

**ANTISCHISTOSOMAL AND IMMUNE ENHANCING
POTENTIAL OF *AZADIRACHTA INDICA* AND
EKEBERGIA CAPENSIS IN MICE INFECTED WITH
*SCHISTOSOMA MANSONI***

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Antischistosomal and Immune Enhancing Potential of *Azadirachta Indica* and *Ekebergia Capensis* in Mice Infected with *Schistosoma Mansoni*

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A thesis submitted in partial fulfillment for the Degree of Master of Science in Molecular Medicine in the Jomo Kenyatta University of Agriculture and Technology.

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DECLARATION

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

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The thesis has been submitted for examination with our approval as the university supervisors.

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DEDICATION

I dedicate this work to my children Sammy and Stephanie and my husband Kennedy Gakami who have been very patient and supportive to see my progress to victory. A special dedication to my parents Daniel and Sellah Musili for the inspiration, prayers and encouragement they have given me throughout my studies. May God bless you all. Most of all I give glory and praise to God for making this possible.

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LIST OF ABBREVIATIONS AND ACRONYMS

CBRD	Centre for Biotechnology Research and Development
KEMRI	Kenya Medical Research Institute
JKUAT	Jomo Kenyatta University of Agriculture and Technology
MKU	Mount Kenya University
PZQ	Praziquantel
FACS	Fluorescence-activated cell sorting
CBA	Cytometric Bead Array
Th2	T helper 2
Th1	T helper 1
Th17	T helper 17
WHO	World Health Organization
D.R.C	Democratic Republic of Congo
NTD	Neglected Tropical Diseases
CDC	Centre for Disease Control
MDA	Mass Drug Administration
AIDS	Acquired Immune Deficiency Syndrome
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IgD	Immunoglobulin D
SANBI	South African National Biodiversity Institute

CQ	Chloroquine
SEA	Soluble Egg Antigen
SWAP	Soluble Worm Antigen Preparation
CD4	Cluster of Differentiation 4
BD	Becton Dickinson
PE	Phycoerythrin
IL	Interleukin
FL3 or FL4	Flourescent Light 3 or 4
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Flow Cytometer Standard Files
DMSO	Dimethyl sulfoxide
IFN-γ	Interferon gamma
ART	Artemether
TNF-α	Tumor Necrosis Factor alpha
Pi	Post infection
EC	<i>Ekebergia capensis</i>
AI	<i>Azadirachta indica</i>
SD	Standard deviation

ABSTRACT

Schistosomiasis is a parasitic disease of great socio-economic and public health importance in tropical and sub-tropical countries. Praziquantel (PZQ) is effective against all schistosome species, it is ineffective on larval stages and concerns of drug resistant strains developing prompts the need for alternative antischistosomal drugs. Plants have over the years provided a rich source of novel drugs for a wide range of diseases afflicting man and domestic animals. Swiss albino mice were infected with 90 cercariae each and treated orally with aqueous extracts of *Ekebergia capensis* or *Azadirachta indica* at doses of 25, 50, 100, 200 and 400 mg/kg at 2 weeks (juvenile worms), 4 weeks (immature worms) and 7 weeks (adult worms) post infection. Total reduction of worm and egg loads was used as an indicator of drug activity, relative to the infected but untreated control groups. Both *E. capensis* and *A. indica* showed significant dose-dependent percentage worm load reduction ($P < 0.05$) at different doses ranging from 100 mg/kg to 400 mg/kg. These extracts also significantly reduced tissues (liver and intestine) egg load counts at doses ranging from 50 mg/kg to 400 mg/kg which was also dose-dependent. *E. capensis* was more potent than *A. indica* in reducing both the worm burden at all the stages and tissue egg load ($P < 0.05$). Immune enhancing potential of the medicinal plants was determined by analyzing the levels of cytokines in serum samples that were collected before and after treatment. A BD-Cytometric Bead Array (CBA) mouse Th1/Th2/Th17 kit was used to quantitate the levels of cytokines using flow cytometer (FACS Calibur) and analysis of the data was done using FCAP software. Results from two doses (400mg/kg and 200mg/kg) in 2 weeks and 7 weeks pi experiments (to represent juvenile and adult worms respectively) indicated that the two medicinal plant extracts have immunomodulatory effect. There was a significant increase ($P < 0.05$) in Th1 cytokines (IL-2, IFN- γ and TNF- α), a decrease in Th2 cytokines (IL-4, IL-6 and IL-10) and an increase in Th17 (IL-17) following treatment with the plant extract at both 2 weeks and 7 weeks pi. These findings confirm the potential use of medicinal plants in the management of schistosomiasis.

CHAPTER ONE

INTRODUCTION

1.1 Schistosomiasis

Schistosomiasis is an acute and chronic parasitic disease caused by blood flukes (trematode worms) of the genus *Schistosoma*. Schistosomiasis (bilharziasis or snail fever) is second only to malaria in terms of socio-economic and public health importance in tropical and subtropical areas (The Carter Center, 2008) and it is prevalent in poor communities without potable water and adequate sanitation. Schistosomiasis affects almost 240 million people worldwide, and more than 700 million people live in endemic areas (WHO, 2015). It is endemic in 78 countries with over 90% of cases occurring in sub-Saharan Africa (WHO, 2015) where prevalence rates can exceed 50% in local populations (Montgomery, 2013).

Human schistosomiasis is caused by *Schistosoma haematobium*, *S. intercalatum*, *S. japonicum*, *S. mansoni* and *S. mekongi* (Chitsulo, Loverde & Engels, 2004). Two main schistosome species cause human schistosomiasis in Africa namely; *S. mansoni* and *S. haematobium* of which *S. mansoni* is the most-wide spread in human populations in endemic areas. *S. mansoni* causes intestinal schistosomiasis while *S. haematobium* causes urinary schistosomiasis (Brooker *et al.*, 2009).

Schistosomiasis is endemic in Kenya with more than 6 million infected people and many more at risk of infection (Chitsulo, Engels, Montresor & Savioli, 2000). Over 16 million people are at risk of infection with the disease (Neglected Tropical Diseases Kenya, 2015). Schistosomiasis is mainly found within the Mwea irrigation scheme in Kirinyaga district, Machakos, Kitui, Taita Taveta and Nyanza counties with both *S. mansoni* and *S. haematobium* being the most predominant (WHO, 2006). In areas surrounding Lake Victoria and along the coastal belt of Kenya prevalence ranges between 50 and 90% (Odiere *et al.*, 2012). The highest infection rates are in adolescents between ages 10-19 years but also adult workers in rural areas employed in activities associated with water contact are also affected (Grzych *et al.*, 1987; Karanja *et al.*, 1997). The overall prevalence of schistosomiasis ranges

from 5% - over 65% in communities in Kenya and contributes to significant morbidity (Karanja *et al.*, 1997; 1998; Mwinzi *et al.*, 2004).

1.2 Signs and symptoms of Schistosomiasis

Symptoms of schistosomiasis are caused by the host's reaction to the worms' eggs. Intestinal schistosomiasis can result in abdominal pain, diarrhea and blood in the stool. Liver enlargement is common in advanced cases, and is frequently associated with an accumulation of fluid in the peritoneal cavity and hypertension of the abdominal blood vessels. In such cases there may also be enlargement of the spleen. The classic sign of urogenital schistosomiasis is hematuria (blood in urine). Fibrosis of the bladder, ureter and kidney damage are sometimes diagnosed in advanced cases. Bladder cancer is another possible complication in the later stages. In women, urogenital schistosomiasis may present with genital lesions, vaginal bleeding, pain during sexual intercourse and nodules in the vulva. In men, urogenital schistosomiasis can induce pathology of the seminal vesicles, prostate and other organs. This disease may also have other long-term irreversible consequences, including infertility (WHO, 2015). Other disease manifestations include eosinophilia, fever and fatigue (NTD Kenya, 2015). In children, schistosomiasis can cause anaemia, stunting and a reduced ability to learn, although the effects are usually reversible with treatment. Chronic schistosomiasis may affect people's ability to work and in some cases can result in death (van der Werf *et al.*, 2003).

1.3 Schistosomiasis control

The WHO strategy for schistosomiasis control focuses on reducing disease through periodic, targeted treatment with praziquantel (PZQ). This involves regular treatment of all people in at-risk groups. Groups targeted for treatment are: school-aged children in endemic areas; adults considered to be at risk in endemic area for example pregnant and breastfeeding women, people with occupations involving contact with infested water – such as fishermen, farmers, irrigation workers – and women whose domestic tasks bring them into contact with infested water and entire communities living in endemic areas. Other control strategies include snail control, improved sanitation and health education (WHO,2012).

1.4 Problem statement

The drugs that are used to treat schistosomiasis include metrifonate which is effective against *S. haematobium*, Oxamniquine which is effective against *S. mansoni* and artemisinin which exhibits highest activity against 1-3 week old liver stages of the parasite. Praziquantel has been the main drug of choice for treatment of all species of schistosomes because of its efficacy, ease of administration, safety, and cost (Cioli & Pica-Mattocchia, 2003). A single dose of 40 mg/kg has been widely accepted as the standard dosage, resulting in cure rates of 60–95% (Magnussen, 2003). The main disadvantage of PZQ is that it is only effective on adult and ova of schistosomes. PZQ has been in use for more than two decades and evidence of parasite resistance to it has been reported based on low cure rates (CDC, 2012). The effectiveness of PZQ is also dependent on an intact immune system (Brindley & Sher, 1987a). There is therefore need for development of alternative antischistosomal drugs that are cheap, readily available and possibly effective on all stages of schistosomes or that can be used in combination with PZQ effectively.

1.5 Justification

S. mansoni which causes intestinal schistosomiasis is widespread in endemic communities. The drug of choice for treatment of *S. mansoni* is PZQ that has been in use for many years and there are recent reports of possible resistance developing. It is ineffective against larval stages of the parasite and therefore for effective treatment and sustainable control, PZQ has to be administered on regular basis. Experimental studies have also demonstrated that successful therapy with PZQ is dependent on a normal and functional host immune system. The development of resistance by target parasites and high cost of drugs paves way for herbal remedies as reasonable alternatives.

Herbal medicines are natural products that are environmentally friendly and inexpensive. These traditional medicines are still in use and available especially to people who do not have access to conventional drugs. There are several plants which have been reported to have antischistosomal activity by traditional practitioners. Studies on antischistosomal effects of artemether either alone or in combination with

praziquantel have been reported where artemether showed more effect on juvenile worms than on adult worms.

This study determined the antischistosomal and immune enhancing potential of *A. indica* and *E. capensis* in Swiss albino mice infected with *S. mansoni*. The *in vivo* antischistosomal effect of these herbs against both juvenile and adult worms of *S. mansoni* were tested and compared to PZQ and artemether as the standard drugs. The immune enhancing effect of these herbs was assessed by measuring levels of circulating cytokines before and after treatment with the herbal extracts. The results of this study have provide useful information that the two plants are effective against juvenile or adult schistosome worms and therefore these plants have the potential to be used in the treatment of *S. mansoni*. This study has also provided additional information on the effects of these herbs on the immune system of the host. Advancing knowledge of mechanisms of immune regulation in infections could lead to effective and targeted therapies.

1.6 Research questions

1. What are the effects of *A. indica* and *E. capensis* on both juvenile and adult stages of *S. mansoni* infection?
2. What are the effects of these medicinal plants on the immune system of the *S. mansoni* infected hosts?

1.7 Hypothesis

1.7.1 Null hypothesis

There are no antischistosomal nor immune enhancing effects of *A. indica* and *E. capensis* in *S. mansoni* infection.

1.7.2 Alternative hypothesis

There are antischistosomal and immune enhancing effects of *A. indica* and *E. capensis* in *S. mansoni* infection.

1.8 Objectives

1.8.1 General objective

To determine antischistosomal and immune enhancing potential of *A. indica* and *E. capensis* in mice infected with *S. mansoni*.

1.8.2 Specific objectives

1. To determine the *in vivo* antischistosomal activity of *A. indica* and *E. capensis* against adult worms in comparison to PZQ.
2. To determine the *in vivo* antischistosomal activity of *A. indica* and *E. capensis* against juvenile worms in comparison to artemether.
3. To determine the *in vivo* immune enhancing potential of *A. indica* and *E. capensis*.

CHAPTER TWO:

LITERATURE REVIEW

2.1 Geographical distribution of Schistosomes

Schistosomes (blood flukes) are trematodes in the family Schistosomatidae that are known to infect a range of hosts including man. Schistosomes are dioecious (have separate sexes), blood dwelling helminth parasites of human and animals in the tropics and subtropical region of the world. Schistosome species in the genus *Schistosoma* cause human schistosomiasis (or bilharziasis), a debilitating disease characterized by a variety of symptoms including abdominal pain, hepatosplenomegally, diarrhea or blood urine, and in complicated cases may cause death.

Five species of schistosomes are prevalent in different parts of the world. *S. mansoni* is widespread in Africa (Plate 2.1), the Eastern-Mediterranean, the Caribbean, and South America and infects humans and rodents. *S. mekongi* is prevalent in the Mekong river basin in Asia. *S. japonicum* is limited to China and the Philippines and in addition to humans it infects other mammals such as pigs, dogs, and water buffalos. *S. intercalatum* is found in central Africa while *S. haematobium* occurs predominantly in Africa and the Eastern Mediterranean. The majority of infections with *S. haematobium*, *S. mansoni* and *S. intercalatum* are found in sub-Saharan Africa. *S. mansoni* remains endemic in parts of Brazil, Venezuela, and the Caribbean. *S. japonicum* infection occurs in the People's Republic of China, the Philippines, and small pockets in Indonesia, despite substantial and largely successful control measures (Ross *et al.*, 2002). *S. mekongi* is found along the Mekong River in Cambodia and Lao People's Democratic Republic (Laos) (Ferrari, 2004).

