schistosomiasis is presinusoidal and presumably related to the portal zone reaction (Coutinho, 1968). In advanced schistosomiasis, hepatic arterial hypertension contributes to increased sinusoidal pressure (Alves et al., 1977). Retrograde flow develops in the portal vein. Hepatic blood flow is not significantly reduced. At the stage when haemorrhage occurs from varices, the granulomatous reaction may have subsided and the picture is predominantly that of fibrosis (Sherlock, 1989).
Portal hypertension is considered present when the portal vein pressure is raised to 5 mmHg above inferior vena caval pressure, when the intra-splenic pressure is above 15 mmHg, or when the portal vein pressure measured directly at surgery is above 30 mmHg (Boyer, 1982). While portal hypertension is a prerequisite for the development of a collateral circulation, in cirrhosis the risk of bleeding cannot be directly correlated with the exact portal vein pressure, although haemorrhage is unlikely in cases where the portal vein pressure is less than 10 mmHg above inferior vena caval pressure (Lebrec et al., 1980). Cirrhosis is defined anatomically as a diffuse process with fibrosis and nodule formation (Anthony et al., 1977).

Urinary schistosomiasis is caused by *S. haematobium* and affects the genito-urinary system. The stage of oviposition is manifested by genitourinary trouble such as cystitis, dysuria with terminal haematuria, dull suprapubic pain, spermatorrhea and haemospermia. Spontaneous recovery is rare and the condition may be complicated by the bladder ulcer, calculi, polyps, fistulae, hydroureters or hydronephrosis or carcinomatous changes of the bladder (Cook, 1980). The association of bladder cancer with *S. haematobium* has been discussed in the context of the involvement of urinary tract infestations by species of nitrate-reducing bacteria. The urine of patients infected with *S. haematobium* contained higher levels of nitrosamines, in association with nitrate-reducing bacteria, than the urine of either Egyptian or German controls, and this may result in the endogenous formation of carcinogenic N-nitroso compounds in the urine (Mostafa. et al., 1999). The involvement of gynaecological organs may be observed in *S. hematobium* infestation.
As a disease entity, female genital schistosomiasis has been neglected, despite the fact that vaginal schistosomiasis was reported from Egypt as early as in 1899. It has generally been considered that the presence of *S. haematobium* eggs is not as common in female genital organs as in male genital organs, although in the female lesions are found in the vulva, vagina, cervix and less commonly the ovaries, fallopian tubes or uterus (Wright *et al*., 1982). However, *S. haematobium* may migrate through the network of female pelvic vasculature during puberty and especially during pregnancy make ectopic localization of the parasites possible (Feldmeier *et al*., 1995). Because sexually transmitted diseases increase the probability of HIV transmission, presumably through lesions in the genital mucosa, female genital schistosomiasis may be an important risk factor for transmission of HIV (Feldmeier *et al*., 1994).

### 2.7 Diagnosis of Schistosomiasis

Decisions on individual or community treatment, estimations on prognosis, assessment of morbidity, evaluation of chemotherapy and control measures all depend on the results from diagnostic tests. Selection and application of methods should, therefore, correspond to the type of information sought by the clinician or the epidemiologist (Feldmeier, 1993). Specific diagnosis of schistosomiasis can be made by detection of the characteristic eggs in the stools or urine under microscopic examination (Harries *et al*., 1986). In *S. mansoni*, where eggs are excreted in faeces, simple concentration and sedimentation of faecal specimens are reliable. Many concentration techniques have been described (Knight *et al*., 1976). These involve removal of fat, faecal debris and mucus and require more sophisticated laboratory facilities. They find their optimum use
in the detection of “light” infestations where few eggs are excreted or, in some cases, eggs are excreted intermittently. Currently, the cellophane thick faecal smear, the Kato technique (Komiya and Kobayashi, 1966) or one of its numerous modifications (Kartz et al., 1972, Peters et al., 1980) have become standard diagnostic tools in epidemiological studies. They are simple microscopic methods which examine about 50 mg of stool and are quantitative thus permitting comparison of data.

Infestation with all human schistosome species are efficiently diagnosed through microscopic examination of minute biopsies of the rectal mucosa (Badran et al., 1955). Snips are taken from suspicious lesions or if absent, from the plica transversalis recti. It is rarely necessary to resort to liver biopsy for diagnosing infestation with intestinal schistosomes, but where this has been done the examination of hepatic tissue in crush preparations is more efficient than sectioning of the material. The probability of aspirating tissue that contains egg granulomas is rather low in conventional and even ultrasound guided fine needle liver puncture (Farid, 1993). Moreover, diagnosis of peripheral fibrosis is made with similar efficiency by means of ultrasonography and, does not require biopsies with histological sectioning (Kartz et al., 1972).

Bladder biopsy and cystoscopy is normally not necessary except when carcinoma of the bladder is considered as differential diagnosis. In contrast, filtration of several 24 h. urine samples is commonly available in hospital and frequently leads to the detection of ova in urine (Feldmeier et al., 1983). Burki et al. (1986) demonstrated that ultrasonography compares favourably with cystoscopy and pyelography to detect
specific pathology. Indirect methods for the diagnosis depend on clinical symptoms and signs, and biochemical or immunological analyses. In diagnosis of urinary schistosomiasis, haematuria is a suggestive sign and microhaematuria or proteinuria may correlate well with the intensity of infestation in endemic areas (Savioli. et al., 1989). In intestinal schistosomiasis, the repeated presence of blood in stool is indicative of high intensity of infestation (Proiett and Antunes, 1989). Immunodiagnosis may be useful for demonstration of active or chronic schistosomiasis. A unique immunological method for the diagnosis of schistosomiasis is the circumoval precipitin (COP) test in which precipitate is formed around the eggs containing live miracidia after incubation in the serum of infected individuals (Hairstone, 1973).

The enzyme linked immunosorbent assays (ELISA) is also widely used in diagnosis. ELISAs for the detection of circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) in serum and urine have been developed and applied as an epidemiologic tool in a recent, intense focus of S. mansoni in Senegal (Polman. et al., 1995). CAA and CCA in serum and CCA in urine were found in 94%, 83% and 95%, respectively, of the population of which 91% were positive on stool examination. Circulating antigens were also detectable in sera and urine of most egg-negative individuals. The sensitivities of the urine CCA and serum CAA ELISA were substantially higher than that of a single egg count and increased with egg output. The CAA and CCA levels correlated well with egg counts and with each other. The age related evolution of antigen levels followed a similar pattern to egg counts, providing supplementary evidence for a genuine reduction
of worm burden in adults in spite of the supposed absence of acquired immunity in this exposed community (Cioli et al., 1993).

2.8 Control of Schistosomiasis

There are a large number of environmental variables involved in schistosomiasis transmission (Mott, 1987); hence no single method of control, regardless of location, has been shown to be effective. The most successful control programmes have been those that included some method of curbing transmission, including mollusciciding, even at a reduced level. Transmission control delays reinfection and extends the period between treatment, thereby reducing drug delivery costs (Webbe and El Hak, 1990). At least four approaches to controlling infection have proven effective at the community level. These are: control of snails (both molluscicidal biological control), public health education, sanitation, and community-based chemotherapy employing praziquantel (Jolcelyn, 2004).

2.8.1 Sanitation and public health education

The classic solution involving only basic sanitation and clean water supply would, in theory, be an effective method, but it is very difficult and expensive to achieve such a solution in large endemic areas. Simple methods of water supply and sanitation can reduce the prevalence and morbidity of schistosomiasis (Molgaard et al., 1999), but only in the long term if associated with permanent health education programs. According to Pitchford (1958, 1972), in some parts of Africa where water was supplied for domestic purposes and other simple and relatively cheap measures were encouraged
in order to reduce human contact with water, schistosomiasis infection rates declined progressively. In north-eastern Brazil, Barbosa et al. (1971; 1992) showed that schistosomiasis could be effectively controlled with relatively limited intervention involving community-organized sanitation programs. The case of Saint Lucia, reported by Jordan et al. (1975), is very impressive because it shows that, with simple introduction of household water; a significant reduction in human contact with water can be achieved leading to reduced schistosomiasis prevalence and incidence (Jordan et al., 1975). Control of schistosomiasis can be possible with changes in human behaviour through health education, with the improvement of the basic social and economic standards of the involved communities and with enforcement by public health services (WHO, 2004).

2.8.2 Chemotherapy

There have been great advances in chemotherapy of schistosomiasis during the past 2 decades. Compared to antimonials, which were the only available chemotherapeutic agents for schistosomiasis from the 1920s to the 1960s, new drugs are more consistently effective, less toxic and applicable to oral rather than parenteral administration, thereby making field trials of mass chemotherapy feasible (Kojima, 1998). The major anti-schistosomal drugs that have been or still are in use against infestation with schistosomes are metrifonate, oxamniquine and praziquantel and all three are included in the World Health Organization list of essential drugs (Cioli et al., 1993). The classification of anti-schistosomal drugs can now be simplified into two categories: i) the one drug of choice against all species of schistosomes infecting man (praziquantel).
ii) the other drugs effective against one species of schistosomes *i.e.* the monospecific
drugs: oxamniquine, effective only against *S. mansoni* and metrifonate, used in *S. haematobium* infestations (Davis, 1993).