Infection is usually acquired through activities such as swimming, bathing, fishing, farming, and washing clothes (Ross *et al.*, 2002; Gryseel, Polman, Clerinx & Kestens, 2006). Important transmission sites include Lake Malawi and Lake Victoria in Africa, the Poyang and Dongting Lakes in China, and along the Mekong River in Laos.



Map courtesy of CDC.

Plate 2.1: Map of Distribution of Schistosomiasis.

2.2 Life cycle of Schistosomes

Schistosomiasis is contracted through direct contact with fresh water infested with the free-living form of the parasite known as cercariae during normal activities like bathing, doing domestic chores, fishing, swimming and farming (CDC, 2010). The adult worms inhabit the mesenteric veins (*S. mansoni*, *S. japonicum*, *S. mekongi*, *S. intercalatum*) or the veins of the vesicles and pelvic plexuses (*S. haematobium*). The life cycle (as shown in the Figure 2.1 below) is common in all the species but differs in the nature of the intermediate snail host and the duration of the cycle. Schistosomes have a sexual generation in vascular system of the definitive host and

an asexual generation in the intermediate host (snails). Newly developed adult females and males find each other and pair up. Adult blood flukes are 1-2 cm long. Males make a gynaecophoric canal for the longer and thinner females to reside. The worm pair then travels to mesenteric veins. They attach to the venous wall with oral and ventral suckers and can live for many years (an estimated average of 5 years up to 20 years if not treated). Females lay eggs on the endothelial lining of the venous capillary walls at the rate of 300–3000 eggs per day depending on the *Schistosoma* species. Embryonated eggs are discharged in faeces and urine. In water, miracidia hatch from the eggs and penetrate the intermediate hosts. The intermediate hosts of *S. mansoni* are snails of the genus *Biomphalaria* (Planorbidae family). The intermediate hosts of *S. haematobium* are snails of the genus *Bulinus* while the intermediate hosts of *S. japonicum* are snails of the genus *Onchomelania* (CDC, 2010).

After penetrating the snail miracidia develops into mother sporocysts and then daughter sporocysts and in 4 weeks for *S. mansoni*, thousands of cercariae are produced (asexual multiplication). Infection of the definitive host occurs by penetration of the skin. During the penetration process the cercariae lose their tail and transform into the larval stage; the schistosomulum. After penetration schistosomula migrate to the lungs (3-4 days for *S. mansoni*) and after penetration in the pulmonary capillaries they are carried to the systemic circulation and to the portal system. In the hepatic circulation schistosomes mature to adult and in pairs they migrate to the mesenteric veins (*S. mansoni* and *S. japonicum*) and to the vesicle plexus (*S. haematobium*). After 35 days (*S. japonicum* and *S. mansoni*) and 60 days (*S. haematobium*) embryonated eggs are excreted in faeces and/or urine (CDC, 2010).

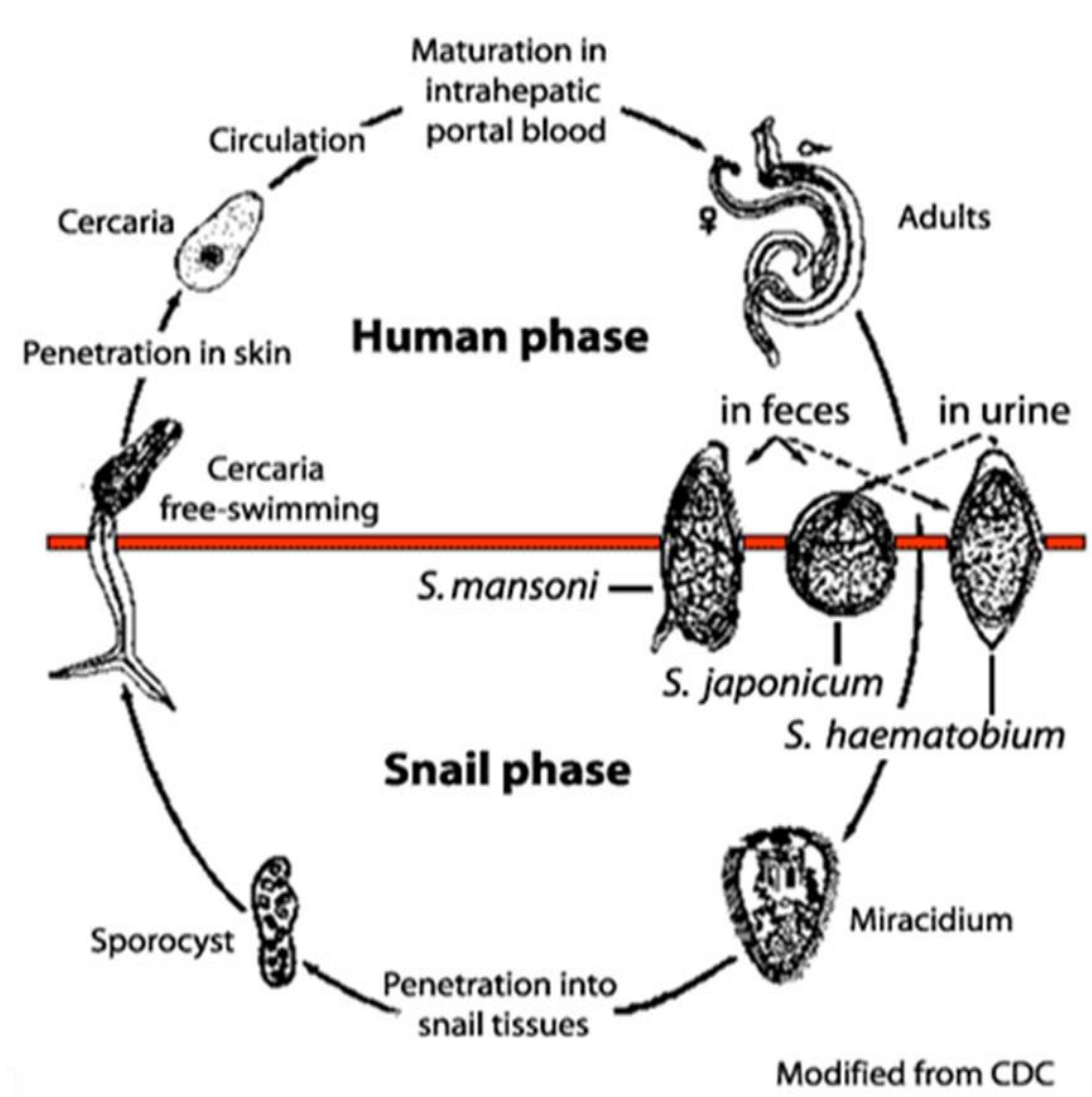


Figure 2.1: Life cycle of Schistosomes

Source: CDC's Parasite and Health page on Schistosomiasis

2.3 Approaches in the control of Schistosomiasis

The World Health Assembly in 2001 drafted resolution 54.19 that endorsed chemotherapy as the main strategy for control of schistosomiasis through mass drug administration (MDA) (WHO, 2013). The aim of MDA is to lessen morbidity and mortality due to the infection as well as prevent new infection by limiting transmission through reduction of the overall prevalence in the population (Humphries, Nguyen, Boakye, Wilson & Capello, 2012). MDA is assumed to lead to

reduction in excretion of schistosome eggs, contamination of the environment, and infection of the snail population and this would lead to less source of infection for humans. MDA was first implemented in Egypt in the 1920s using intravenous tartar emetic in adults and children. Safe drugs like niridazole, metrifonate, oxamniquine, and praziquantel for treating human infection were later discovered and this refocused the control strategy to chemotherapy. MDA has been implemented as part of national schistosomal control programs in Uganda, Burkina Faso, Sierra Leone Niger, Mali, Brazil, the People's Republic of China, and the Philippines (Humphries *et al.*, 2012; Hodges *et al.*, 2012; Koukounari *et al.*, 2007; Sarvel, Oliveira, Silva, Lima & Katz, 2011; Sesay *et al.*, 2014; Garba *et al.*, 2009). Praziquantel is the drug of choice for MDA because it is safe, inexpensive, low cost, with a significant impact on disease prevalence and intensity (Fenwick & Webster, 2006). Despite the low cost of the drug, the large number of the people at risk of infection makes the overall cost of implementing MDA difficult for affected countries to sustain. Therefore, MDA is implemented in combination with other strategies like snail control.

Snail control is a vital component of schistosomiasis control because it targets the intermediate host using chemical compounds called molluscicide (Yang, Li & Sun, 2010). Mollusciciding was introduced in 1940s using copper sulphate and sodium pentachlorophenate in 1955 which was a more efficient drug in Egypt (Barbosa & Coimbra, 1992). Four compounds namely Yurimin, sodium pentachlorophenate, N-tritylmorpholine, and niclosamide were listed as molluscicides in the WHO expert report in 1972 (McCullough *et al.*, 1980). Niclosamide is the only WHO recommended molluscicide at present and its dosing has to be done at least twice a year to be effective for eliminating snails (Yang *et al.*, 2010). The most economical and effective way of snail control is focal and seasonal application of molluscicide based on the transmission cycle (Madsen, 1992) This need for repeat treatment makes this strategy time-consuming and less cost effective especially in large areas (Yang, Sun & Hong, 2012). Molluscicides are toxic to other macro-organisms and micro-organisms and cause environmental pollution (Mazigo *et al.*, 2012; Wang *et al.*, 2009). Environmental methods like burying snail habitat, flooding the snail waters up to several meters in depth and digging ditches or water drainage tunnels are also snail control strategies.

Promotion of health education, access to safe water and sanitations are methods that were emphasized in the World Health Assembly resolution 54.19 (WHO, 2013). The health education will lead to behavioural changes like avoidance of contact with schistosome-infested waters and contamination of the environment with feces. For this to be feasible, alternative must be provided and complemented with the provision of portable water and toilet facilities (Colley, Bustunduy, Secor & King, 2014).

2.4 Antischistosomal drugs: Mode of action and Immunomodulation effects

2.4.1. Praziquantel

Infections with all major schistosome species can be treated with praziquantel. The timing of treatment is important since praziquantel is most effective against the adult worm and requires the presence of a mature antibody response to the parasite (CDC, 2010). Limited evidence of parasite resistance to praziquantel has been reported based on low cure rates in recently exposed or heavily infected populations; however, widespread clinical resistance has not yet occurred (CDC, 2012). Thus, praziquantel remains the drug of choice for treatment of schistosomiasis. It is also active against other trematode and cestode infections but generally not against nematodes (Andrews, 1985). The main advantages of PZQ include convenient oral administration, high safety and efficacy as well as short treatment course (Xiao, 2007). The major weakness of PZQ is its lack of efficacy against juvenile schistosomes (2 to 4 weeks old). This has been clearly shown in *in vitro* tests (Xiao, Catto & Webster, 1985) and it has been confirmed by clinical data (Gryseels *et al.*, 2001). PZQ causes side effects that include stomach discomfort, dizziness, diarrhea, nausea, headache, vomiting, itchy skin, lethargic and sleepy swollen face. The majority of the side-effects develop due to the release of the contents of the parasites as they are killed and the consequent host immune reaction. The heavier the parasite burden, the heavier and more frequent the side effects (Midzi *et al.*, 2008). It is unpalatable (bitter), the size and shape of the tablet discourages mass drug administration to include small children (Aronson, 2012).

Praziquantel(2-cyclohexylcarbonyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinolin-4-one) was synthesized in the 1970s (Seubert, Pohlke & Loebich, 1977). Despite considerable efforts, the mechanism of action of praziquantel has yet to be fully elucidated (Andrews, 1985; Cioli, Pica-Mattocia & Archer, 1995). However, three central features are observed in schistosomes following the administration of praziquantel, and these effects are directly or indirectly associated with Ca^{2+} redistributions between worm tissues and the surrounding environments. First, worm motor activity is immediately stimulated, followed by strong muscular contraction. Second, praziquantel induces extensive tegumental damage, commencing 5 minutes post treatment. Third, treatment is accompanied by metabolic changes altering glycogen content and energy metabolism (Cioli *et al.*, 1995). The drug induces a rapid shift of worms from the mesenteric veins to the liver, known as hepatic shift (Becker, Mehlhorn, Andrews, Thomas & Frenkel, 1981). Studies *in vitro* have shown that PZQ initially affects the outer tegument of worms, causing rupture of the membranes with release or exposure of internal antigens (Harnet *et al.*, 1986; Redman *et al.*, 1996). Studies in mice have shown that by 17 hours after PZQ treatment cells of the host immune system have adhered to and penetrated the worm's tegument (Brindley *et al.*, 1987b; Doenhoff *et al.*, 1987; Mehlhorn, 1981).

2.4.2 Metrifonate

Metrifonate has been shown to cause reversible paralysis to adult worms *in vitro*. The drug exhibits activity against *S. haematobium* more effectively but has recently been withdrawn from the market because of medical, operational and economic criteria (Feldmeier & Chitsulo, 1999). The metrifonate treatment schedule has a low rate of compliance, which leads to a generally lower cure rate than praziquantel. Metrifonate tablets need to be kept in tightly closed containers and stored at temperatures not exceeding 25⁰C, preferably in refrigerators (WHO, 1993).

2.4.3 Oxamniquine

In contrast to PZQ, which displays activity against all human schistosome species, the activity of oxamniquine is confined to *S. mansoni* (Cioli *et al.*, 1995; Katz, 1980). The stage-specific susceptibility of *S. mansoni* to oxamniquine exhibits a pattern

very similar to that of susceptibility to PZQ: the invasive stages and the adult worms are significantly more affected than the liver stages (Foster, Mesmer, Cheetham & King 1971). Adult male worms are considerably more affected by oxamniquine than adult females (Foster *et al.*, 1971), and Central and East African strains of *S. mansoni* are significantly less susceptible than South American strains. Oxamniquine is the only alternative antischistosomal drug to PZQ, but its use is declining (Cioli, 2000).

Oxamniquine (6-hydroxymethyl-2-isopropyl-aminomethyl-7-nitro-1, 2, 3, 4-tetrahydroquinoline) was first described in the late 1960s (Richards *et al.*, 1969). The mechanism of action of oxamniquine is reasonably well understood, and the body of evidence suggests that it is closely associated with an irreversible inhibition of the nucleic acid metabolism of the parasites. The following working hypothesis has been put forth: the drug is activated by a single step, in which a schistosome enzyme converts oxamniquine into an ester (probably acetate, phosphate, or sulfate). Subsequently, the ester spontaneously dissociates, while the resulting electrophilic reactant is capable of alkylation of schistosome DNA (Cioli *et al.*, 1995).

2.4.4 Artemisinin

The antischistosomal activities of artemisinin, artemether, and artesunate were described in the early 1980s, with the initial experiments focusing on *S. japonicum* (Le *et al.*, 1982; Le *et al.*, 1983). More recent studies confirmed that arteether and dihydroartemisinin also display antischistosomal properties against *S. japonicum* (Abdel & el-Badawy, 2000). Dihydroartemisinin is the principle active metabolite (Mordi, Mansor, Navaratnam & Wernsdorfer, 1997). Laboratory experiments conducted so far in different animal models found that artemether is active against the three major human schistosome parasites (Utzing, Xiao, N'Goran, Bergquist & Tanner, 2001, Xiao *et al.*, 2002). In contrast to PZQ and oxamniquine, artemether exhibits the highest level of activity against 1-3 week old liver stages, while the invasive stages and the adult worms are less susceptible. Adult female worms are somewhat more susceptible to artemether than male worms (Utzing *et al.*, 2001), which is opposite the activity of oxamniquine.

Artemisinin, a sesquiterpene lactone with a peroxide group, is the active principle derived from the leaves of *Artemisia annua* and is best known for its antimalarial

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

The stage-specific susceptibility of a Liberian strain of *S. mansoni* harbored in mice treated with a single oral dose of artemether (Xiao, Chollet, Weiss, Bergquist & Tanner, 2000a) was very similar to the stage-specific susceptibilities that have also been reported for *S. japonicum* (Xiao, You, Yang & Wang, 1995). There is a need for additional *in vivo* experiments to assess the stage-specific susceptibilities of *S. haematobium* to artemether and to comparatively assess the species- and stage-specific susceptibilities of schistosomes to other artemisinin derivatives. A first comparative appraisal revealed that artemether exhibits consistently higher levels of activity against *S. mansoni* parasites of different ages than artesunate (Utzing, Chollet, Tu, Xiao & Tanner, 2002).

The exact mechanism of action of artemether against schistosomes remains elusive, but progress has been made in recent years (Utzing *et al.*, 2001). A typical biochemical feature is that, following artemether treatment, adult worms showed significant reductions in their glycogen contents (Xiao, Hotez & Tanner, 2000). As with praziquantel, artemether also induces severe and extensive tegumental damage; however, the onset of tegumental alterations is considerably slower (Utzing *et al.*, 2001). Another important finding is that *in vitro* exposure of schistosomes to a medium containing artemether plus hemin results in parasite death, while exposure to artemether or hemin alone showed no effect. Therefore, it has been suggested that artemether might be activated by hemin and sequentially cleaves the Endoperoxide Bridge and generates free radicals that might form covalent bonds with schistosome-specific proteins (Xiao *et al.*, 2001).

The hepatic shift commences within 8 hours after artemether administration and is completed within 7 days (Xiao & Catto, 1989); hence, it is much slower than that after praziquantel treatment but only somewhat slower than that after oxamniquine

treatment. Oral formulations of artemisinin derivatives are absorbed rapidly but incompletely (the peak concentrations of most artemisinins in plasma are reached 1 to 2 hours post treatment) and have short half-lives in plasma of 1 to 3 h before undergoing hepatic metabolism (van Agtmael *et al.*, 1999). *In vivo* studies in different animal models revealed brain stem neurotoxicity after repeated treatment with high doses of some artemisinin derivatives over at least 7 days (Genovese, Newman & Brewer, 2000). However, repeated treatments with high doses of artemether once every 2 weeks, the recommended dose schedule for the prevention of *S. japonicum* infection, revealed no neurotoxicity (Xiao *et al.*, 2002). Most importantly, there is no clinical evidence of neurological lesions, although several million people have been treated with an artemisinin derivative for malaria (Price *et al.*, 1999).

2.5 Immune dependency of treatment of schistosomiasis

2.5.1 Immunopotentiality

Successful therapy with praziquantel in schistosomiasis is dependent on normal as well as specific host immune responses (Snyman & Sommers, 1998). Experimental studies have demonstrated reduced efficacy of praziquantel against schistosome infections in immunodeficient animals (Sabah, Fletcher, Webbe & Doenhoff, 1985) but have shown that restoration of efficacy could be achieved by passive transfer of immune serum from immunocompetent animals (Brindley *et al.*, 1987; Doenhoff, Modha, Lambertucci & McLaren, 1988; 1987), indicating that a functional immune response is necessary for drug efficacy (Doenhoff, Modha, Lambertucci & McLaren, 1991). This has also been demonstrated in a study by Doenhoff (1989) where the drugs hycanthone, oxamniquine, and praziquantel have been found to kill fewer adult *S. mansoni* worms in T-cell-deprived CBA mice (Doenhoff, 1989). In the mouse model, praziquantel efficacy depends on specific host antibody responses and has been shown to increase the exposure of schistosome antigens at the worm surface (Brindley *et al.*, 1987b; Doenhoff *et al.*, 1988; Harnett *et al.*, 1986). The efficacy of schistosomicides is decreased in mice infected with *S. mansoni* and immunosuppressed by thymectomy and administration of rabbit anti-mouse thymocyte serum (Brindley & Sher, 1987a; Lambertucci *et al.* 1989). The

schistosomicidal activity of antimony, oxamniquine, and praziquantel has been shown to be enhanced by passive transfer of immune serum simultaneously with drug administration to *S. mansoni* infected mice, thus indicating a role for humoral immune effector mechanisms in this phenomenon (Brindley & Sher, 1987a, Lambertucci, Modha & Doenhoff, 1989).

In humans however, it has proved difficult to identify the specific host related factors that influence the efficacy of praziquantel (Karanja *et al.*, 1998; van Lieshout *et al.*, 1999). Human immune response differences may impact individual response to treatment with praziquantel (CDC, 2010).

2.5.2 Immunosuppression

Immunosuppressed individuals by drugs (cytotoxic chemotherapy, other immunosuppressive agents including steroids and irradiation) or affected by diseases that cause immunodepression (AIDS, neoplasia, malnutrition, and chronic renal failure) tend to have disseminated infection with *S. mansoni* (eggs) involving lung, liver, spleen, intestine, pancreas, and testis (Hillyer & Climent, 1988, Lambertucci & Neves, 1993). The migration of worms to different organs in the human body may explain the finding of a great number of eggs in unusual places. This implies that the immune system is important in keeping the adult worms of *S. mansoni* confined to the mesenteric vessels.

2.6 Medicinal plants in treatment

Herbal plants have been used traditionally to treat or manage schistosomiasis in Eastern and Southern Africa (Kokwaro, 1993; Ndamba, Nyazema, Makaza, Anderson & Kaondera, 1994; Sheir, 2001). However limited information is available on the beneficial effects of herbal preparations in the treatment and management of the disease and few attempts have been made to scientifically verify the antischistosomal properties of such preparations. The active ingredients of such plants are not known. Kokwaro (1993) reported that at least 19 plant species occurring in East Africa region and belonging to 10 families and 16 genera have been used traditionally as remedies for schistosomiasis in this region. Of these species, 5 of them comprising 5 families and 5 genera, namely *Dissotis rotundifolia*

(Melistomataceae), *Iboza multiflora* (Labiatae), *Macaranga kilimandscharia* (Euphorbiaceae), *Ozoroa mucronata* (Amatanthaceae) and *Rhynchosia hirta* (Leguminosae) are known to exist in Kenya. Examination of the potential of plants in management of schistosomiasis by various investigators like Ndamba *et al.* (1994), Sheir *et al.* (2001) and Sparg, van Staden & Jager (2000) revealed several plants with appreciable antischistosomal activities.

Artemether derived from the plant *Artemisia annua* has been investigated for its activity on *S. mansoni* and it showed efficacy on early stages of the infection with 75.3- 82.0% worm reduction which was boosted to 97.2 - 100% on treatment in mice experiments (Xiao *et al.*, 2000). More recently, artemisinin derivatives, either alone or in combination with praziquantel, have been shown to be effective against immature stages of *S. mansoni*, *S. haematobium*, and *S. japonicum* in laboratory studies and limited field studies. There is need to further evaluate and document effectiveness of artemisinin derivative monotherapy or combination therapy (CDC, 2010).

2.6.1 *Azadirachta indica* (Neem)

Azadirachta indica is commonly known as neem, margosa or Indian lilac and it was introduced to Africa by Indian immigrants and the colonial administration (Schmutterer, 1995). Neem is from the family Meliaceae and it is a tree found in Western Himalayas of India and Iran and its biological names are *Melia azadiracta* or *Azadirachta indica*. Neem's Swahili name, 'mwarobaini', translates literally as 'of forty' and reflects the popular belief in East Africa that the tree can cure forty diseases. It is a medium sized to large tree characterized by its short, straight pole, furrowed, dark brown to grey bark and dense rounded crown of pinnate leaves (Plate 2.2). It is an evergreen of tropics and subtropics and is widely planted throughout Asia and Africa. Neem is considered a very valuable herb for a variety of folk applications (Neem foundation). Neem is bitter in taste and one of the most effective medicinal plants in India. Environmentally it has a reputation as an air purifier and hence it is called a "wonder tree" in India. Different parts of this tree are used for example the bark, leaves, flower and seeds. These are used as infusions, powder,

medicated ghee or oil. The herb is used in inflammatory and febrile diseases as well as for worm infestations.

Neem has been used widely in Indian traditional medicine for various therapeutic purposes for many centuries. Scientific investigations carried out during the last two decades have reported antibacterial, antifungal, antiviral and antifertility properties of neem with a view to develop neem based products for human use (Neem foundation). The efficacy of neem as a broad spectrum pesticide, non-toxic to humans has important implications for its application to parasitic diseases. The bark, seeds (Neem Foundation), leaf juice and roots are all traditionally used for the treatment of worms. In particular, tender leaves are used in combination with *Piper nigrum* for intestinal helminthiasis (Chatterjee & Pakrashi, 1994). A decoction of leaves is used as a head wash to treat lice and scabies (Cole, Blackwell, Evans & Strang, 2002) and has shown good results in clinical trials among school children with headlice. There is significant phytochemical evidence for the efficacy of neem leaf and seed extracts, and isolated compounds, in the treatment of malaria, as reviewed by Wilcox & Chamberlain (2004).



Plate 2.2: Photos of mature Neem tree, leaves and mature fruits.

There is also evidence of immunomodulatory activity of neem. Willcox & Chamberlain (2004) reported that the effects of neem on the immune system are complex with some evidence pointing to an immune-stimulant effect, while other

evidence suggests that neem can act as an anti-inflammatory agent. In the case of humoral immunity, malaria infection leads to the production of specific anti-malarial IgM, IgG and IgD antibodies and it has been shown that passive transfer of IgG across the placenta protects newborns against malaria. Willcox & Chamberlain (200) reported that the mice fed on *A. indica* leaf extracts (in peanut oil) produced more IgM and IgG than controls in response to challenge with ovalbumin, and this effect was dose-related. And in rats and mice, production of antibodies in response to challenge with sheep red blood cells is increased after intraperitoneal injection of 100 mg / kg of aqueous neem bark or leaf extract, and immunosuppression due to stress was reduced (Willcox & Chamberlain, 2003). In the case of cell-mediated immunity, Upadhyay , Dhawan, Garg & Talwar (1992) found that neem seed oil injected intraperitoneally in mice induces the production of IFN- γ by spleen cells. Upadhyay *et al.*, 1992 also report that neem seed oil increases peritoneal leucocyte counts, enhances the phagocytic activity of macrophages, enhances the lymphocyte proliferative response to *in vitro* mitogen challenge, and enhances the cellular immune response to tetanus toxoid.

People in India believe that neem can purify blood, so they clean their teeth with neem twigs, apply neem leaf juice to skin disorders and drink neem tea as a tonic. It is also an ideal source of timber for carpentry as its wood is termite repellent (Neem foundation, n.d). Many bioactive ingredients have been identified and isolated the most important ones being azadiractin and meliantriol. Neem derivatives such as azadiractin and nimbicidin and other compounds are now used as pesticides. Neem is best known for its multi-malady curing powers of its leaves, twigs, bark and roots. In recent years, the ‘tree of forty cures’ has taken its rightful place as a major source for effective, eco-friendly and low cost natural pest control products. Fruit, seeds, oil, leaves, roots, bark and almost every part of the tree is bitter and contain compounds with proven antiviral, antiretroviral, anti-inflammatory, anti-ulcer and antifungal, anti-bacterial, anti-plasmodial, anti-septic, anti-pyretic and anti-diabetic properties (Subaprya& Nagini, 2005); El-Hawary, El-Tantawy, Raheh & Badr, 2013); Pandey, Ahmed, Chimwal & Pandey, 2012). Aqueous-extracts of fresh matured leaves of *A. indica* (neem) to screen for pharmacologically active chemical constituents showed high scores of saponins, tannins and glycosides (moderate scores), while alkaloids,

terpenes, flavonoids, reducing sugars, pentoses and whole carbohydrates showed low scores. Anthraquinones, ketones and monosaccharides were not detected from the extract (Biu, Yusufu & Rabo, 2009).