Praziquantel is the newest and relatively effective drug for treating schistosomiasis
occurring in man (Andrews *et al.*, 1983). It is effective orally in a single dose (40
mg/kg) yielding 70% to 95% cure rates against all species of schistosomes infecting
man. With few significant side effects and no adverse reactions on liver, renal,
haematopoietic or other body functions, praziquantel is undoubtedly the most advanced
in anti-helminthic chemotherapy of recent decades (Becker *et al.*, 1980). The mode of
action of praziquantel is unknown. Most evidence implicates the susceptibility of
muscle and tegumental systems as important sites of action. Praziquantel’s effect on
worms is very dramatic. Worms exposed to 1µM praziquantel *in vitro* show almost an
instantaneous and sustained contraction with a half-maximal effect time of 12 seconds
(Clement *et al.*, 1989). The effect of praziquantel on worm muscle tension seems to
result from the ability of the drug to increase the permeability of the worm muscle cells
to calcium ions. Praziquantel also, causes severe destruction of the worm’s tegument
(Brindley and Sher, 1987). However, the possible existence of an *S. mansoni* isolate
tolerance to praziquantel has been reported from Senegal where the parasitologic cure
rate 12 weeks after treatment was as low as 18% (Stelma *et al.*, 1995). The tolerance of
the Senegalese isolate to praziquantel may be defined as tolerance, indicating an innate
insusceptibility of a parasite to a drug to which it has never been previously exposed
(Fallon *et al.*, 1996).
In contrast, a genetically transmitted loss of susceptibility in a parasite population that was previously susceptible to a given schistosomicidal drug has been termed resistance (Cioli et al., 1993). Indeed, in some work carried out in Egypt, where praziquantel has been extensively used, it has been demonstrated that a small percentage (1-2.4%) of villagers may harbour parasites which cannot be killed even after repeated administration of high doses of praziquantel (Ismail et al., 1996). When isolates obtained from these uncured individuals were examined in the mouse model, the ED50 values of the isolates were found to be 3 times higher than that of one reference control isolate (Fallon et al., 1996). The reduced susceptibility of *S. mansoni* to praziquantel in infected human populations has important implications for current schistosomiasis control programs. Praziquantel failure on initial treatment in *S. mansoni* endemic areas of Senegal suggests primary resistance to praziquantel (Guisse et al., 1997; Stelma et al., 1995) and a high prevalence of resistance genes in the local *S. mansoni* strain.

More recent reports indicate, however, that in this area of Senegal, retreatment after 40 days adequately reduced infection levels and achieved better cure rates (Renganathan and Cioli, 1998). The latter results suggest that immature schistosomes, which are known not to be susceptible to praziquantel, were typically present in Senegalese patients at the time of treatment. If the treated patient has been recently exposed to infection (≤6 weeks ago) in an area with continuous rather than seasonal transmission, apparent treatment failure may be observed when unaffected juvenile worms reach maturity and pass eggs several weeks after praziquantel treatment (Renganathan and Cioli, 1998).
Oxamniquine is widely used in the treatment of infestation due to *S. mansoni*. It is a well-known, highly useful drug for the treatment of all forms of *S. mansoni* infestation including many complicated syndromes (Davis, 1991). Studies show that oxamniquine irreversibly inhibits nucleic acids and protein synthesis in adult worms. Males were more susceptible than females to the drug and showed a higher degree of inhibition of protein synthesis (El-Kouni, 1992). Metrifonate, an organophosphorus cholinesterase inhibitor, is used for the treatment of urinary schistosomiasis. Metrifonate, like other organophosphorus compounds, inactivates the enzyme that destroys acetylcholine.

This action allows the chemical neurotransmitter to persist, and therefore cholinergic symptoms might be expected during treatment. The symptoms include fatigue, muscular weakness, abdominal colic, nausea, diarrhoea and vomiting. All of these symptoms are a reflection of stimulation of cholinergic synapses in the autonomic nervous system, ganglionic sites in both parasympathetic and sympathetic divisions, the neuromuscular junction and several sites in the cardiovascular system (Davis, 1991).

Studies carried out in some endemic areas in Brazil have shown that specific chemotherapy for schistosomiasis, delivered either to individuals or in mass treatment campaigns, reduces the severity of the disease's clinical forms and also temporarily reduce the prevalence of infection (Kloetzel 1963; Bina 1977; Santos 1978; Katz *et al.*, 1978; 1980). Unfortunately, the long-term worldwide application of Praziquantel coupled with the discovery of Praziquantel-tolerant schistosomes and drug resistance has generated concern over the development of drugs-resistant schistosoma strains.
(Shengliang et al., 2001; Appiah and DeVlas, 2002). However, because of frequent re-infections in endemic areas, mass treatment, even when repeated several times, cannot completely control schistosomiasis (Katz et al., 1980; Prata et al., 1980; Santos & Coura, 1986; Coura et al., 1987; Cutrim and Coura, 1992; Coura et al., 1992). As shown in figure 2.5, after the first mass treatment with oxamniquine there is a sudden drop in the prevalence of infection and in the egg load, but this is followed by rapid re-infection and egg load reconstitution, and even after five mass treatment an important residual infection remains (Coura et al., 1980; 1987).

**Figure 2.5:** Attempt to control Schistosomiasis by mass treatment with Oxamniquine. Note the initial drop in the prevalence and the rate of re-infection in 24 months. Coura et al. (1980; 1987).
2.9 Snail Control

2.9.1 Biological control

An alternative method used in the control of snail vectors is the use of predatory organisms or competitors, which can control the expansion of the snail population and eventually eliminate the snails from the breeding site (Milward and Antunes, 1969). The biological control of schistosoma vectors with microbial agents, parasites, predators and competitors has sometimes given satisfactory results (Abdallah and Nars, 1973) in the laboratory, but has failed to deliver decisive gains in the field. Biological control has been undertaken principally with snails such as *Marisa cornuarietis* (Ruiz-Tiben *et al.*, 1969) and *Pomacea haustrum* (Milward, 1972). In the northeast of Brazil a *Biomphalaria straminea* strain resistant to *S. mansoni* has been used to out-compete *B. glabrata* (Barbosa *et al.*, 1981). Another snail used as a competitor is *Helisa duryi* (Abdallah and Nars, 1973). Several competitors and predators, such as *Pomacea lineata*, *Marisa cornuarietis*, *Helisoma duryi*, *Tilapia*, among others, have been used in pilot snail control studies, sometimes leading to an initially drastic reduction of population, but with disappointing final results (Paraense, 1987).

A relatively effective biological agent against *Schistosoma mansoni* is *Ribeiroia marini*, described by Marin (1928) as Cercaria IV. It destroys the ovotestis of parasitized Biomphalarias (Harry, 1965), causing castration. Golvan *et al.* (1974) and Golvan (1978) observed natural *R. marini* infection in 90% of *B. glabrata* during the dry season in the island of Guadeloupe, with a complete disappearance of the snails in some parts of the island. Combes *et al.* (1975) showed that the common rats *Rattus rattus* and *Rattus
norvergicus are the definitive host of *C. marini* in Guadeloupe, a finding confirmed by Nassi (1978). Despite a significant decline in population, however, the snails reappeared in the following rainy season.

A number of fish species such as *Tilapia melanopleura* and *Astronotus ocellatus*, have been used to control snail (Motta and Gouvea, 1971; Feitosa and Milward, 1986) and aquatic birds such as ducks (Michelson, 1957) and chelonian (Coelho et al., 1975) have also been employed as snail predators. In addition, a number of other types of predators such as mosquito larvae and other diverse insects have also been described (Berg, 1964).

In the laboratory a small leeche, *Helobdella trise-rialis lineata* and ostracods crustacia have been found to be good snail predators (Sohn and Hornicker, 1972; Guimar et al., 1983). In the field, however, these animals are found in snail breeding sites in an ecological equilibrium with the snails. Some aquatic plants such as *Characeae* have been used to combat snail vectors (Renno, 1958). The pathological action of bacteria such as *Bacillus pinotti* against *B. glabrata* has also been studied (Texera and Vicente, 1954). However, biological control requires involvement of trained people who are familiar with the biology of the different snail species. In addition, it can take a long time for the competitor/predator organisms to reach a population density sufficient to tackle the snail host problem.
2.9.2 Medicinal plants

Medicinal plants have been used in virtually all cultures as a source of medicine since time immemorial. The widespread use of herbal remedies and healthcare preparations, as those described in ancient texts such as the Vedas and the Bible, and obtained from commonly used traditional herbs and medicinal plants, has been traced to the occurrence of natural products with medicinal properties (Chopra, 1956). The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed (UNESCO, 1996). Furthermore, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies (UNESCO, 1998). Moreover, in these societies, herbal remedies have become more popular in the treatment of minor ailments, and also on account of the increasing costs of personal health maintenance. Indeed, the market and public demand has been so great that there is a great risk that many medicinal plants today, face either extinction or loss of genetic diversity (UNESCO, 1996).