2.6.2 *Ekebergia capensis* Sparm

Ekebergia capensis Sparm from the family Meliaceae (The Mahogany Family) is a large, attractive and deciduous tree attaining a height of up to 30 m that has been used as a street tree in many towns and cities (Plate 2.3). It is also a good ornamental garden tree and its fruits are enjoyed by birds and mammals. It is widely distributed in the central and Nyanza regions of Kenya (Gachathi, 2007; Beentje, 1994), and is also widespread in South Africa, Swaziland, Zimbabwe, Uganda and Ethiopia. The Zulu community in South Africa uses its wood to facilitate childbirth (Sewram, Raynor, Mulholland & Raidoo, 2000). In Kenya, the Sabaot community uses its leaf macerations internally and externally to treat headache, fever, cough and skin diseases, while the Agĩkũyũ community treats diarrhea with its stem bark (Gachathi, 2007; Okello, Nyunja, Netondo & Onyango, 2010). Pharmacological studies have indicated antiplasmodial, antiinflammatory, hypotensive, uterotonic and antituberculous activities of the crude extracts of this plant (Sewram *et al.*, 2000; Kamadyapa *et al.*, 2009; Lall and Meyer, 1999; Mulaudzi, Ndhlala, Kulkarni, Finnie & Staden., 2013; Muregi *et al.*, 2004), providing scientific support for their indigenous use. Phytochemical investigations of its stem bark led to isolation of triterpenoids, steroids and flavonoids (Sewram *et al.*, 2000; Murata *et al.*, 2008; Nishiyama *et al.*, 1996).



Plate 2.3: Photos of *Ekebergia capensis* tree, mature fruits and stem bark

The safe application of *E. capensis* in traditional medicine requires the presence of metabolites with useful pharmacological properties and low toxicity levels.

The main stem of *E. capensis* is characterized by a rough light grey to almost black bark, with few buttress roots at the base. The large drooping glossy green leaves that are often tinged with a pinkish patch, or pink edges are pinnate (Figure 2.3). In favorable conditions, the trees flower (white flowers, occasionally with a pink tinge) conspicuously every year but in other localities they may only flower once every few years (SANBI, 2004). The large, round, fleshy fruits, 1 - 2 cm in diameter containing four seeds, with an onion-like taste, turn bright pink to red in autumn, attracting Knysna and Purple-crested louries, barbets, bulbuls, mouse birds and hornbills (Kumbula indigenous nursery, 2012).

Among the studies that have been carried out using this herb extract include; it's anti-plasmodial activity singly or in combination with chloroquine. *E. capensis* extract was among the plants that showed anti plasmodial activity and may be used as a source of novel anti-plasmodial compounds (Muregi *et al.*, 2004). Uterotonic properties of extracts from this tree were also evaluated in an *in vivo* study. The extract yielded compounds of which two viz, oleonic acid and 3-epioleonic acid displayed uterotonic activity. The herb has also been included in a study where 20 South African medicinal plants used to treat pulmonary diseases were screened for activity against drug resistant and drug sensitive strains of *Mycobacteria*

tuberculosis. *E. capensis* extract was among the plants that were active against the resistant strain (Lall & Meyer, 1999). The stem bark of *E. capensis* was isolated and resulted in 10 new triterpenoid compounds, ekeberins A (1), B (2), C1 (3), C2 (4), C3 (5), D1 (6), D2 (7), D3 (8), D4 (9), and D5 (10), together with 17 known compounds. Several of these compounds were screened *in vitro* against both chloroquine (CQ)-sensitive and -resistant *Plasmodium falciparum* isolates and were found to exhibit moderate anti-plasmodial activity (Murata *et al.*, 2008).

2.7 The role of cytokines in Schistosome infection

The immunopathology of schistosomiasis in humans is mediated and orchestrated by CD4 T cells specific for schistosome egg antigens (SEA), and its severity varies from person to person, as well as among inbred mouse strains. Mature *S. mansoni* worm pairs live in the portal vasculature, producing eggs, which are able to transit the lumen of vasculature to the intestines. Eggs excreted with the feces allow transmission of the infection. Since blood flows toward the liver in the portal system, many eggs fail to engage the intestine and are instead carried to the liver where they become trapped in the sinusoids (CDC, 2010). The eggs elicit a strong Th2 polarized cellular response that orchestrates the development of granulomatous lesions around tissue trapped eggs (Pearce & MacDonald, 2002). The Th2 response is essential for survival (Brunet, Finkelmann, Cheever, Kopf & Pearce, 1997; Fallon, Richardson & McKenzie, 2000; Herbert *et al.*, 2004) but also leads to hepatic fibrosis during chronic infection due to profibrotic effects of IL-13, a major Th2 cytokine (Fallon *et al.*, 2000; Chiaramonte, Donaldson, Cheever & Wynn, 1999; Wynn *et al.*, 2004). Murine schistosomiasis is characterized by Th1 reaction (with a predominant secretion of IFN- γ and minimal levels of IL-4 and IL-5) occurring during prepatency and then shifting to a Th2-based profile which develops after the onset of oviposition and persists throughout the acute phase of infection (with high IL-4 and IL-5, but low IFN- γ) (Davies *et al.*, 2004). Ironically, egg induced Th2 responses are an immunologic double-edged sword, participating in protection of host tissues from egg-induced injury (Brunet *et al.*, 1997) and in the development of the egg-induced pathology and fibrosis associated with chronic schistosome infection (Wynn and MacDonald, 2004). The natural and induced forms of severe schistosomiasis correlates with high levels of pro-inflammatory cytokines IFN- γ and IL-17 (Rutitzky

et al., 2008). This is indicative of the Th1 and Th17 subpopulations of CD4 T lymphocytes.

In the context of cytokine milieu triggered by *S. mansoni* infection, it has been suggested that IL-4 is a crucial cytokine for granuloma formation because it upregulates fibroblast chemokine, matrix protein expression, and collagen (Liu *et al.*, 2002) and reduces the cellular proliferative response to soluble egg antigens (SEA) (Corrêa-Oliveira *et al.*, 1998). IL-5 in schistosomiasis induces liver fibrosis (Reiman *et al.*, 2006). IL-10 has been shown to be a major cytokine during infection with downregulatory activity of both Th1 and Th2 T cell subpopulations (Mosmann & Moore, 1991). IFN- γ is related to the activation of macrophages and plays a key role in the protective mechanism against periportal fibrosis, whereas the proinflammatory tumor necrosis factor-alpha (TNF- α) may aggravate the disease (Booth *et al.*, 2004). Furthermore, we demonstrated that IFN- γ secretion correlates with resistance to infection, supporting a role of the Th1 response in immunity to *S. mansoni* (Viana *et al.*, 1994).

2.8 Effect of treatment on cytokine levels

As regards the cytokine profile after specific chemotherapy, it has been suggested that peripheral blood mononuclear cells (PBMC) from patients with acute infection responded to SEA and soluble worm antigen preparation (SWAP) by producing significantly higher amounts of IFN- γ and IL-10. However, IL-5 was detected only in SEA-stimulated cultures, and little or no IL-4 was detected in SEA or SWAP-stimulated cells (Montenegro *et al.*, 1999). De Jesus *et al.* (De Jesus *et al.*, 2002) suggested that most patients after specific chemotherapy for schistosomiasis spontaneously released high levels of TNF- α , IL-1, and IL-6. In addition, detectable levels of IFN- γ were present in the supernatants of unstimulated PBMC from these patients. Stimulation of PBMC from patients with acute disease only induced higher levels of IFN- γ upon SEA stimulation (De Jesus *et al.*, 2002).

Several studies have shown that treatment affects the levels of cytokines. A study to determine the effect of treatment with either oxamniquine or praziquantel on *S. mansoni* specific IFN-gamma, IL-4, IL-5 and IL-10 was compared on PBMC which were collected pretreatment, 6 and 18 weeks post treatment. The results from this

study showed a general increase in immuno-modulation post-treatment with elevated immune reactivity and cytokine production in both treatment groups. Treatment induced significant increases in levels of IL-4, IL-5 and IL-10 cytokines 6 and 18 weeks after treatment (Mduluzza *et al.*, 2009).

The production and regulation of IFN- γ , IL-2 (Th1 cytokines), IL-4, IL-5, and IL-10 (Th2 cytokines) were evaluated in urinary schistosomiasis, egg-positive patients with chronic and acute urinary schistosomiasis in comparison to a control group. The cytokines response in chronic *S. mansoni* infected patients represented significantly high level of IFN- γ , IL-2, IL-4, IL-5, and IL-10 before and after treatment compared to a control group. After treatment there was a reduction in the cytokine levels. In acute infected patients, the treatment group had the level of cytokines reduced after treatment compared with control group (Elfadil *et al.*, 2015).

2.9 Cytometric Bead Array (CBA) assay

BD-CBA assays provide a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry. Each capture bead in a BD CBA kit has been conjugated with a specific antibody. The detection reagent provided in the kit (Plate 2.4 and Plate 2.5) is a mixture of phycoerythrin (PE)-conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) are formed. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector.

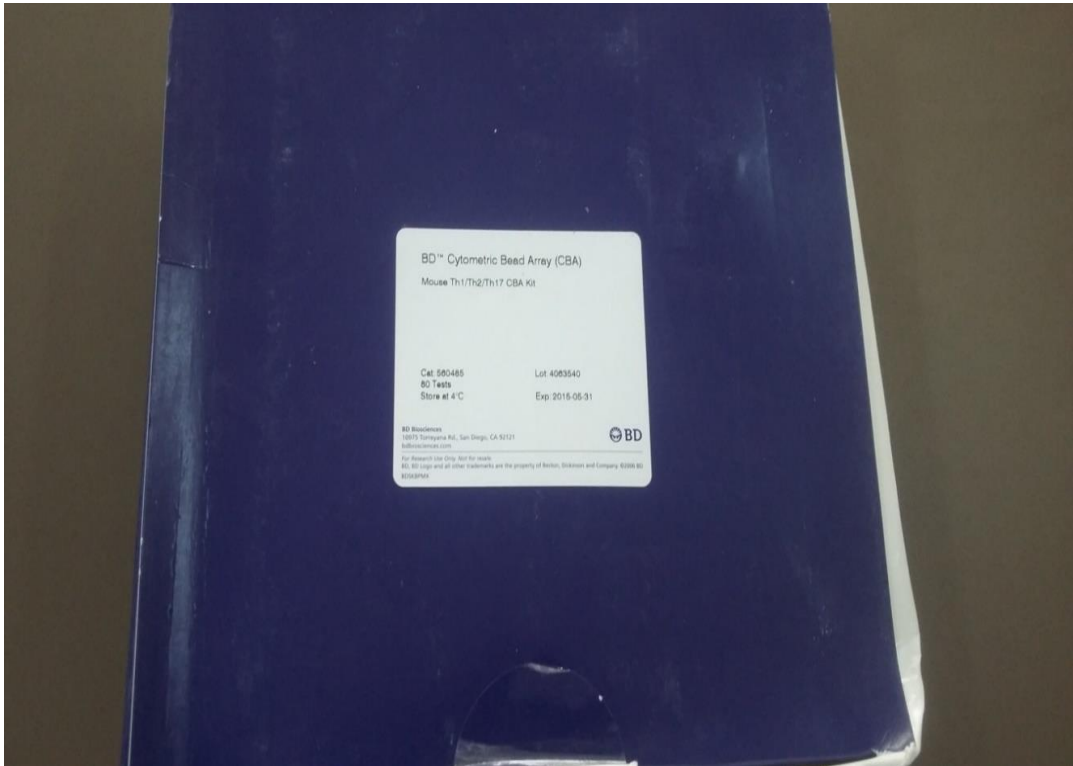


Plate 2.4: BD™ Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Kit.



Plate 2.5: The reagents in the BDTM Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Kit.

The BD CBA Mouse Th1/Th2/Th17 Cytokine Kit uses bead array technology to simultaneously detect multiple cytokine proteins in aqueous samples. Seven bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-2, IL-4, IL-6, IFN- γ , TNF, IL-17A, and IL-10 cytokines. The seven bead populations are mixed together to form the bead array, which is resolved in a red channel (FL3 or FL4) of a flow cytometer. During the assay procedure, cytokine capture beads are mixed with recombinant standards or unknown samples and incubated with the PE-conjugated detection antibodies to form sandwich complexes. The intensity of PE fluorescence of each sandwich complex reveals the concentration of that cytokine. After acquiring samples on a flow cytometer, FCAP ArrayTM software is used to generate results in graphical and tabular format (Plate 2.6). The BD CBA Mouse Th1/Th2/Th17 Cytokine Kit can be used to measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interferon- γ (IFN- γ), Tumor Necrosis Factor (TNF- α), Interleukin-17A (IL-17A), and Interleukin-10 (IL-10) protein levels in a single sample. The kit performance has been optimized for analysis of physiologically relevant concentrations (pg/mL levels) of specific cytokine proteins in tissue culture supernatants and serum samples.

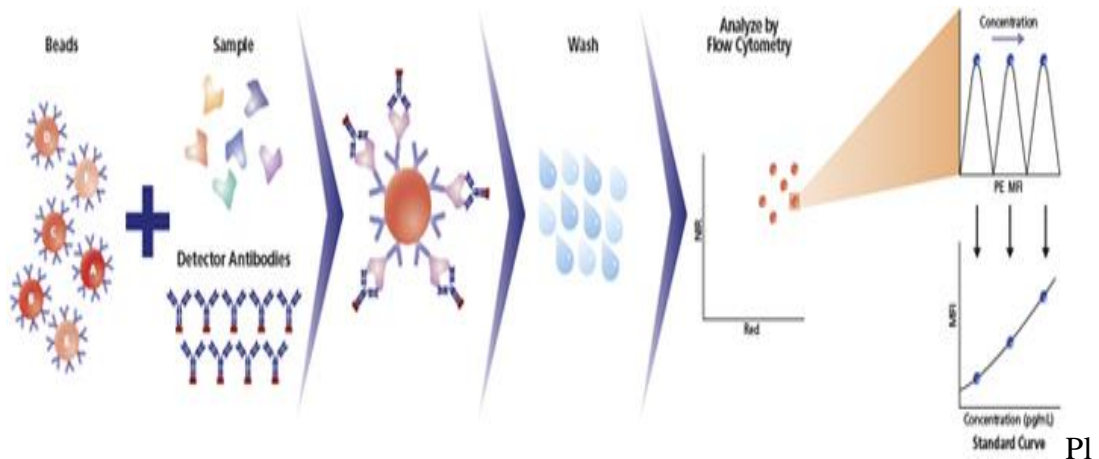


Figure 2.6: The steps in BD™ Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 assay.

Generally, ELISA is considered as the gold standard for measuring the secreted proteins from biological fluids, such as plasma or serum (Frank, Carroll, Allee & Zannelli, 2003; Elshaland & McCoy, 2006; Williams, Collier, Carroll, Welsh & Laurenz, 2009). However, only one cytokine for one ELISA can be detected; thus, ELISA is costly in terms of monetary expense, time, and the number of sample aliquots required. Recently, an increasing number of CBA techniques, which have showed more advantages than ELISA, have been reported (Wyns *et al*, 2013; Williams, Steffens, Reinecke & Meyer, 2013; Yujie *et al*, 2012; Talat, Shahid, Perry, Dawood & Hussain 2011; Sadakane, Takano, Ichinose, Yanagisawa & Shibamoto, 2002). CBA assay has advantages over ELISA. The broad dynamic range of fluorescent detection via flow cytometry and the efficient capturing of analytes via suspended particles enable the BD-CBA assay to measure the concentration of an unknown in substantially less time and using fewer sample dilutions compared to conventional ELISA methodology. The required sample volume is approximately one seventh the quantity necessary for conventional ELISA assays due to the detection of seven analytes in a single sample. A single set of diluted standards is used to generate a standard curve for each analyte. A BD CBA experiment takes less time than a single ELISA and provides results that would normally require seven conventional ELISAs (BD-mouse Th1/Th2/Th17 kit manual). In addition, cytokine standards of known concentration are run alongside the samples to allow for quantitation. The CBA kit decreases handling steps which translates into less

handling errors. Using a commercial kit with internal standards helps in standardizing the data.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Maintenance of *S. mansoni*

Schistosoma mansoni infected Swiss albino *Mus musculus* mice from Schistosomiasis Laboratory's life cycle maintenance at the KEMRI animal maintenance facility was the source of the parasite used in this study. The livers of 3 mice were processed in order to infect thirty *Biomphalaria pfeifferi* snails in the laboratory. Briefly, the mice were injected intra peritoneally (i.p) with 0.2ml of sodium pentobarbitone (1:20) to humanely kill them. The mice were then dissected to expose and remove the livers with the use of dissecting scissors and forcep. The liver tissues were homogenized and filtered through a bank of sieves (750, 250 and 125 μ m). The filtrate containing eggs was poured into a large side-arm flask filled with water and placed in light for 5 minutes to illuminate the eggs and stimulate hatching. This flask was then covered with foil leaving the side-arm (MacInns, 1970). Freshly hatched miracidia (less than 1hour old) were used to infect *B. pfeifferi* snails obtained from snail maintenance facility at KEMRI. Thirty snails of 4mm diameter in size were infected using the routine optimized technique used for schistosome cycle maintenance at the facility. After this the infected snails were transferred into freshly prepared and well labeled aquariums of standard size (18cm x 30 cm x 24 cm) with a stocking rate of 48 snails and maintained with lettuce and bone meal for 28 days.

3.2 Infection of mice

The infected snails were placed under a lamp for illumination to enhance cercariae shedding. Protective clothing: laboratory coat, gloves and forceps, were used while handling infected snails and cercariae to avoid infection by cercariae. Five, 50 μ l subsamples of the cercariae suspension were stained with Lugol's iodine and enumerated under a dissecting microscope to estimate the number of cercariae in the suspension. Male Swiss albino *Mus musculus* mice weighing between 20-22g were obtained from the KEMRI animal maintenance facility. Each murine model was

infected with 90 cercariae using the abdominal ring method as described by Smithers and Terry 1965. Freshly shed cercariae less than 1 hour old were used in order to ascertain their viability. Briefly, the mice were restrained by anaesthetizing them by injecting them with 0.06ml of sodium pentobarbitone (1:20 dilution) intra peritoneally using a 1ml syringe and 25G needle. Once they were fully anaesthetized, the abdominal skin area was shaved using a barber's clipper and laid on their back on an exposure board (Plate 3.1).



Plate 3.1: Infection of rodents using the ring method.

The shaved abdominal skin area was wiped with cotton wool soaked in dechlorinated water and metal rings placed on the shaved area. The volume of cercariae suspension containing 90 cercariae was then poured into the ring using a micro-pipette. These mice were left undisturbed for 60 minutes to allow cercariae to penetrate. The remaining water in the rings was removed using a micropipette and the ring removed using a pair of forceps. The infected mice were transferred into already prepared holding cage containing wood shavings as bedding and maintained with mice pellets and water ad libitum.

3.3 Experimental Design

This was an experimental study design. Infected mice were randomized into groups of 6 and placed in separate well labelled cages. Three experiments were carried out to target different developmental stages. Treatments with the plant extracts were done at 2, 4 and 7 weeks post infection (p.i) to represent juvenile, immature adults and adult worms respectively. The plant extracts were administered at doses of 25, 50, 100, 200 and 400 mg/kg. An infected untreated group (negative control) and group treated with the standard drug (positive control) were included in each experiment setup. Each experiment set up (i.e. 2 weeks, 4 weeks and 7 weeks) had 6 mice randomly allocated in each of the 7 treatment cages for 25, 50, 100, 200 and 400 mg/kg for plant extract and an infected untreated group (negative control) and group treated with the standard drug (positive control) giving a total of 42 mice per experiment. A total of 126 mice were used for the 3 experiments. The experiments were done in duplicates. This sample size was arrived at by following similar studies on *in vivo* effects of drugs. Each mouse was marked individually using ear tags containing different numbers and records kept to avoid mixing up the animals during the experiments.

3.4 Extraction of plant parts

The test plants *E. capensis* and *A. indica* were collected from central Kenya (Mount Kenya Forest) and Southern Rift Valley (Nguruman Escarpment in Magadi) respectively. The plants were identified by a taxonomic botanist from East African Herbarium in Nairobi, where they were catalogued and voucher specimens deposited (*E. capensis*: Stem bark (Ec-SB/04) 26 and *A. indica*: Leaf (Ai-L/04) 10). The plant samples were then air dried at room temperature under shade and ground to powder using an electric mill. The powder was packed into one kilogram packs and stored in a dry and well ventilated room until use. The plant parts used were bark for *E. capensis* and leaves for *A. indica*. The dried chaff of the plant parts to be used (50 g of each) was weighed and soaked in 500 ml of water (water extract). This was followed by thorough mixing on a shaker and soaking for 12 hours. The mixture was then filtered and filtrate freeze dried to give at least 2g of dry solid material (water extract).

3.5 Antischistosomal effect of the herbs

Treatment was done by administering the mice with different doses of the herbal extracts orally by gavage using stainless steel needles (Plate 3.2). A control group was treated with artemether at 200mg/kg orally by gastric gavage at a dose volume of 0.2ml for 3 consecutive days (Utzinger *et al.*, 2002).



Plate 3.2: Oral treatment of experimental mice.

Another control group was treated with PZQ at a concentration of 200mg/kg body weight orally at a dose volume of 0.05ml for 5 consecutive days to give a total of 1000mg/kg body weight (Gönnert & Andrews, 1977). An infected control group was included and was administered 0.2 ml of 1% DMSO (1% of DMSO was used to prevent harmful effects of DMSO on the animal) for 3 consecutive days because it was used to dissolve all the drugs. The experiments were not blinded. This treatment was done at 14 (to target juvenile worms), 28 days (to target immature adults worms) and 49 days post infection (to target adult worms).

3.6 Collection of blood

On the day of treatment, blood samples were collected from the tail ends of all the infected mice on days 14, 28 and 49 post infection (pi) from juvenile and adult worm groups respectively then treatment was started on the same day (few hours after bleeding). Blood samples were collected again on day 20 (one day before perfusion) that is on day 34, 48 and 69 from juvenile, immature adults and adult worm groups respectively. Briefly, the tip of the tail was nicked off (2-3mm) using a sterile sharp pair of scissors. The tail was gently massaged and blood collected drop by drop into a sterile 300µl eppendorf tube. This procedure was performed aseptically to avoid infection. To enhance blood flow the mice placed near a table lamp or their tails were dipped in warm water to dilate the tail vein. All blood samples were placed on ice to enhance blood clotting and spun in a microfuge at 1500rpm for 30 minutes to separate serum. The serum was pipetted out using a micropipette and sterile tips into 200µl storage tubes, labeled and stored at -80°C until analysis of cytokine profiles. A maximum volume of 300µl was collected from each mouse. For collection of the second blood sample (before perfusion), the wounds at the tips of the tails were reopened by cutting 2-3mm using a sterile pair of scissors and bled the same way as before.

3.7 Worm load counts

Adult worms were recovered by perfusion as described by Smithers and Terry (1965). Perfusion was done 21 days post treatment on day 70 for adult worm groups, day 49 for immature adults and 35 for juvenile worm groups. Briefly, mice were injected with 0.25ml of heparinised Sagatal (heparin + sodium pentobarbitone) intraperitoneally to euthanize them. Once the animals were under deep anesthesia, the mice were dissected and perfusion fluid (citrate saline) pushed through the left ventricle of the heart using a gauge 18 needle and 50ml syringe. All the worms were collected in a sieve placed in position below the mouse on the perfusion stand as shown in Plate 3.3. These worms were washed off into labeled petri dishes. The number of male and female worms were counted using a dissecting microscope and recorded. Livers and guts from the perfused mice were removed and placed in separate labeled petri dishes and later wrapped with aluminium foil paper and

labelled for storage in a freezer at -20°C . The efficacy of the drug regimens was calculated as the mean number of recovered worms from each animal relative to the control groups as shown in the formula below by Xiao *et al* (2000):

$$\% \text{ worm burden reduction} = \frac{(\text{Mean\#worm in control group}) - (\text{Mean\#worms in treatment group})}{(\text{Mean\#worm in control group})} \times 100$$



Plate 3.3: Perfusion for recovery of adult worms from infected rodents.

3.8 Tissue egg load count

The liver and guts from the perfused animals that had been frozen were processed for tissue egg load count by trypsin method. Briefly, the tissues were weighed, chopped using a sharp blade and poured into a kitchen blender. 100ml of normal saline was added and the tissue homogenized into a fine emulsion. 10mg of trypsin was added into the emulsion and mixed thoroughly. The emulsion was poured into 200ml beaker, covered using a Para film, labeled and place into an incubator at 37°C for 2 hours with occasional shaking. After digestion the emulsion was poured into 50ml centrifuge tubes and spun at 1500 rpm for 10 minutes. This was repeated and supernatant was poured off to remain with an egg pellet. The egg pellet was

resuspended in 15ml normal saline. This was shaken and four 50 μ l subsamples picked using a micro pipette and placed onto glass slides. Coverslips were placed on top of these subsamples and eggs counted under a compound microscope at 10X magnification. A mean was calculated and multiplied by 20 to get the number of eggs per ml. This was multiplied by the volume of the egg suspension to get the number of eggs in the tissue.

3.9 Cytometric Bead Array (CBA) assay

Cytokine analysis was done using the BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit which allowed for the simultaneous detection of IL-2, IL-4, IL-6, IL-10, TNF- α , IFN- γ and IL-17A. Aliquots of sera that had been frozen at -80 $^{\circ}$ C were thawed and CBA analysis performed as per the manufacturer's instructions.

3.9.1 Reconstitution and serial dilution of standards

Cytokine standards were serially diluted to facilitate the construction of calibration curves necessary for determining protein concentrations of test samples. Briefly, one vial of lyophilized Mouse Th1/Th2/Th17 standards was opened and the contents transferred into a 15ml conical polypropylene tube. This tube was labeled "Top Standard". The standards were reconstituted using 2ml of Assay Diluent and allowed to equilibrate for at least 15 minutes at room temperature. The reconstituted protein was mixed by gentle pipetting. 12 x 75-mm tubes were labeled and arranged in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256. Assay Diluent (300 μ l) was pipetted into each of the labeled tubes. A serial dilution was performed whereby 300 μ l was transferred from the Top Standard to the 1:2 dilution tube, mixed thoroughly and transferred 300 μ l to the 1:4 tube and so on to the 1:256 tube. Mixing was done using gentle pipetting only and not vortexing. One 12 x75-mm tube containing only Assay Diluent was prepared to serve as the 0 pg/ml negative control.