Medicine, in several developing countries, using local traditions and beliefs, is still the mainstay of health care. The practice of traditional medicine is widespread in China, India, Japan, Pakistan, Sri Lanka and Thailand. In China about 40% of the total medicinal consumption is attributed to traditional tribal medicines. In Thailand, herbal medicines make use of legumes encountered in the *Caesalpiniaceae*, the *Fabaceae*, and the *Mimosaceae*. In the mid-90s, it is estimated that receipts of more than US$2.5
billion have resulted from the sales of herbal medicines. In Japan, herbal medicinal preparations are in more demand than mainstream pharmaceutical products (Singh et al., 1996).

Africa is a rich source of medicinal plants. Perhaps, the best known species is *Phytolacca dodecandra*. Extracts of the plant, commonly known as endod, have been used as molluscicide to control schistosomiasis (Lemma, 1991). Other notable examples are *Catharanthus roseus*, which yields anti-tumour agents such as vinblastine and vincristine; and *Ricinus communis*, which yields the laxative-castor oil. In Botswana, Lesotho, Namibia and South Africa, *Harpagophytum procumbens* is produced as a crude drug for export. Similarly, *Hibiscus sabdariffa* is exported from Sudan and Egypt. Other exports are *Pausinystalia yohimbe* from Cameroon, Nigeria and Rwanda, which yields yohimbine; and *Rauwolfia vomitoria*, from Madagascar, Mozambique and Zaire, which is exploited to yield reserpine and ajmaline. The use of medicinal plants like *Eupatorium perfoliatum* (bonest), *Podophyllum peltatum* (mayapple), and *Panax quinquefolium* (ginseng) in the USA has long been associated with the American Indians. These plants have also been appreciated and recognised for their aesthetic and ornamental value. In Central America medicinal plants have been widely used - by the Maya Indians in Mexico, the Miskitos and Sumus in Honduras and Nicaragua, the Pech, Lencas, and Xicaques in Honduras, the Pipiles in El Salvador, the Talamancas in Costa Rica, and the Guaymis and Kunas in Panama (Singh et al., 1996).
In Europe, some 1500 species of medicinal and aromatic plants are widely used in Albania, Bulgaria, Croatia, France, Germany, Hungary, Poland, Spain, Turkey, and the United Kingdom. The Maltese islands constitute an apt example where medicinal plants are widely used in every day life as part of folk medicinal remedies (Lanfranco, 1992).

2.9.3 Molluscicides

The use of molluscicides as one of the strategies to control schistosomiasis began in Brazil in 1976, with the creation of the Special Schistosomiasis Control Program by the National Health Foundation (Machado, 1982). The product used in the program was niclosamide, an ethanolamine salt of 2', 5-dichloro-4'-nitrosalicylanilide, manufactured under the trade name Bayluscide®, whose efficacy had previously been established (Gonnert, 1961). Selective molluscicide treatment in snail-infested bodies of water at main human contact points is the preferred way to approach controlling snail populations.

2.9.3.1 Synthetic molluscicides

Metallic salts, such as copper sulfate, were among the first agents sed, and were most effective when applied to standing bodies of water (Bailey et al., 1989). Copper sulfate worked well enough, but it also limited algal growth, that in turn affected growth patterns of fish that served as primary sources of protein. Newer molluscicides, such as nicotinanilide, organotin, dibromo-nitraozo-benzene, sodium pentachlorophenate, tritylmorpholine, sodium dichloro-bromopheno, niclosamide, and acetamide analogs replaced copper sulfate, as these were deemed safer to the environment (Agarwal and Singh, 1988).
While niclosamide is biodegradable, its “side effects” included the death of many fish species, as well as the targeted snail populations. It acts by depleting glycogen stores, and is the drug of choice for some adult tapeworm infections in humans. It also used be a drug of choice for schistosomiasis, but too many people suffered from the same side effect of depletion of glycogen stores. This led in some cases to coma, an unacceptable outcome of treatment. Its use is limited by cost, as well (Kloos and McCullough, 1982).

Synthetic organic molluscicides have been widely used for the control of harmful snails (Agarwal and Singh, 1988). It was realized that these molluscicides are toxic to non-target animals and have a long-term detrimental effect on the aquatic environment (US Congress, 1976; Singh et al., 1996). Synthetic molluscicides have proved too expensive for most countries wanting to include snail control in their anti-schistosomiasis programmes. Other setbacks of using synthetic molluscicides to control Schistosomiasis include: need for repeated applications since snail eradication is difficult, implementation and evaluation of control is time consuming and technical capacity is required to decide appropriate application procedures in view of the great variation in transmission sites (US Congress, 1976). An alternative, which is not only cheaper but also promotes self-reliance and empowerment of the affected communities, is the use of molluscicidal plants

2.9.3.2 Plant molluscicides

The use of natural products in health management throughout the world is increasing, as well as the interest for research in this area. Molluscicides of plant origin have become
the focus of attention since they are less expensive and less hazardous to the environment than their synthetic counterparts (Agarwal and Singh, 1988). The study of plants exhibiting snail toxicity has been encouraged with the aim of finding alternative for use in the fight against snail vectors. Plants and compounds with molluscicide activity were reviewed by McCllough (1992), and have shown to exhibit low toxicity for snails’ embryos. For instance, both the flowering parts and the fruits of *Ammi majus* (Umbilliferae) have been shown to have marked molluscicidal activity against *Biomphalaria alexandrina* snails. Upon histopathological examination of the hermaphrodite gland of *Biomphalaria alexandrina* snail vectors, this plant was shown to deprive most of the gland acini at different stages of gametogenesis. The plant was also shown to decrease the hepatopancreas aminotransferase and alkaline phosphatase activities as well as to adversely affect glycogen stores of the snail vectors (McCllough, 1992)

However, some questions have arisen in the selection of plant molluscicides, such as: toxicity, availability, annual growth, adaptability to different local conditions, and location of molluscicidal activity in parts of the plant that easily regenerate, such as the leaves. To be eligible for use, a product must be storable and remain viable for at least one year; be physically and chemically stable, have ethnobotanical value, and be easy to extract and apply, preferably in aqueous extracts (Kloos and McCullough, 1982).
2.9.4 Antischistosomal Plants

Many community-based programmes depend on Praziquantel for treating patients with schistosomiasis and other fluke infections. There is however, loss of Praziquantel efficacy (Charles et al., 2000) and its inaccessibility (Anthony et al., 1994), drug resistance and high cost requiring hard currency (Kloos and McCollough, 1982) which set back helminthes control efforts. There is the need to screen local plants as other alternative source of schistosome chemotherapeutic agents. This claim is due to the frequent use of plants by most people in the third world countries in primary health care (Fransworht, 1993; Houghton, 1995). Plants are known to offer excellent perspective for the discovery of new therapeutic products (Cox and Balik, 1994) whose subsequent development may lead to discovery of a safe and therapeutically effective form of useful drugs (Philipson, 1994). In several trials using different plants, promising antischistosomal compounds were reported by several workers (Aliou et al., 1986; Canxi and Enst, 1982; Kucera and Kucerova, 1975; Per et al., 2001; Istifanus and Adamu, 2001).

In Mali, the Office du Niger area constitutes the main zone of schistosomiasis transmission where both Schistosoma mansoni and Schistosoma haematobium are encountered. An ethnopharmacological survey, using questionnaire, was conducted in the Office du Niger area of the Niono District to determine the plants used against schistosomiasis amongst traditional healers. Forty healers from 21 villages of six different health areas were interviewed. Fifty-five plants belonging to 30 families were reported to be used alone for treating urinary and intestinal schistosomiasis, while nine
combinations of plants were used against the urinary form of the disease. *Cissus quadrangularis* and *Stylosanthes erecta* were the plants most frequently used and were reported for the first time, to be used against schistosomiasis in Mali (Lemma, 1991). *Nigella sativa* seeds oil was found to have antihelmintic activity against *S. mansoni* infection (Mahmoud et al., 2002). Similar results have been recorded on the effect of other plants against schistosomes at different stages; cercariae, schistosomula and adult worms (Naples et al., 1992; Ahmed and Ramzy, 1997; Molgaard et al., 2001; Lyddiard et al., 2002).

In addition, Mahmoud et al. (2002) found that treatment of mice infected with *S. mansoni* parasite, by using black *N. sativa* seed oil, was effective in reducing egg count in both liver and intestine. Also, goyazensolide, a natural compound isolated from the plant *Eremanthus goyazensis*, showed a significant inhibitory effect on egg laying capacity of *S. mansoni* female worm during in vitro cultivation (Barth et al., 1997). The promising results obtained from plants, encourages the screening of more plants for their antischistosomal activity so as to come up with more natural products that are effective schistosomicides (Lewisa and Lewis, 1977). Thus, a good substitute to the current singularly used synthetic anti-chistosomal drug, Praziquantel, may be obtained for chemotherapeutic treatment of Schistosomiasis especially in rural communities where these plants abound. It therefore becomes very important to screen and evaluate the potency of some local plants that are active on parasitic organisms such as schistosomes (WHO, 2004).
2.9.5 Plants used in this study

- *Aloe secundiflora* (AS): The vernacular name is kiluma (Kamba). The expressed leaf sap is used as an antiseptic, disinfectant and a wound healing agent. The root decoction treats colds, Newcastle disease in poultry, labour pains in women, and treats insect bites. Heated leaves are applied to various body parts as pain killers (Kokwaro, 1993; Beentjie, 1994).