3.9.2 Mixing Mouse Th1/Th2/Th17 Cytokine Capture Beads

The number of assay tubes required for the experiment was determined including standards and controls for example 70 unknowns, 9 cytokine standard dilution and 1

negative control = 80 assay tubes. Each Capture Bead suspension was vigorously vortexed for 3 to 5 seconds before mixing. Vortexing the vial before taking a bead suspension aliquot was done because the antibody-conjugated beads settled out of suspension over time. A 10 μ l aliquot of each Capture Bead for each assay tube to be analyzed was added into a single tube labeled “mixed Capture Beads” (10 μ l of IL-2 capture beads x 80 tubes = 800 μ l of IL-2 Capture Beads required). The beads were vortexed thoroughly.

3.9.3 Performing the Mouse Th1/Th2/Th17 Cytokine Assay

After vigorous vortexing, 50 μ l of the mixed Capture Beads was added to all the assay tubes. To the control tubes, 50 μ l of the Mouse Th1/Th2/Th17 Cytokine Standard dilutions was added. Unknown samples (50 μ l) were added to the appropriately labeled sample assay tubes. 50 μ l of the Mouse Th1/Th2/Th17 PE Detection Reagent was added to all the assay tubes. The assay tubes were incubated for 2 hours at room temperature, protected from light and during the incubation time the cytometer setup was performed. 1ml of washing buffer was added to the assay tubes and centrifuged at 200g (1500rpm) for 5 minutes. The supernatant was carefully aspirated and discarded from each tube. 300 μ l of the Wash Buffer was added to each assay tube to re-suspend the bead pellet.

The samples were acquired on the flow cytometer the same day they were prepared since prolonged storage of the samples once the assay is complete would result to increased background and reduced sensitivity. To facilitate analysis of samples using FCAP Array software, the manufacturer’s recommendations were followed in that the samples were acquired from the lowest (0 pg/ml) to the highest (Top Standard) concentration followed by the test samples. All the Flow Cytometer Standard (FCS) files (standards and samples) were stored in a single folder for analysis using FCAP v3 software.

3.10 Data Analysis

All the data collected was entered in both a laboratory record book and Microsoft excel sheets for storage. All the data was backed up in both flash disks and hard drive storage devices to avoid loss of the data. The number of worms recovered from the

experimental groups was expressed as mean \pm SD. The efficacy of the drug regimens was calculated as the mean number of recovered worms from each group relative to the control group (Xiao *et al.*, 2000). The mean number of worms and eggs recovered from the different groups were subjected to Student's t-test using Microsoft Excel® to determine their statistical significance in comparison with the control groups. The data was considered significant if $P < 0.05$, highly significant if $P < 0.01$ and very highly significant if $P < 0.001$.

The Mouse Th1/Th2/Th17 Cytokine data was analyzed using FCAP v3 Array Software and the results saved in Excel sheets for statistical analysis to be conducted. The mean level of cytokines post treatment in each of the groups was used to calculate percentage reduction and was also subjected to Student's t-test using Microsoft Excel® to determine their statistical significance in comparison with the control groups. The data was considered significant if $P < 0.05$, highly significant if $P < 0.01$ and very highly significant if $P < 0.001$.

CHAPTER FOUR

RESULTS

4.1 *In vivo* antischistosomal activity of *A. indica* and *E. capensis* against adult worms (7 weeks p.i) in *S. mansoni* infected mice.

The worm burden and distribution for all the *S. mansoni* infected mice treated with *E. capensis* and *A. indica* 7 weeks p.i at doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg is shown on Table 4.1. These results are representative of two similar experiments. PZQ (at a dose of 1000mg/kg) treated mice showed a very highly significant difference ($P<0.001$) in the mean total worm burden, number of eggs in liver and number of eggs in the intestines (Table 4.1). The total worm burden was reduced by 94% while the egg load reduction for liver and intestines was 99% and 99% respectively (Figure 4.1) for the PZQ treatment group in comparison with the infected untreated (control) group.

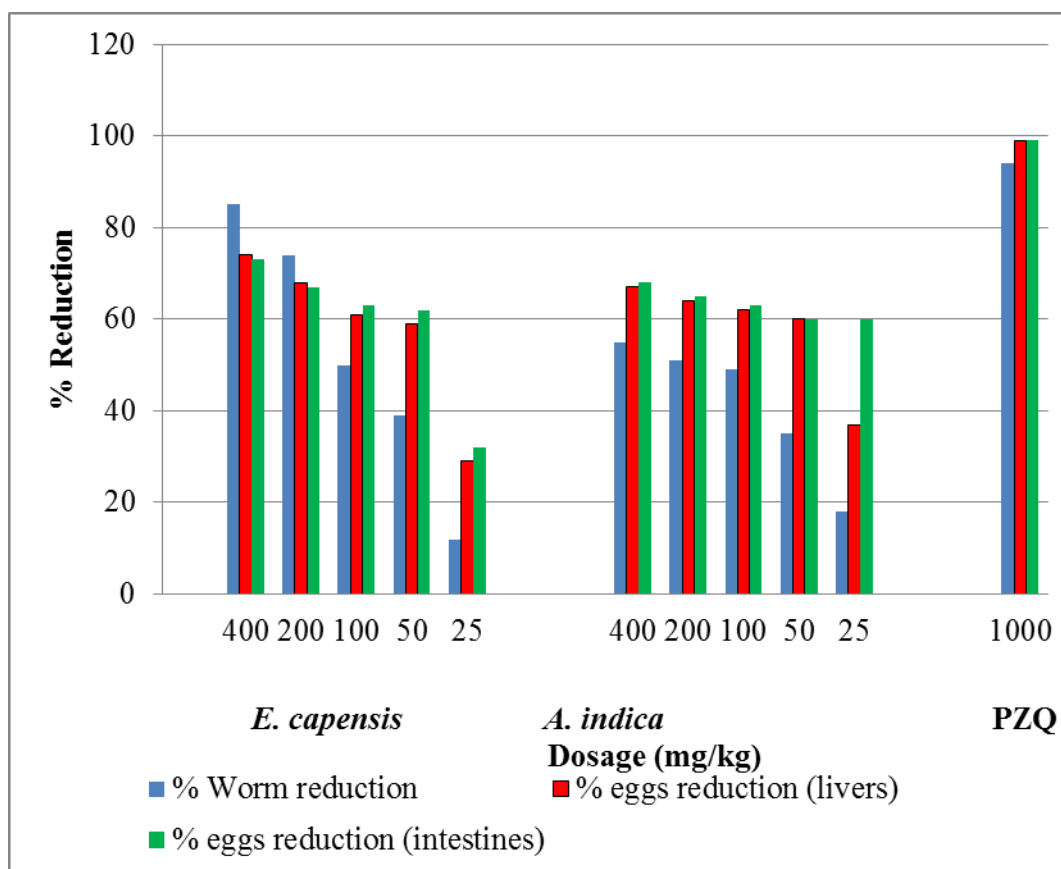
Mice treated with *E. capensis* 49 days p.i induced mean worm burden reductions as follows: 12%, 39%, 50%, 74% and 85% at 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively (Figure 4.1). This was statistically significant ($P<0.05$) at 25mg/kg, highly significant ($P<0.01$) at 50mg/kg and very highly significant ($P<0.001$) at 100mg/kg, 200mg/kg and 400mg/kg in the mean worm burden reductions in comparison with the infected untreated (control) group (Table 4.1). The percentage egg load reduction in the livers was recorded as 29%, 59%, 61%, 68% and 74% at doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively (Figure 4.1). This was highly significant ($P<0.01$) at 25mg/kg, 50mg/kg, 100mg/kg and very highly significant ($P<0.001$) difference at 200mg/kg and 400mg/kg when compared to the infected untreated (control) group (Table 4.1). The corresponding intestinal percentage egg load reduction was recorded as 32%, 62%, 63%, 67% and 73% at doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively (Figure 4.1). This reduction was highly significant ($P<0.01$) at 25mg/kg and very highly significant reduction ($P<0.001$) at 50mg/kg, 100mg/kg,

200mg/kg and 400mg/kg compared to the infected untreated (control) group (Table 4.1). In comparison to the PZQ (positive control) group, *E. capensis* at 25mg/kg, 50mg/kg, 100mg/kg and 200mg/kg showed very highly significant ($P<0.001$) difference in the mean worm loads but no significant difference ($P>0.05$) at a dose of 400mg/kg. In the liver mean egg load, *E. capensis* at doses of 25mg/kg, 50mg/kg, 100mg/kg and 200mg/kg showed very highly significant ($P<0.001$) difference but no significant difference ($P>0.05$) at 400mg/kg. In the intestinal egg loads, *E. capensis* at all the doses (25mg/kg-400mg/kg) showed very highly significant ($P<0.001$) difference in comparison the PZQ group (Table 4.1).

Table 4.1. Mean number of worms and eggs recovered from livers and intestines following treatment with *E. capensis* and *A. indica* at 7 weeks post infection

Drug	Number of Male worms (Mean±SD)	Number of Female worms (Mean±SD)	Total Number of worms (Mean±SD)	Number of eggs in the livers (Mean±SD)	Number of eggs in the intestines (Mean±SD)
<i>E. capensis</i>					
400mg/kg	5.8±3.7	4.8±3.3	10.6±6.9 ^{a***b}	10292±1101 ^{a***,b}	11744±1604 ^{a***,b***}
200mg/kg	10.4±1.1	8±1.6	18.4±2.7 ^{a***,b***}	12450±1766 ^{a***,b***}	13428±1858 ^{a***,b***}
100mg/kg	18.8±4.1	16.4±4.4	35.2±8.2 ^{a***,b***}	15158±1970 ^{a***,b***}	15694±1323 ^{a***,b***}
50mg/kg	22.2±2.3	21.4±1.5	43.6±3.5 ^{a***,b***}	15772±1371 ^{a***,b***}	16110±3079 ^{a***,b***}
25mg/kg	29.4±3.1	33±1.6	62.4±4.1 ^{a*,b***}	27858±5442 ^{a***,b***}	29298±5725 ^{a***,b***}
<i>A. indica</i>					
400mg/kg	17±4.6	14.6±2.8	31.6±4 ^{a***,b***}	12876±2643 ^{a***,b}	13603±2819 ^{a***,b***}
200mg/kg	17.4±4.4	17.4±4.1	34.8±8.4 ^{a***,b***}	14005±2666 ^{a***,b***}	14872±3046 ^{a***,b***}
100mg/kg	19±5.3	16.6±6.3	35.6±11.3 ^{a***b**}	14932±3546 ^{a***,b***}	15974±5229 ^{a***,b***}
50mg/kg	23.8±2.4	22.4±3.6	46.2±5.4 ^{a***b***}	15577±1448 ^{a***,b***}	17222±1759 ^{a***,b***}
25mg/kg	30.8±2.1	27.2±3.7	58±5.6 ^{a*,b***}	24545±3326 ^{a***,b***}	27254±3181 ^{a***,b***}
PZQ	1.8±1.7	1.8±1.6	3.6±3.3 ^{a***}	278±288 ^{a***}	408±314 ^{a***}
Control	36.4±4.1	34.6±2.6	71±6.4	39142±4963	42790±4827

Data are expressed as Mean±SD, n=5. Data were analyzed by t-test: two sample assuming unequal variance. Values with superscript letters: ^a compared to the infected untreated (negative control) group and ^b compared to PZQ (positive control) group. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$



Percentage reduction (mean numbers of recovered worms and eggs relative to the infected untreated control group, n=5. A representative of 2 similar experiments.

Figure 4.1: Percentage worm and egg reduction after treatment with aqueous extracts of *A. indica* and *E. capensis* 7 weeks post infection.

Mice treated with *A. indica* 49 days post infection showed 18%, 35%, 49%, 51% and 55% at 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively in the mean worm burden reduction in comparison to the infected untreated (control group) (Figure 4.1). This was highly significant ($P < 0.01$) worm load reduction at 25mg/kg and very highly significant ($P < 0.001$) worm load reduction at 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg when compared to the infected untreated (control group) Table 4.1. The percentage egg load reduction in the livers of mice treated with *A. indica* at 7 weeks p.i was 37%, 60%, 62%, 64% and 67% at doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively (Figure 4.1). This was very highly significant ($P < 0.001$) difference at all the doses when compared to the infected untreated (control) group (Table 4.1). On the other hand, the corresponding percentage intestinal egg load reduction was 60%, 60%, 63%, 65% and 68% at the

doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively (Figure 4.1). This was very highly significant ($P<0.001$) at all the doses when compared to the infected untreated (control) group (Table 4.1). In comparison with PZQ group, treatment with *A. indica* showed very highly significant difference ($P<0.001$) in mean worm load reduction at 25mg/kg, 50mg/kg, 200mg/kg and 400mg/kg but highly significant difference ($P<0.01$) at 100mg/kg. The liver mean egg load reduction at 25mg/kg, 50mg/kg, 100mg/kg and 200mg/kg showed very highly significant ($P<0.001$) difference but no significant difference ($P>0.05$) at 400mg/kg. In the intestinal mean egg load reduction, *A. indica* at 25mg/kg and 50mg/kg, 200mg/kg and 400mg/kg showed very highly significant difference ($P<0.001$) and highly significant difference ($P<0.01$) at 100mg/kg (Table 4.1).

4.2 *In vivo* antischistosomal activity of *A. indica* and *E. capensis* against juvenile worms (2 weeks p.i) in *S. mansoni* infected mice

The worm burden and distribution for all the *S. mansoni* infected mice treated with *E. capensis* and *A. indica* at 2 weeks p.i at doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg is shown in Table 4.2. Artemether (ART) was used in this experiment as a positive control drug since studies have reported that it is effective on juvenile schistosome infections. These results are representative of two similar experiments. ART at a dose of 200mg/kg showed a very highly significant ($P<0.001$) difference in the mean worm load for total number of worms, number of eggs in the liver and number of eggs in the intestines (Table 4.2). The total worm load was reduced by 88%, while the egg load reduction for liver and intestines was 85% and 89% respectively (Figure 4.2) in comparison with the infected untreated (control) group. This difference was very highly significant ($P<0.001$) (Table 4.2).

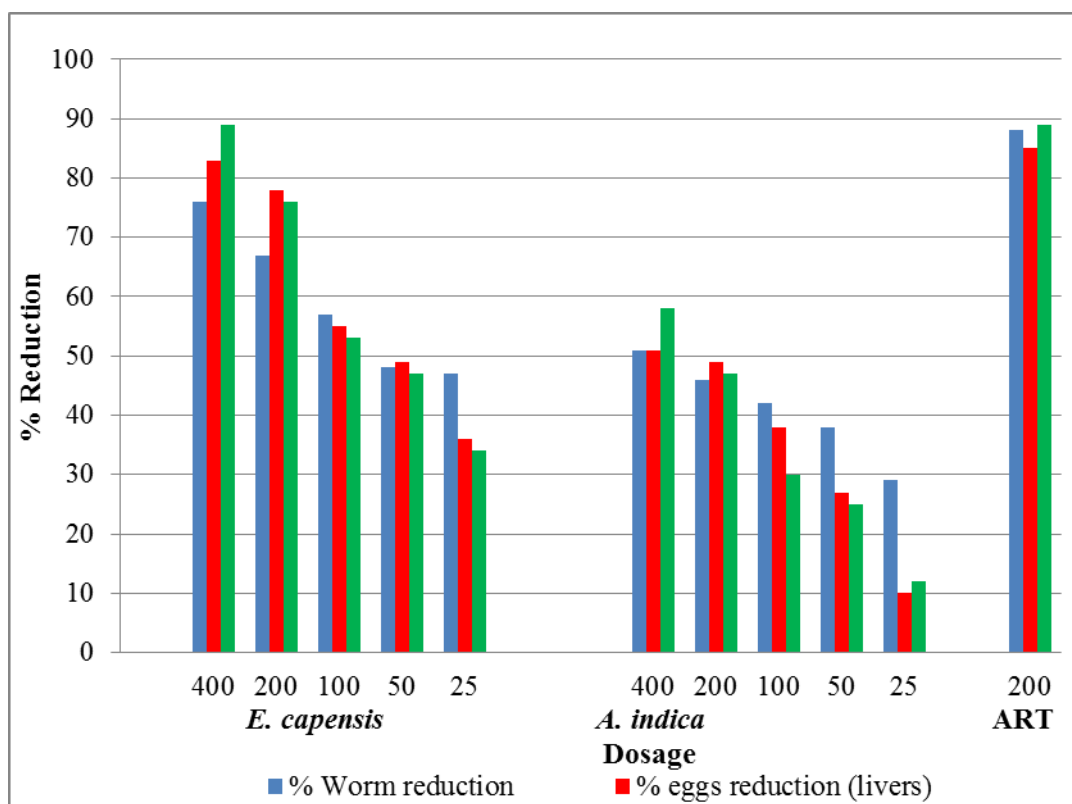
Mice treated with *E. capensis* 2 weeks p.i at doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg induced worm load reduction of 47%, 48%, 57%, 67% and 76% respectively. This was very highly significant ($P<0.001$) at doses of 100mg/kg, 200mg/kg and 400mg/kg, highly significant ($P<0.01$) at 25mg/kg and 50mg/kg when compared to the infected untreated (control) group (Table 4.2). The percentage mean egg load reduction in the liver was recorded as 36%, 49%, 55%, 78% and 83% at 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively (Figure 4.2).

This reduction was very highly significant ($P<0.001$) at the doses of 200mg/kg and 400mg/kg, highly significant ($P<0.01$) at the doses of 50mg/kg and 100mg/kg and significant ($P<0.05$) at 25mg/kg when compared to the infected untreated (control) group (Table 4.2). The percentage mean egg load reduction in the intestines was 34%, 47%, 53%, 76% and 89% at the doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively. This reduction was very highly significant ($P<0.001$) at 200mg/kg and 400mg/kg, highly significant ($P<0.01$) at 50mg/kg and 100mg/kg and significant ($P<0.05$) at 25mg/kg (Table 4.2) compared to the infected untreated (control) group.

Table 4.2. Mean number of worms and eggs recovered from livers and intestines following treatment with *A. indica* and *E. capensis* at 2 weeks post infection

Drug	Number of Male worms (Mean±SD)	Number of Female worms (Mean±SD)	Total Number of worms (Mean±SD)	Number of eggs (livers) (Mean±SD)	Number of eggs (intestines) (Mean±SD)
<i>E. capensis</i>					
400mg/kg	6.6±2.7	4.6±3	10.6±5.3 ^{a***b}	2580±753 ^{a***b}	1640±474 ^{a**b}
200mg/kg	8.4±1.1	6±2.1	14.4±3 ^{a***b}	3370±744 ^{a***b}	3735±1545 ^{a**b}
100mg/kg	11.2±2.3	8±2.5	19.2±4.3 ^{a***b}	6900±897 ^{a**b}	7284±406 ^{a***b}
50mg/kg	12±3.4	11±4.8	23±7.8 ^{a**}	7772±586 ^{a**b}	8176±1368 ^{a***b}
25mg/kg	14±1.6	9.6±1.1	23.6±2.6 ^{a**b}	9752±934 ^{a b}	10330±556 ^{a b}
<i>A. indica</i>					
400mg/kg	10.4±2.1	11.2±3.3	21.6±4.1 ^{a***b}	7350±1199 ^{a**b}	6572±724 ^{a**b}
200mg/kg	11.4±2.1	12.4±4	23.8±3.8 ^{a***b}	7784±811 ^{a**b}	8190±1097 ^{a**b}
100mg/kg	12.6±2.7	13±2.1	25.6±3.8 ^{a***b*}	9440±854 ^{a**b}	10895±1998 ^{ab}
50mg/kg	13.8±2.8	13.6±2.9	27.4±3.4 ^{a***b*}	11128±859 ^{a b}	11697±1103 ^{ab}
25mg/kg	15.6±3	16±2	31.6±4.8 ^{a***b**}	15172±1302 ^{ab}	13709±581 ^{a b}
Artemether	9.8±2.4	8±3.1	17.8±5.3 ^{a***}	2300±245 ^{a***}	1638±385 ^{a***}
Control	29±3.5	25±4.3	54±6.9	15307±549	15572±1342

Data are expressed as Mean±SD, n=5. Data were analyzed by t-test: two sample assuming unequal variance. Values with superscript letters: ^a compared to the infected untreated (negative control) group and ^b compared to ART (positive control) group. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$



Percentage reduction (mean numbers of recovered worms and eggs relative to the infected untreated control group. n=5. A representative of 2 similar experiment.

Figure 4.2: Percentage worm and egg reduction after treatment with aqueous extracts of *A. indica* and *E. capensis* 2 weeks post infection.

The mice treated with *A. indica* 2 weeks p.i showed 29%, 38%, 42%, 46% and 51% reduction at 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively in the percentage mean worm burden reduction in comparison to the (infected untreated) control group (Figure 4.2). This reduction was very highly significant ($P < 0.001$) at 200mg/kg and 400mg/kg, highly significant ($P < 0.01$) at 25mg/kg, 50mg/kg and 100mg/kg (Table 4.2). When compared to the ART (positive control) group, there was no significant difference ($P > 0.05$) in the reduction of worm load at 200mg/kg and 400mg/kg but a significant mean worm reduction ($P < 0.05$) was recorded at 25mg/kg, 50mg/kg and 100mg/kg (Table 4.2).

The percentage reduction in mean egg load in the livers of the mice treated with *A. indica* at 2 weeks p.i was 10%, 27%, 38%, 49% and 51mg/kg at 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively (Figure 4.2). This reduction was highly significant ($P < 0.01$) at 100mg/kg, 200mg/kg and 400mg/kg doses but not

significant ($P>0.05$) at 25mg/kg and 50g/kg when compared to the infected untreated control group (Table 4.2). This reduction was not significantly different from the ART (positive) control group. The corresponding percentage mean egg load reduction in the intestines was 12%, 25%, 30%, 47% and 58% at the doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively. This reduction was significantly different ($P<0.05$) at the doses of 200mg/kg and 400mg/kg but not significantly different ($P>0.05$) at 25mg/kg, 50mg/kg and 100mg/kg when compared to the control (infected untreated) group. This reduction was not significantly different when compared to the ART (positive) control group (Table 4.2).

4.3 *In vivo* antischistosomal activity of *A. indica* and *E. capensis* against immature worms (4 weeks p.i) in *S. mansoni* infected mice

The mean worm burden and distribution for all the *S. mansoni* infected mice treated with *E. capensis* and *A. indica* at 4 weeks p.i at doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg is shown in Table 4.3. ART was also used as a positive control in this experiment. These results are representative of two similar experiments. Treatment with ART at a dose of 200mg/kg showed mean percentage reduction of 60%, 88% and 89% egg load in worm load, egg load in livers and egg load in intestines respectively. This reduction was very highly significantly different ($P<0.001$) when compared to the control (infected untreated) group (Table 4.3).

Mice treated with *E. capensis* at 4 weeks p.i recorded percentage mean worm load reductions of 4%, 34%, 52%, 77% and 82% at the doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively (Figure 4.3). This reduction was very highly significantly different ($P<0.001$) at 100mg/kg, 200mg/kg and 400mg/kg, highly significantly different ($P<0.01$) at 50mg/kg and not significantly different ($P>0.05$) at 25mg/kg (Table 4.3). When compared to the ART group, the results showed very highly significant ($P<0.001$) difference at 25mg/kg, 50mg/kg and 100mg/kg but no significant difference ($P>0.05$) at 200mg/kg and 400mg/kg. The percentage mean egg load reduction in the livers was 37%, 54%, 73%, 89% and 91% at the doses of 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively.

This reduction was a very highly significant different ($P<0.001$) at 200mg/kg and 400mg/kg, highly significant different ($P<0.01$) at 50mg/kg and 100mg/kg and significantly different ($P<0.05$) at 25mg/kg in comparison to the infected untreated (control) group. When compared to the ART (positive control) group, the results showed very highly significant ($P<0.001$) difference at 25mg/kg, 50mg/kg, 100mg/kg and 400mg/kg but highly significant ($P<0.01$) at 200mg/kg (Table 4.3). Percentage mean egg reduction in the intestines was 40%, 51%, 67%, 87% and 91% at 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg. This reduction was very highly significantly different ($P<0.001$) at 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg (similar to the reduction in ART group) when compared to the infected untreated (control) group. Reduction was not significantly different ($P>0.05$) at 25mg/kg. In comparison to the ART (positive control) group, the reduction was very highly significantly ($P<0.001$) different at 50mg/kg, 100mg/kg and 200mg/kg and highly significantly different ($P<0.01$) at 400mg/kg.