![Aloe secundiflora plant growing in Makueni district](image)

*Figure 2.6 Aloe secundiflora* plant growing in Makueni district

- *Aspilia pluriseta* (AP): The vernacular name is wuti (Kamba). It is used to cure gastrointestinal diseases, stomach pain, diarrhoea, and constipation. It is also used to treat snakebites. The leaves are chewed and then rubbed between the palms into a ball which is applied to the bite. During this procedure, the patient keeps the eyes
shut. It is believed to possess anti-giardial activity. The plant leaves are used as mosquito repellents. This study used flowers and the leaves of *Aspilia pluriseta*.

![Aspilia pluriseta plant](image)

**Figure 2.7** *Aspilia pluriseta* plant growing in Kibauni area of Makueni district

- **Psidium guajava (PG):** The vernacular name is Kivela (Kamba). The roots are used as a slow acting medicine in stomach troubles and headaches. A bark decoction is mixed with milk and given to children as a tonic. Pounded young leaves are mixed with ghee and rubbed on skin to cure skin diseases. Roots may be pounded and soaked in water, or boiled and the infusion drunk for indigestion. Macerated roots and leaves are used for treatment of hepatic diseases (Kokwaro, 1993).
• *Balanites aegyptiaca* (BA): The vernacular name is Kilului (kamba). Root bark is used traditionally to wash clothes because it produces foam. The roots are cleaned and boiled and the brew taken as such or mixed with soup. Its alleged to cleanse blood. Traditionally, this preparation may be used also to prevent ‘loss of male strength’ that is to prevent loss of libido. It is also recommended as a remedy for headache, urinogenital infections and urethral pain (Personal communication, 2006).
Figure 2.9 *Balanites aegyptiaca* plant growing in Mbumbuni-Makueni district

- *Azadirachta indica* (AI): The vernacular name is *mwaluvaini* (Kamba). Boiled leaves and roots are used to treat malaria and cure joint pain. Leaves, roots’, stems’ and barks’ decoction is used to diminish the fever in children and pregnant women. The plant has also been used to cure amoebas, bilharzias and small intestinal worm infestation in children (Kokwaro, 1993).
- *Amaranthus hybridus* (AH): The vernacular name is musavula (Kamba). The leaves are employed as an inhalant for fever. The flowers are pounded, water added and the resultant red liquid is drunk as remedy for diarrhoea and dysmenorrhoea. The seed oil of *Amaranthus hybridus* is used in traditional medicine for its antidiabetic, spermicidal, antifertility, antibacterial, and wound healing properties (Molgaard *et al.*, 1999).
Figure 2.11 *Amaranthus hybridus* plant growing in Makueni district
CHAPTER THREE
MATERIALS AND METHODS

3.1 Preparation of plant extracts

3.1.1 Collection of medicinal plants

The plants used in this study were collected from their natural habitats on the basis of ethnobotanical information. They were collected with bio-conservation aspects in mind from Kisau location, Makueni County. An acknowledged authority in taxonomy from the National Museums of Kenya (NMK) authenticated the botanical identity of the plants and a voucher specimen was deposited at the National Museums of Kenya Herbarium, Nairobi. A traditional medical practitioner provided the information on which plant to collect, what part to collect, the precise locality where it grows, when curative potency is maximal and the mode of preparation.

3.1.2 Plant parts collected and initial preparation for extraction

The plant parts collected for use in this study were: leaves of Aloe secundiflora (AS), roots of Balanites aegyptiaca (BA), leaves and flowers of Aspilia pluriseta (AP) and Amaranthus hybridus (AH), and roots and leaves of Psidium guajava (PG) and Azadirachta indica (AI). The stems and roots were harvested and their barks peeled off while still fresh and cut into small pieces and then dried at room temperature for different periods of time depending on the succulence of the plant materials. Leaves were collected while green and dried in the same way. The root barks, stem barks and the leaves were separately ground when completely dry using an electric mill at The Technical University of Kenya (formerly Kenya Polytechnic). The powdered plant
materials were kept at room temperature away from direct sunlight in closed dry plastic bags.

3.1.3 Extraction procedure

Plant extraction was carried as per method described by Kofi-Tsekpo et al. (1985). A hundred grammes of each powdered plant material were boiled in one litre of distilled water for 2 h. After boiling, the solution was allowed to cool. The extract was then decanted into a 1-litre clean dry conical flask and filtered through a No. 1 Whatman filter paper under vacuum pump into a 500mL clean dry conical flask. Decantation and filtration processes were repeated until the sample became clear. The filtrate was freeze-dried, weighed and stored in an airtight container at room temperature until it was used for bioassay.

3.1.4 Preparation of extracts for molluscicidal bioassay

The plant extracts for testing molluscicidal activity were prepared using the procedure described by Ndamba and Chandidawa, (1989). A stock solution of 80 ppm was prepared for each of the plant preparations. This was achieved by weighing separately, 80 mg of the freeze-dried plant materials, which was placed in 1000 mL of chlorine free water and boiled for 5 minutes. This gave 80 mg/L that is 80ppm. Half of the prepared plant extract (0.5L) was frozen and stored at -20°C and the remaining stock was diluted to obtain concentrations of 40 and 10 mg/L for each preparation.
3.2 Laboratory animals

3.2.1 Collection, screening and maintenance of the snail intermediate host

*B. pfeifferi* snails were collected from Mwea Irrigation Scheme in Mbeere District. They were screened for schistosomes under strong light (100 watts) for 2h for six consecutive weeks to exclude infections from the wild. Negative snails were housed in temperature controlled (25-27ºC) and light-controlled (12h light /12h dark) snail room at the Institute of Primate Research (IPR). They were placed in water tanks containing non-chlorinated tap water and the water was changed twice a week. Soft lettuce (dipped in hot water and cooled) was added to feed the snails.

3.2.2 Maintenance of definitive host (BALB-C mice)

BALB/c mice are permissive definitive hosts of *S. mansoni*. They were bred at the Animal Resources Department, Rodent facility at IPR. They were fed on nutrient pellets (Laboratory chow, Unga Feeds ® Co.) and supplemented with carrots and kale leaves. Water was supplied to animals *ad libitum*.

3.2.3 Isolation of miracidia

Faecal samples were collected from, *Papio anubis* (Olive baboons) with chronic *S. mansoni* infection that are maintained at Animal Resource Department at IPR. The faecal material was thoroughly mixed with PBS (x1) in a plastic jar and the slurry was poured on a standard test sieve (Arthur Thomas Co. USA) of 600 μm placed on top of another test sieve of 125 μm. A collecting tray was placed under the 125-μm sieve. Chlorine free water was poured onto the faecal sample and after sieving the debris was
discarded. The faecal suspension from the collecting tray was poured into urine glass jars, which were then placed in the dark and left to sediment for at least 30 minutes. The supernatant was poured out and the pellet re-suspended in water and then allowed to sediment in the dark. The procedure was repeated three times until the supernatant was clear. The sediment was transferred into a Petri dish and placed under a 100 watts lamp for 30 minutes. Freely swimming larvae (miracidia) were hatched from the eggs as observed under a dissecting microscope. They were used to infect the snails.

3.2.4 Infection of snails and isolation of cercaria

Six to eight miracidia were picked from the Petri dish under dissecting microscope using a drawn out glass pipette mounted on a rubber bulb. The miracidia were then dispensed into each well of a 24 well culture plate (Nunclon, Denmark). Biomphalaria pfeifferi snails were transferred individually into each of the wells containing miracidia. The plates were then covered with their lids to prevent the snails from crawling out. The plates were left for 30 minutes to allow for miracidia penetration. The snails were transferred into newly prepared snail tanks and left for 5 weeks; one week before end of this period, they were placed in the dark.

Fifty infected snails were removed from the dark and transferred to a 100 mL glass beaker containing 20 mL of water. They were illuminated with 100 watts lamp for one hour in order to shed cercariae for the infection of mice. The number of cercariae infected per snail was determined as follows: A 20µL aliquot cercariae suspension was picked and drops put into a 1cm squared ruled Petri-dish. The drops were covered with Lugol’s iodine to stain the cercariae to make them easy to count. Cercariae in three such
aliquots were counted. The average number of cercariae was then calculated. This number was then used to determine the volume that would contain 200 cercariae.

3.2.5 Infection of mice with *S. mansoni* cercariae

Mice were anaesthetized intra-peritoneally with ketamine/xylazine (20:1) mixture made by adding 0.5 mL of xylazine to 9.5 mL of ketamine) mixture. Once unconscious, the mice were shaved on the stomach area and arranged on a wooden infecting rack. Cotton wool was dipped in water and used to clean and wet the shaven area to allow easy penetration of the cercariae. 1 cm diameter metal rings were placed on the shaven area and a suspension containing 200 cercariae was dispensed into the metal ring using the 1mL micropipette. Cercariae were allowed 30 minutes to penetrate.

3.3 Evaluation of molluscicidal activity

This objective was achieved using the method of WHO (1965) and Duncan and Sturrock (1987). Fifty snails were exposed to each extract in triplicate at concentrations of 80, 40 and 10 ppm in a total volume of 200 mL chlorine-free water for 24 hours. Fifty other snails were also exposed to 200 mL of dechlorinated water for 24 hours as control. After exposure, the snails were washed, placed in fresh non-chlorinated water, provided with lettuce to feed on, and allowed a recovery period of 24 hours after which mortality was accessed by probing the snails with a wooden spatula to elicit typical withdrawal movements. Death was ascertained by observing the snails under a dissecting microscope for any heart activity. The number of dead snails was recorded.
3.4 Evaluation of antischistosomal activity

The antischistosomal activity of the plant extracts was determined as follows:

3.4.1 Dosage administration to the mice

Extracts at doses of 50mg per kg body weight (50mg/kgbw), 100mg per kg body weight (100mg/kgbw) and 300mg per kg body weight (300mg/kgbw) were given twice to *S. mansoni* infected mice *per ose* at a two days interval. This was achieved by introducing a yellow tip (fixed on micropipette) into the mouth of the mouse. The micropipette was then gently pressed to release the drug, which was swallowed by the mice. Each dose was given to five mice. Five mice were given normal saline to serve as infected control (IC); five mice were given praziquantel at 450mg/kgbw to serve as positive control (PZQ) and five uninfected mice were kept untreated in order to serve as not infected control (NC) for immunological tests.

3.4.2 Serum preparation

On week six post infection, the 5 mice from each group were anaesthetized with 0.02 mL per 30 g body weight Ketamine/Rompun® (2:1) mixture. A transverse mid-ventral nick was made on the skin of the abdomen using a pair of scissors and the skin peeled off upwards and downwards. The thoracic cavity was opened and ribs on either side of the sternum snipped off leaving the two veins on either side of the sternum intact. The left ventricle was located and a 1 mL syringe inserted. Blood was drawn in small jerks in order to create a vacuum in the syringe and to prevent collapsing of the heart. About 0.5 mL of blood was collected. The blood collected from each group of five mice was
pooled together in centrifuge tubes and allowed two hours to clot at room temperature. After clotting, it was left overnight at 4°C. The following day, it was spun in a centrifuge at 1000g revolutions per minute for 20 minutes to obtain serum. The sera was then transferred to clean Eppendorf tubes and stored at -20°C ready for analysis.

3.4.3 Gross pathology

The abdominal wall was opened up without cutting the viscera. Gross pathology of the mice livers and spleens was observed. Observations included: liver enlargement, adhesions and presence of granulomas. The gross pathology of the liver was then subjectively categorized as normal (no granulomas), few (≤3 granulomas per lobe), moderate (3-10 granulomas per lobe), and severe (more than 10 granulomas per lobe).

3.4.4 Worm burden determination

The worm burden determination was done using a modified method of Yole et al. (1996). The mice were perfused to recover the adult worms as follows: the hepatic portal vein was located and cut. A perfusion needle containing perfusion fluid (a mixture of 0.85% sodium chloride and 1.5% sodium citrate) was inserted in the left ventricle of the heart and perfusion carried out until the liver and the mesenteries were clear. Briefly, the perfusate was collected in glass Petri-dish (20 cm in diameter) and then transferred to urine jars to settle. After settling, the supernatant was carefully sucked out. Phosphate buffered saline (PBS) was dispensed into the urine jars and the worms allowed to sediment before sucking out the supernatant. This process was repeated until the perfusate became clear. Once clear, the supernatant was discarded and worms transferred to a 10 cm plastic Petri-dish, ruled at the bottom, on the outside for
ease of counting. The worms were counted and the mean and SEM. for each group calculated. The livers were collected and fixed in 10% buffered formalin ready for histological processing.

3.4.5 Histopathological examination of the liver tissues

The liver tissues were dehydrated using ethyl alcohol, and embedded in paraffin wax. Sections, 7 µm thick, were cut using a rotary microtome. Tissue sections were placed on slides and stained with haematoxylin and counter-stained with eosin. Slides were observed under X25 and X40 objective lenses. The number of granulomas was noted and the sizes established by measuring the length and width of each granuloma with a centrally placed schistosome egg, and then getting the average (Farah et al., 2000). Ten granulomas were measured for each of the infected groups. Microscopic examination was also done to check for immune cell infiltration, fibrosis and periportal infiltrations.

3.4.6 Determination of immunological responses

3.4.6.1 Schistosome Worm Antigen Preparation (SWAP)

Adult *S. mansoni* worms were obtained from infected baboons perfused at week five post-infection. The worms were washed thoroughly in PBS, placed in a tube containing PBS, and sonicated 923 KHZ, 16 µm amplitude for 10 minutes and the homogenate centrifuged for 1 hour at 100 000 g at 4°C to obtain the soluble protein. The protein estimation was done based on the Bio-Rad method of Bradford (1976). This method utilizes bovine serum albumin (BSA) as a standard protein. The optical densities of serial dilution of BSA were read at 595 nm using a Cecil spectrophotometer. Optical densities of different dilutions of SWAP were then calculated using the equation of the
curve given by the spectrophotometer (Cecil Instruments CE 6600R, England). The protein was aliquoted and sterilized by exposure to UV light (10 minutes, 5 cm from a 30 watt ultra violet OSRAM bulb) before use in *in vitro* assays. The aliquots were stored at \(-70^\circ C\). Protein concentration was adjusted to 100\(\mu\)g/mL in PBS before use in *in vitro* assays.

### 3.4.6.2 0-3 hr Antigen Preparation

*S. mansoni* cercaria were shed from infected snails with a patent infection of five weeks. Heads and tails of the cercariae were separated and the heads isolated on a discontinuous percoll gradient and washed three times in complete media RPMI 10 (RPMI 1640, 10% foetal serum, 0.1% gentamycin and \(5 \times 10^{-5}\) \(\beta\)-mecarptoehtanol). Gradient for separating heads and tails was prepared using two concentrations of percoll in RPMI 1640: 70% and 45%. The 45% percoll was layered over the 70% percoll to create a discontinuous gradient. Cercarial suspension was chilled for one hour in the cold room (\(4^\circ C\)) to make cercariae settle at the bottom of the beaker. A 5% glucose solution was made in double distilled water. Excess water from the chilled cercarial suspension was discarded and the cercariae re-suspended by gently sucking in and out using a glass Pasteur pipette. The cercariae were placed in chilled glass tubes and centrifuged for 10 seconds at 100 g. The supernatant was sucked out and 0.5 mL of glucose added. The chilled cercariae were vortexed for 90 seconds to separate the heads from the tails. The separated heads and tails of cercariae were dispensed gently on the percoll gradient using a Pasteur pipette and centrifuged at 450 g for 10 minutes. The tails formed a band at inter-phase of the gradient while the heads formed a band on top
of the inter-phase. These heads were aspirated and washed three times in RPMI/10. The heads were re-suspended in RPMI/10 and transferred to bijou tubes and incubated at 37°C, 5% CO₂ for 3 h. After 3 h of incubation, the suspension was centrifuged for 10 minutes at 450 g at 37°C. The supernatant containing the proteins released by penetrating schistosomula between 0-3 hours of penetration was obtained and aliquoted in cryovials and then the protein concentration determined. The protein was sterilized by exposure to ultraviolet light for 10 minutes by placing the vials 5 cm from a 30 watt ultraviolet OSRAM bulb. Aliquots were stored at 20°C until use in in vitro assays.

3.4.6.3 Serum IgG level determination

Specified wells of Nunc-Immuno™ plates (MaxiSorp™ Surface) ELISA plates were coated overnight with 10 µg/mL SWAP and others with 0-3 hr antigen, diluted in bicarbonate buffer pH 9.6. The plates were incubated overnight at 4°C. The antigen was dispensed off on a blotting paper. The plate was washed six times using the washing buffer (0.05% Tween 20 in PBS). This was followed by blocking off the non-specific binding sites with 100 µl 3% BSA in PBS for 1 hour at 37°C and washing off unbound BSA six times with washing buffer. Dilute (1:20) serum samples (50 µL) were dispensed into different wells in duplicates, incubated for 1 hour at 37°C and then washed as above. After washing the unbound serum, 50 µL of 1:2000 peroxidase conjugated rabbit anti-mouse IgG was dispensed in all the wells and incubated for 1 hour at 37°C. The unbound conjugate was washed off as before. After washing off the unbound conjugate, 50µL orthophenyldiamine substrate (0.4µg/mL) in citrate buffer
was added to each of the wells, and the plates incubated at 37°C in the dark for 30 minutes. Optical density was read at 630 nm in an ELISA microplate reader.

3.5 Data Analysis

The experimental data was analyzed using unpaired Student t-test. This test determined whether there were any significant differences between the data obtained from animal groups treated with plant extracts and animal groups treated with Praziquantel/Niclosamide and infected controls. A value $p<0.05$ was considered significant. Instat statistical computer package was used.
CHAPTER FOUR

RESULTS

4.1 Molluscicidal effect of the plant extracts

The efficacy of the plant extracts against *B. pffeiferi* was evaluated by counting the number of dead snails in the treated and untreated water. The results were expressed as mean total ± S.E as shown in table 4.1. The Niclosamide treated (positive control) group had the highest number of deaths (48.67±0.7) while dechlorinated water had the lowest (2.3±1.9). All plant extracts had significantly higher snail deaths (t-test; \(p \leq 0.05\)) than dechlorinated water at all concentrations. AH at 80ppm had similar value of snail mortality to Niclosamide (t-test; \(p = 0.05\)). The rest of the plant extracts had snail mortality significantly lower than Niclosamide (t-test; \(p > 0.05\)) but higher than dechlorinated water (t-test; \(p < 0.05\)). Mortality increased with increasing concentration of the extract for all the plant extracts. Snails exposed to AH and Niclosamide were weak and could not retract into their shells. Excess mucus secretion was observed and the snails hardly fed. They attempted to crawl out of the test solution.
Table 4.1: Mean total *B. pffeiferi* mortality after treatment with crude aqueous extracts of *Aloe secundiflora, Aspilia pluriseta, Psidium guajava, Balanites aegyptiaca, Azadirachta indica* and *Amaranthus hybridus*

<table>
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<th>GROUP</th>
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<th>AS</th>
<th>AP</th>
<th>PG</th>
<th>BA</th>
<th>AI</th>
<th>AH</th>
<th>Niclosamide</th>
<th>Dechlorinated water</th>
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</thead>
<tbody>
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<td>10ppm</td>
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<td>7.6±1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.7±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.3±3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.3±4.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.3±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>9.6±2.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>44.0±2.3&lt;sup&gt;a&lt;/sup&gt;</td>
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</tbody>
</table>

All values are expressed as Mean±SEM for FIVE animals per group. *P < 0.05 for extract versus Niclosamide by T-test; *P < 0.05 for extract versus IC by T-test. AS - *Aloe secundiflora; AP - *Aspilia pluriseta; PG - *Psidium guajava; BA - *Balanites aegyptiaca; AI - *Azadirachta indica; AH - *Amaranthus*
4.2 Antischistosomal effect

4.2.1 Worm recovery

The efficacy of the plant extracts against *S. mansoni* in BALB-C mice was evaluated by comparing the number of worms recovered from treated and untreated mice. The worms recovered by portal perfusion at week 6 after infections were counted and results expressed as mean total ± S.E as shown in table 4.2. The PQZ treated (positive control) group had the lowest worm count (4.8±1.3) while IC had a worm recovery of 24.4 ± 2.3. The plant extracts that had similar or higher (t-test; p ≤ 0.05) worm recovery than IC were: AS$_{50}$, AS$_{100}$, AP$_{50}$, AP$_{100}$, PG$_{50}$, BA$_{50}$, BA$_{100}$, AI$_{50}$ and all concentrations of AH. The worm recovery in AH$_{300}$ was significantly higher than IC (t-test; p < 0.05).

AI$_{300}$ had similar value of worm recovery to PZQ (t-test; p = 0.05). The rest of the plant extracts had worm recovery significantly lower than IC (t-test; p > 0.05) but higher than PZQ (t-test; p < 0.05). They included AS$_{300}$, AP$_{300}$, PG$_{100}$, PG$_{300}$ and BA$_{300}$. The total worm recovery decreased with increasing concentration of the extract for all the plant except AH whose worm recovery increased with increasing concentration of the extract.
Table 4.2: Mean total worm recovery from *S. mansoni* infected mice after treatment with crude aqueous extracts of *Aloe secundiflora, Aspilia pluriseta, Psidium guajava, Balanites aegyptiaca, Azadirachta indica* and *Amaranthus hybridus*

<table>
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<th>Dose</th>
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<th>PG</th>
<th>BA</th>
<th>AI</th>
<th>AH</th>
<th>PZQ</th>
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</table>

All values are expressed as Mean±SEM for FIVE animals per group. *P < 0.05 for extract versus PQZ by T-test; aP < 0.05 for extract versus IC by T-test. AS - *Aloe secundiflora; AP - Aspilia pluriseta; PG - Psidium guajava; BA - Balanites aegyptiaca; AI -Azadirachta indica; AH - Amaranthus hybridus*
4.2.2 Gross pathology and histopathology of the infected liver

Upon examination of the sectioned and processed tissues, the observations made for mice exposed to the six plant extracts tested for schistosomicidal effects are described below and the obtained photographs of the tissues are compared to the normal tissues. The findings were divided into gross pathology and histopathology.

4.2.2.1 Gross pathology

Detailed gross pathology is shown in table 4.3. The gross pathology was categorized as few (0-3 granulomas per lobe), moderate (3-10 granulomas) or severe (> 10 granulomas). In the group treated with PZQ, all the livers were not inflamed and there were no adhesions. All mice in the IC group had granulomas; 3 had few and 2 had moderate granulomas. All their livers were inflamed and had adhesions.

All mice treated with various plant extracts showed none, few or moderate granulomas. All the livers for the groups treated with plant extracts were inflamed except for AS$_{50}$ and AS$_{100}$ and majority had adhesions.
Table 4.3: Gross pathology on livers of groups of *S. mansoni* infected mice subjected to different concentrations of extracts of *Aloe secundiflora*, *Aspilia pluriseta*, *Psidium guajava*, *Balanites aegyptiaca*, *Azadirachta indica* and *Amaranthus hybridus*.

<table>
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<tr>
<th>Group extract/conc.</th>
<th>Granuloma status</th>
<th>Observations</th>
<th>Adhesions</th>
<th>Inflammation</th>
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<td>None</td>
<td>Few</td>
<td>Moderate</td>
<td>Severe</td>
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<td>4</td>
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<td>-</td>
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<tr>
<td>IC</td>
<td>-</td>
<td>3</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>PZQ</td>
<td>5</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

Legend: 1-5 indicates the number of mice found to have granulomas at varying degree of severity for each dose.
4.2.2.2 Histopathological examination of the liver

Histopathological examination of liver tissues from the mice treated with PZQ appeared normal but with slight peri-portal infiltration (figure 4.1 A). Liver section from the group of mice treated with PG, AP$_{300}$ and AI extracts showed small egg granulomas, surrounded by some mononuclear inflammatory cells and few eosinophils (figure 4.1B). The sections from the infected and untreated mice showed large portal egg-granulomas, formed of central ova surrounded by large number of eosinophils, neutrophils and histiocytes (figure 4.1C).

Ten liver granulomas were counted and their diameters were measured using graduated eyepiece lens, considering only lobular granulomas containing central ova. Two perpendicular maximal diameters were measured, getting the mean diameter for each granuloma, and then calculating the mean granuloma diameter for the group. The mean granuloma sizes for the PZQ group and negative control were 5.6±1.12 and 26.4±3.2 respectively. The plant extracts whose mean granuloma sizes were equal (t-test; p≤0.05) or greater than that of IC were AS$_{50}$, AS$_{100}$, AP$_{50}$, AP$_{100}$, BA$_{50}$, and all the concentrations of AH. Those whose mean granuloma sizes were equal or less than PZQ include only AI$_{300}$. Plant extracts whose mean granuloma sizes were intermediate between IC and PZQ were AP$_{300}$, all concentrations of PG, BA$_{100}$ and BA$_{300}$. All extracts showed a decreasing mean granuloma size with increasing concentration of plant extract except AH which had very slight increase in mean granuloma size in the successive concentrations.
Figure 4.1: Histopathological examination of control infected and treated mice using Haematoxylin and Eosin stain. 

A: Section in mouse liver infected by *S. mansoni* cercariae and treated using PZQ, showing slight infiltration round portal triad (X400). Vn = Vein; Ar = artery; Bd = bile duct.

B: Section in mouse liver infected by *S. mansoni* cercariae and treated using PG, showing smaller egg granuloma formed around ova in the center, surrounded by some mononuclear inflammatory cells and few eosinophils (x 400).

C: Section in mouse liver infected with *S. mansoni* and untreated showing a large portal egg-granuloma, formed of central ova surrounded by large number of eosinophils, neutrophils and histiocytes (x 400).
Table 4.4: Mean granuloma sizes for the various groups of mice subjected to different concentrations of extracts of *Aloe secundiflora*, *Aspilia pluriseta*, *Psidium guajava*, *Balanites aegyptiaca*, *Azadirachta indica* and *Amaranthus hybridus* on *S. mansoni* infected mice

<table>
<thead>
<tr>
<th>Dose</th>
<th>AS</th>
<th>AP</th>
<th>PG</th>
<th>BA</th>
<th>AI</th>
<th>AH</th>
<th>PZQ</th>
<th>IC</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mg/kgbw</td>
<td>43.3±12.1a</td>
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<td>16.1±9.9*</td>
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</table>

All values are expressed as Mean ± SEM for FIVE animals per group. *P* < 0.05 for extract versus PQZ by T-test; †*P* < 0.05 for extract versus IC by T-test. AS - *Aloe secundiflora*; AP - *Aspilia pluriseta*; PG - *Psidium guajava*; BA - *Balanites aegyptiaca*; AI - *Azadirachta indica*; AH - *Amaranthus hybridus*
4.2.3 Immunoglobulin G levels

4.2.3.1 SWAP - Specific IgG responses

The IgG responses to SWAP are shown in figure 4.2 for each of the various concentrations of plant extracts. The naive control (NC) showed the lowest IgG response (0.031). The infected control (IC) group showed an intermediate immune response between NC and positive control (PZQ), that is, 0.342. PZQ had the highest response (0.416) compared to IC and NC. The plant extracts that had IgG responses similar to or lower than IC were AS$_{50}$, AP$_{50}$, AP$_{100}$, PG$_{50}$, BA$_{50}$, BA$_{100}$, AI$_{50}$, and all concentrations of AH. Those whose IgG responses were similar to or above PZQ were AS$_{100}$, AS$_{300}$, AP$_{300}$, PG$_{300}$, BA$_{300}$, and AI$_{300}$. However those plants whose responses were intermediate between IC and PZQ included PG$_{100}$ and AI$_{100}$. All extracts exhibited an increasing IgG response with increase in the concentration of plant extract.

4.2.3.2 0-3Hr – Specific IgG responses:

The IgG responses to 0-3 Hr soluble schistosome preparation are shown in figure 4.3. The naive control (NC) showed the lowest IgG response (0.031). The infected control group (IC) showed an intermediate immune response between NC and positive control (PZQ) that is 0.246. PZQ had the highest response (0.381) compared to IC and NC. The plant extracts whose IgG responses were similar to or lower than IC were AS$_{50}$, AP$_{50}$, AP$_{100}$, PG$_{50}$, PG$_{100}$, AI$_{100}$, and all concentrations of AH. Those whose IgG responses were similar to or above PZQ were AS$_{300}$, AP$_{300}$ and AI$_{300}$. However the extracts AS$_{100}$, PG$_{100}$, AI$_{100}$ and all concentrations of BA had responses intermediate between PZQ and
IC. All plant extracts exhibited an increasing IgG response with increase in the concentration of extract except BA and AH whose trends were anomalous.

A comparison of the Swap-specific IgG responses and 0-3 hr responses showed similar pattern for all extracts AH showing similar response to IC in both. However Swap-specific IgG responses were greater than 0-3hr. in all extracts except in AH. The controls showed similar trends with NC having the lowest responses and PZQ having the highest response while IC had intermediate response. The trends were similar for the various concentrations of plant extracts with IgG levels increasing with the increase in the concentration of plant extract except for BA and AH in 0-3hr.
Figure 4.2 Effect of different concentrations of aqueous crude extracts of *Aloe secundiflora*, *Aspilia pluriseta*, *Psidium guajava*, *Balanites aegiptica*, *Azadirachta indica* and *Amaranthus hybridus* on immune responses to *S. mansoni* infected mice (SWAP-speci
Figure 4.3 Effect of different concentrations of aqueous crude extracts of Aloe secundiflora, Aspilia piuriseta, Psidium guajava, Balanites aegyptica, Azadirachta indica and Amaranthus hybridus on immune responses to S. mansoni infected mice (3Hr-sPECIF)
CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

The search for bioactive plants components which can be used as non-conventional molluscicides and anti-helminthes has received considerable attention in recent times because of the increasing, worldwide development of resistance to chemical molluscicides and anthelmintics in molluscs and worm populations respectively. However, scientific evidence to validate the use of plants remains limited (Hoste et al., 2008). Thus, this study was oriented to evaluate the protective and curative capacity of aqueous extracts of \textit{Aloe secundiflora}, \textit{Aspilia pluriseta}, \textit{Psidium guajava}, \textit{Balanites aegyptiaca}, \textit{Azadirachta Indica} and \textit{Amaranthus hybridus} against \textit{S. mansoni}

As intermediate hosts, molluscs play a major role in the transmission of schistosomes; they are the sites of an intense multiplication of parasites. Thus, snail control strategies are considered a priority for the reduction of schistosomiasis transmission (WHO, 1992). A standardized procedure devised for the laboratory screening of synthetic chemical molluscicides (WHO, 1965) is used to evaluate plant molluscides. It recommends that candidate compounds pass through three stages, preliminary screening, definitive screening and comprehensive evaluation. For preliminary screening, five snails are exposed to concentrations of 80, 40 and 10 mg/l of each crude plant extracts (WHO, 1965; Ndamba and Chandiwana, 1989). In the preliminary screening the aim is to
separate materials into those without any molluscicidal activity and those with some, however weak (Duncan and Sturrock, 1987)

In this study preliminary screening of *Aloe secundiflora* (AS), *Aspilia pluriseta* (AP), *Psidium guajava* (PG), *Balanites aegyptiaca* (BA), *Azadirachta indica* (AI) and *Amaranthus hybridus* (AH) for molluscicidal potency was carried out. The aqueous extracts of AS, AP, PG BA and AI exhibited snail mortality above the naïve control, however, this is considered statistically significant compared to the standard molluscicides. The highest molluscicidal effect was exhibited by AH$_{80}$ and this is considered statistically insignificant compared to the standard molluscicide. The mortality increased with increasing concentration of the plant extract.

Plant materials may release active ingredients slowly so that the effect on snail population is delayed, that is, the plant may act as a slow release matrix. In this mode, there may also be effects on feeding and oviposition. Other plant substances affect orientation and feeding behaviour (Thomas and Assefa, 1979). A bioassay of whole plants or parts in which snails are killed within 24 hours at a dosage below 100 mg/L indicates that the molluscicide is released quickly and the material may be a good candidate for LC$_{50}$ determination (Duncan and Sturrock, 1987). This is in agreement with findings of this study in which death of snails was observed within 24 hours exposure to concentrations below 100mg/L of AH.
Snails exposed to *Amaranthus hybridus* (AH) exhibited behaviours that suggested they had been adversely affected by these plants. The snails were weak and could neither eat nor retract into their shells. They exhibited excessive mucus secretion and cessation of feeding. The snails were apparently irritated as observed by their desire to crawl out of the test solution in order to avoid contact with the treated water. Increased mucus production followed by increased mucus secretion as observed in this study is one of the first reactions of gastropods to many stressors, including mechanical stimuli or chemical irritation caused by molluscicidal chemical (Godan 1983; Triebskorn and Ebert, 1989; Triebskorn *et al.*, 1998). One effect of the extruded mucus is to form a protective barrier preventing direct contact between the toxin and the epithelia of the skin or digestive tract, so reducing the toxicity of the chemicals (Port and Port, 1986; Triebskorn and Ebert, 1989).

The responses of *B. pfeifferi* upon exposure to AH$_{80}$ extract suggest that it is possible that this plant may contain molecules with similar effect to Nmethyl-carbamates. Snails poisoned by carbamates become immobilized as the muscle tonus is lost (Godan, 1983). Triebskorn *et al.* (1989) claimed that this compound interferes with the neural control of feeding. Carbamate molluscicides are known to act as nerve toxins by inhibition of cholinesterase (Wilkinson, 1976; Young and Wilkins, 1989). Cytotoxic effects induced by carbamates have been recorded (Triebskorn, 1989; Triebskorn and Kunast, 1990; Triebskorn *et al.*, 1996).
Worm count is the most direct way of determining antischistosomal efficacy of a drug. The present investigation indicated that different concentrations (50-300 mg/L) of the plant extracts were effective in reduction of *S. mansoni* adult worms after exposure in comparison to control untreated worms. Treating infected mice with the plant extracts evoked a significant reduction in the worm load. This reduction in the worm burden observed after treatment with 300mg plant extract per kg body weight of BA (24%), AS (40%), PG (50%), AP (56%) and AI (69%) indicates that these extracts contain antischistosomal constituents. These results are in harmony with Molgaard *et al.* (2001) who reported miracidal and cercaricidal potency of the black seed using different aqueous and organic extracts (petroleum ether, chloroform, ethanol, and water). Mahmoud *et al.* (2002) also reported that administration of the black seed oil to *S. mansoni* infected mice reduced the worm burden. In addition, similar results have been recorded on the effect of other plants against schistosome at different stages; cercariae, schistosomula and adult worms (Naples *et al.*, 1992; Ahmed and Ramzy, 1997; Molgaard *et al.*, 2001; Lyddiard *et al.*, 2002).

The antiparasitic mode of action of these plant extracts may be partly due to their ability to enhance the immunity of the host to attack the parasite. This assumption is verified by the biochemical results of the work of El Shenaway *et al.* (2008) on the antioxidative properties of aqueous garlic extract in *Schistosoma* infected mice. Moreover many workers have documented the ultra-structural alterations encompassing *S. mansoni* as a result of using antibilharzial drugs, such as: Amoscanate (Voge and Bueding, 1980); Oxamniquine (Amin and Mikhail, 1989; Fallon *et al.*, 1996), and Praziquantel
In fact, it can be argued that in this study, the compounds present in the plant extracts may have caused various changes to the tegument of the schistosomes. According to Voge and Bueding, (1980) the tubercles of *S. mansoni* adult worms recovered after treatment with garlic were reduced in number, disrupted, and retracted. It can be assumed in the current study that tubercle distortions may have resulted in the inability of the worms to adhere to the walls of the host blood vessels, consequently causing the schistosome to be dislodged and moved with the blood stream from the mesenteric veins to the portal vein and intravenous hepatic capillaries, and became lodged in the liver (Mehlhorn *et al.*, 1981). In the hepatic blood vessels, the worms may have been trapped and encapsulated with fibrous matrix (da-Silva and Noel, 1990).

In addition, the tegumental changes could have impaired the absorptive functions of the tegument as schistosomes are known to be avid glucose consumers, and glucose particles are mostly absorbed via the tegument. Therefore, such tegumental alterations induced by substances present in the plant extracts could probably have exerted a profound effect on the worm’s metabolism and consequently resulted in their death as evidenced by the reduced worm burden in all treated groups. A similar study on the cestode, *Haemonelephis nana* using garlic extract showed similar lethal effects on the worm (Soffar and Mokhtar, 1991).

The host immune response against schistosome eggs is the basis of chronic pathology in schistosomiasis. Granulomatous lesions form around eggs that are lodged in host tissues
(especially the liver), and this can lead to a generalized fibrosis. Larger lesions are detrimental and small ones ideal. In the case of *S. mansoni*, schistosome eggs release soluble proteins and glycoproteins that are thought to be responsible for granuloma formation and other egg induced reactions (Boros, 1989). In murine schistosomiasis granuloma size is an indicator of morbidity. Granulomas are thought to be host protective, as they wall-off toxic egg products, such as hepatotoxic antigen Omega-1 (Soffar and Mokhtar, 1991), which would otherwise kill the host (and consequently the parasite too). In mice infected with *S. mansoni*, egg deposition begins about 4 to 5 weeks after infection with the first detectable granulomas present by about 6 weeks. This is also in line with findings of the present study. The plants, *Azadirachta indica*, *Psidium guajava* and *Aspilia pluriseta* appeared to have interfered with physiology of the parasites as there were reduction in both the number and size of granulomas. This may be due to separation of adult worm pairs under the effect of the used plant extract indicating that this extract affects the ability of both male and female worms to couple and consequently inhibit egg output by female adult worms. Myrr, a *Commiphora molmol* plant extract exhibits anti-schistosomal properties by causing worm pairs to separate. The female worms shift to the liver where they are destroyed (Hagan *et al.*, 1991).

The live miracidia within each egg secrete antigenic materials through ultra-microscopic pores in the egg shell (Borros and Warren, 1970). These antigens, continually released for 2-4 weeks, induce host sensitization and recruitment of macrophage, lymphocytes, giant cells, fibroblast and numerous eosinophils to
compromise host granulomatous response (Boros and Warren, 1970). This is evident in the present study where the mouse liver infected by *S. mansoni* cercariae and treated using PZQ, showed slight infiltration, and the one treated using PG, showed reduced mononuclear inflammatory cells and few eosinophils while the liver of the untreated mice showed infiltration by a large number of eosinophils, neutrophils and histiocytes.

The pathogenesis of schistosomiasis appears to involve immunologic mechanisms either humoral or cell mediated. The schistosome infection stimulates production of antibodies, IgG₁, IgG₂, IgG₄, IgA, IgE and IgM (Hagan *et al.*, 1991). IgG is important for initiating antibody dependent cell mediated cytotoxicity (ADCC) by eosinophils and macrophages. Some specific antibodies have been shown to enhance the efficacy of drugs. This has been established in PZQ against murine *S. mansoni* infections (Brindley and Sher, 1990). In the present study IgG responses were determined at week 6 using Enzyme Linked Immunosorbent Assay (ELISA). Some of the extracts induced humoral immune response through raising the IgG level at 6 weeks post-infection as compared with infected control. As expected NC had the lowest IgG levels followed by IC while PZQ had the highest IgG levels. This shows that PZQ is not only capable of killing *S. mansoni* worms as demonstrated by low worm counts but it also induces an immunological response. There were plant extracts which did not induce IgG elevation higher than IC for both SWAP and the 0-3h, indicating no involvement of the immune system. They included; AS₅₀, AP₅₀, AP₁₀₀, PG₅₀, and AI₁₀₀ and all concentrations of AH. In addition BA₅₀ and BA₁₀₀ had similar results but only for SWAP.
There were extracts with IgG responses as good as those for PZQ indicating a similar immune response for both SWAP and 0-3hr; these are AS_{300}, AP_{300}, and AI_{300}. Also included in this category but only in SWAP were extracts BA_{300} and AS_{100}. Those that exhibited IgG responses between IC and PZQ – indicating involvement of immune system but not as good as PZQ for both SWAP and 0-3h included PG_{100} and AI_{100}. Others in this category but only for 0-3hr were AS_{100} and all the concentrations of BA.

The plant extract that showed the best immune system involvement was AI_{300} for 0-3h and AP_{300} for SWAP. These responses suggest that the plants *Aloe secundiflora*, *Aspilia pluriseta*, *Psidium guajava*, *Balanites aegyptiaca* and *Azadirachta Indica* boosted the humoral response of mice to schistosome infection. An elevated humoral response could have resulted in less pathology in treated animals than the control (reduction in worm burden and granuloma size). This is because IgG is important in initiating ADCC by eosinophils and macrophages (Hagan *et al.*, 1991).

### 5.2 CONCLUSIONS

In conclusion, the findings of the present study indicate that *Psidium guajava* is not effective against *Biomphalaria pfeifferi*. Exposure of *B. pfeifferi* to the various concentrations of *Psidium guajava* extract did not cause meaningful toxicity to the snails compared to the untreated water. From the observations, it is concluded that *Aloe secundiflora*, *Aspilia pluriseta*, *Balanites aegyptiaca*, *Azadirachta indica* and *Amaranthus hybridus* were found to have molluscicidal effect against *B. pfeifferi*. Crude extracts of these plants caused death of the snails at the varying concentrations. The best
results in terms of toxicity to the vector snail were exhibited by *Amaranthus hybridus* followed by *Azadirachta indica*. Extracts of these two plants could be more preferred for development of a molluscicide as they resulted in high number of dead snails. Crude organic extracts should present LC\(_{90}\) below 20 ppm to be considered a good molluscicide candidate for direct application in infested water (WHO, 1985). However, it is possible that extracts active between 20 and 100 ppm could contain small amounts of very active components, which could be isolated and/or concentrated using simple procedures, or even obtained from other plants known to produce it in larger amounts. Therefore, the above active extracts deserve further studies in order to identify and characterize their molluscicidal components.

The study also found that *Amaranthus hybridus* does not boost humoral response against *S. mansoni*. ELISA results showed that the proliferative response of IgG for the group treated with this plant extract was the same or even less than the infected control (IC) group. If the plant extract had enhanced the stimulation of immune cells, then an elevated immune response could have caused killing of the parasites. This could have resulted in less worm count, fairer gross pathology and less granuloma size in treated mice compared to the untreated mice. The difference in worm count and granuloma size was found to be statistically not significant (p>0.05). Although it was found in this study that mice treated with *Amaranthus hybridus* had some reduction in worm burden, it was not considered a cure since the decrease was insignificant compared to the control. Pathological examination of the liver showed the plant extract had the worst pathology.
However it is concluded that *Aloe secundiflora, Aspilia pluriseta, Psidium guajava, Balanites aegyptiaca*, and *Azadirachta indica* have antischistosomal effect on *S. mansoni*. The study found that these plant extracts boosted the humoral response against *S. mansoni*. ELISA results showed that the proliferative response of IgG for the groups of mice treated with these plant extracts were greater than the infected control (IC) group. The elevated immune responses could have caused killing of the parasites. This then resulted in less worm count, improved gross pathology and less granuloma sizes in the treated mice.

The extracts of *Azadirachta indica, Aspilia pluriseta*, and *Psidium guajava* exhibited encouraging results in terms of worm recovery, IgG response, pathology and granuloma size reduction, the best among them being *Azadirachta indica*. These three plants are therefore considered good candidates for the development of antischistosomal drug.

### 5.3 RECOMMENDATIONS

The promising results obtained from *Amaranthus hybridus, Azadirachta Indica, Psidium guajava and Aspilia pluriseta* encourages further investigations to establish the synergistic effects of the combined plant extracts.

Phytochemical tests should be done to determine the bioactive constituents that are responsible for the schistosomicidal and molluscicidal effects. Toxicity tests should also be conducted to determine the safety of these plant extracts.
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