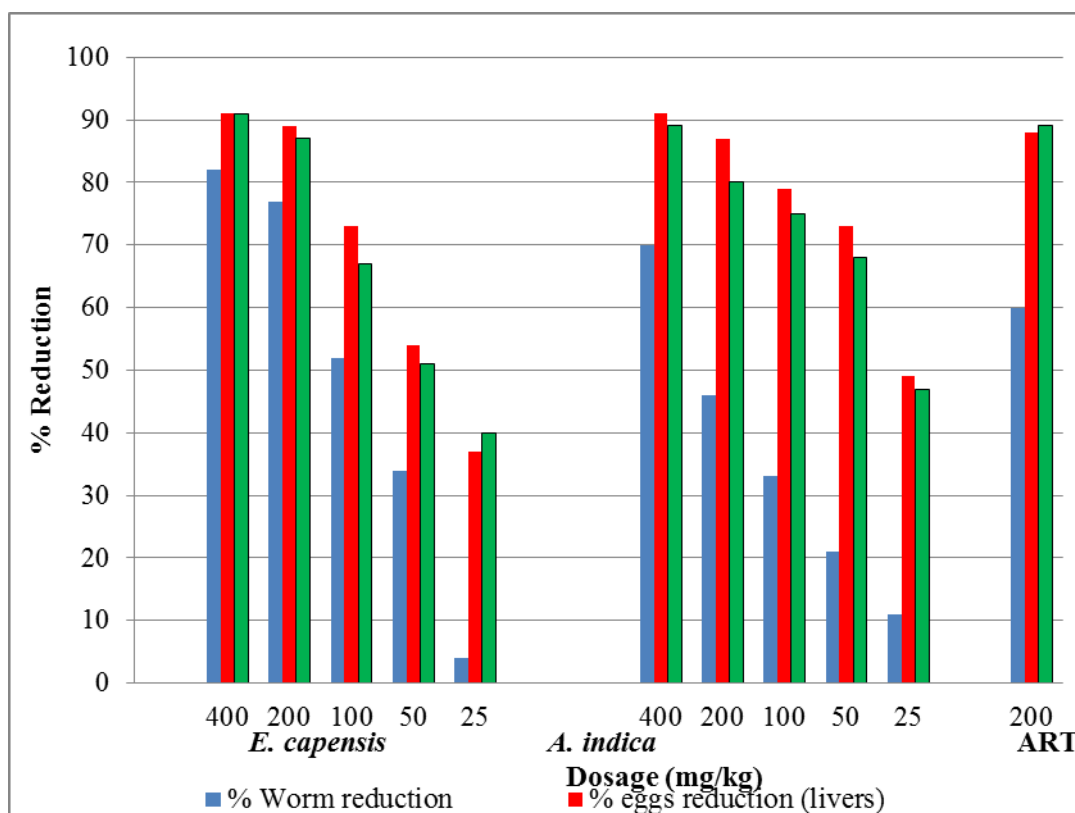
Mice treated with *A. indica* 4 weeks p.i. had 11%, 21%, 33%, 46% and 70% mean worm load reduction at 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg when compared to the infected untreated (control) group. This reduction was very highly significantly ($P<0.001$) different at 200mg/kg and 400mg/kg, highly significantly ($P<0.01$) different at 50mg/kg and 100mg/kg and not significantly ($P>0.05$) different at 25mg/kg. When compared to the ART (positive control) group, the reduction was very highly significantly ($P<0.001$) different at all the doses (Table 4.3). The percentage egg load reduction in the livers was recorded as 49%, 73%, 79%, 87% and 91% at 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively. This reduction was very highly significantly ($P<0.001$) different at 100mg/kg, 200mg/kg and 400mg/kg, highly significantly ($P<0.01$) different at 25mg/kg and 50mg/kg in comparison to the infected untreated (control) group. This reduction was not significantly ($P>0.05$) different at 400mg/kg (similar to the reduction in the ART group), highly significantly ($P<0.01$) different at 200mg/kg and very highly significantly ($P<0.001$) different at 25mg/kg, 50mg/kg and 100mg/kg when compared with the ART (positive control) group. The percentage mean egg load reduction in the intestines was 47%, 68%, 75%, 80% and 89% at 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively relative to the infected

untreated (control) group. This reduction was very highly significantly ($P<0.001$) different at all the doses when compared to the infected untreated (control) group. On the other hand, this reduction was significantly ($P<0.05$) different at 400mg/kg (similar to ART group) and very highly significantly ($P<0.001$) different at all the other doses when compared to the ART group. In generally, worm reduction and egg load reduction in both livers and intestines at 4 weeks p.i at 100mg/kg, 200mg/g and 400mg/kg was comparable to the reduction in the ART group.

Table 4.3. Mean number of worms and eggs recovered from livers and intestines following treatment with *A. indica* and *E. capensis* at 4 weeks post infection

Drug	Number of Male worms (Mean±SD)	Number of Female worms (Mean±SD)	Total Number of worms (Mean±SD)	Number of Eggs (livers) (Mean±SD)	Number of eggs (intestines) (Mean±SD)
<i>E. capensis</i>					
400mg/kg	6.8±1.9	4.6±1.8	11.4±3.6 ^{a***b}	2334±568 ^{a***b***}	2823±878 ^{a***b**}
200mg/kg	8.2±3.3	6.4±3	14.6±6.2 ^{a***b}	3097±1058 ^{a***b**}	4001±1053 ^{a***b***}
100mg/kg	16.8±1.9	13±2.5	29.8±4.2 ^{a***b***}	7434±1150 ^{a***b***}	9562±871 ^{a***b***}
50mg/kg	22.2±3.8	19.2±6.9	41.4±10.5 ^{a***b***}	12552±3279 ^{a***b***}	15232±3680 ^{a***b***}
25mg/kg	30.2±2.6	30.2±2.4	60.4±3.2 ^{a b***}	17320±3439 ^{a*b}	22760±7670 ^{a b**}
<i>A. indica</i>					
400mg/kg	9.2±5.3	9.4±5.8	18.6±10.6 ^{a***b***}	2475±2412 ^{a***b}	3383±2404 ^{a***b*}
200mg/kg	18.6±1.5	15±2.2	33.6±3.5 ^{a***b***}	3666±1196 ^{a***b**}	6186±1289 ^{a*** b***}
100mg/kg	22±2	20.2±2.58	42.2±4.2 ^{a***b***}	5763±1170 ^{a***b***}	7770±1126 ^{a***b***}
50mg/kg	25.4±2.6	24.8±1.3	50.2±2.8 ^{a*b***}	7398±1528 ^{a***b***}	9751±1842 ^{a***b***}
25mg/kg	29±3.2	26.8±4.9	55.8±6.5 ^{a b***}	14007±3324 ^{a***b***}	16402±4265 ^{a***b***}
Artemether	4.6±3.4	2.2±1.5	6.8±4.8 ^{a***}	247±212 ^{a***}	446±293 ^{a***}
Control	33.4±6.9	29.2±3.7	62.6±9.1	27445±6148	30785±3654

Data are expressed as Mean±SD, n=5. Data were analyzed by t-test: two sample assuming unequal variance. Values with superscript letters: ^a compared to the infected untreated (negative control) group and ^b compared to PZQ (positive control) group. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$



Percentage reduction (mean numbers of recovered worms and eggs relative to the infected untreated control group. n=5. A representative of 2 similar experiment.

Figure 4.3: Percentage worm and egg reduction after treatment with aqueous extracts of *A. indica* and *E. capensis* 4 weeks post infection.

4.4 Effects of treatment with *A. indica* and *E. capensis* on cytokine profiles (7 weeks pi)

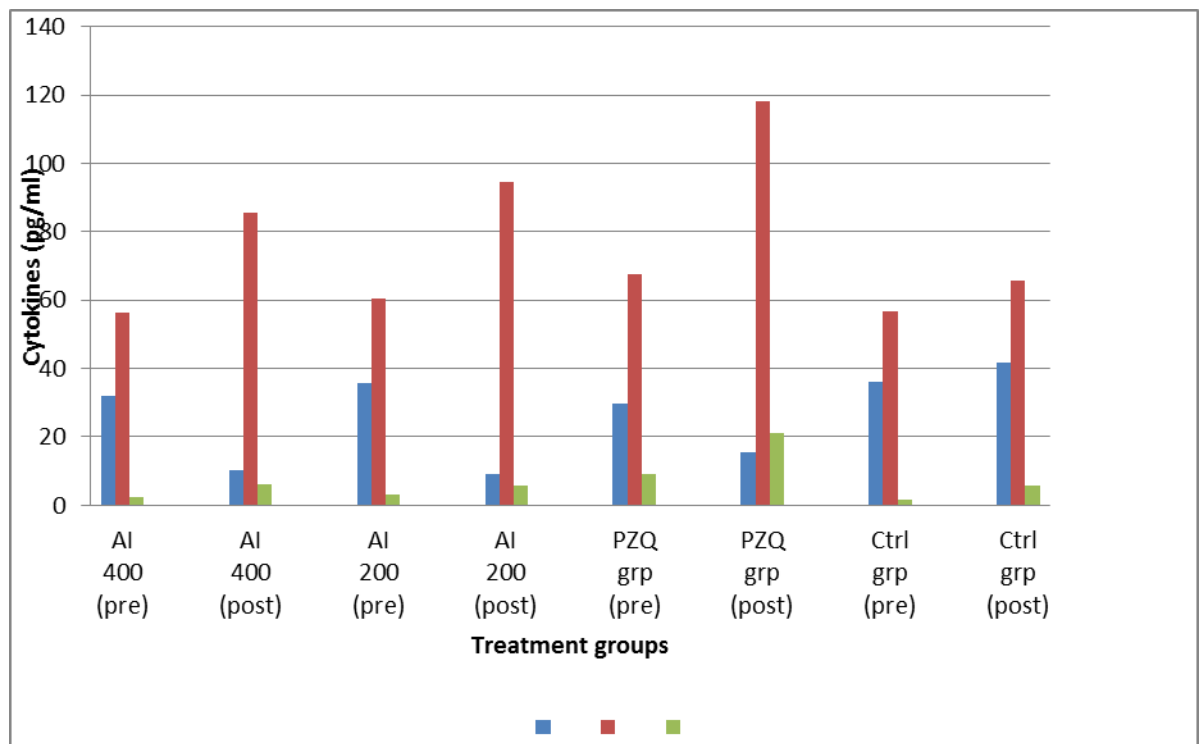
4.4.1 Effect of treatment with *A. indica* at 7 weeks p.i on Th1 cytokines

4.4.1.1 TNF- α

Treatment with *A. indica* at a dose of 400mg/kg resulted in a 68% decrease in the mean serum level of TNF- α when pre-treatment level was compared with post-treatment level. This was a very highly significant ($P < 0.001$) difference in the mean serum level of TNF- α . In comparison with PZQ group, *A. indica* at 400mg/kg was 34% lower in the mean serum level of TNF- α and this was a highly significant ($P < 0.001$) difference. In comparison with the infected untreated control group the

mean serum level of TNF- α was 75% lower and this was a very highly significant ($P<0.001$) difference.

A. indica at 200mg/kg decreased the mean serum level of TNF- α by 74% when the pre-treatment level was compared with post-treatment level and this was a very highly significant ($P<0.001$) difference. In comparison with PZQ group, the mean serum level of TNF- α was 41% lower and this was a high significant ($P<0.01$) difference. On the other hand, the mean serum level of TNF- α was 78% lower when compared with the infected untreated group. This was a very highly significant ($P<0.001$) difference as shown on Table 4.4 and Figure 4.4.



Graph showing the mean serum levels of Th1 cytokines before and after treatment. n=5. PZQ was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post- refers to after treatment. A representative of 2 similar experiments.

Figure 4.4 Mean serum levels of Th1 cytokines after treatment with aqueous extracts of *A. indica* 7 weeks post infection.

4.4.1.2 IFN- γ

Treatment with *A. indica* at a dose of 400mg/kg showed a high significant ($P<0.01$) difference (increase by 34%) in the mean serum level of IFN- γ when the pre-treatment level was compared with post-treatment level. In comparison with PZQ

group, *A. indica* at 400mg/kg showed a very highly significant ($P<0.001$) difference in the mean serum level of IFN- γ . In comparison with the infected untreated control group, the mean serum level of IFN- γ was higher by 23% which was a significant ($P<0.05$) difference.

A. indica at 200mg/kg resulted in 36% increase in the mean serum level of IFN- γ when the pre-treatment level was compared with post-treatment level. This was a very highly significant ($P<0.001$) difference. In comparison with PZQ group, the mean serum level of IFN- γ was lower by 20% and this was a very highly significant ($P<0.001$) difference. When the mean serum level of IFN- γ was 30% higher compared with the infected untreated group, a very highly significant ($P<0.001$) difference as shown on Table 4.4 and Figure 4.4.

4.4.1.3 IL-2

Treatment with *A. indica* at a dose of 400mg/kg resulted in a 60% increase in the mean serum level of IL-2 when pre-treatment level was compared to post-treatment level and this was a very highly significant ($P<0.001$) difference. In comparison with PZQ group, the mean serum level of IL-2 was 70% lower and this was a very highly significant ($P<0.001$) difference. In comparison with the infected untreated control group, there was 7.9% difference and this was not significant ($P>0.05$).

A. indica at 200mg/kg resulted in a 47% increase in the mean serum level of IL-2 when pre-treatment and post treatment levels were compared. This was very highly significant ($P<0.001$) difference. The mean serum level of IL-2 in the PZQ was higher by 72% when compared to this treatment group and this difference was highly significant ($P<0.01$). Treatment with *A. indica* at 200mg/kg showed no significant ($P>0.05$) difference (1.7% higher) in the mean serum level of IL-2 when compared with the infected untreated group as shown on Table 4.4 and Figure 4.4.

Table 4.4. Mean serum levels of cytokines following treatment with *A. indica* and *E. capensis* 7 weeks p.i.

	Th1 TNF	1FN	IL-2	Th2 IL-4	IL-6	IL-10	Th17 IL-17
Uninfected untreated	22.3±1	59.3±1.4	0.2±0.1	43.7±0.7	0.71±0.7	29.51±0.1	35.75±0.3
Infected untreated	41.6±1.0	65.8±0.9	5.8±0.3	86.1±1.8	28.01±1.7	93.1±2.6	55.9±1.2
PZQ before treatment	29.9±1.6	67.5±0.8	8.9±2.5	75.5±0.9	8.8±0.4	67.03±1.2	53.9±1.8
PZQ after treatment	15.5±0.8 a***,c***	118.0±1.9 a***,c***	21.1±1.4 a***,c***	56.2±1.1 a***,c**	18.5±0.9 a***,c***	44.8±1.3 a***,c**	108.6±3.6 a***,c***
AI 400 before treatment	32.1±1.5	56.4±1.4	2.29±0.4	82.5±2	11.8±0.8	66.4±3.2	104.8±4.6
AI 400 after treatment	10.3±0.6 a***,b**,c***	85.7±4.2 a*,b***,c**	6.3±0.6 a ^{ns} ,b***,c**	67.9±1.2 a**,b***,c**	8.4±0.4 a***,b***,c**	64.2±3.1 a***,b**,c ^{ns}	99.02±1.3 a*,b*,c ^{ns}
AI 200 before treatment	35.67±0.6	60.6±1.9	3.1±0.1	85.3±0.9	12.2±0.5	70.9±1.5	97.2±1.9
AI 200 after treatment	9.2±0.3 a***,b**,c***	94.5±2.1 a***,b***,c***	5.9±0.2 a ^{ns} ,b**,c***	72.7±1.3 a***,b***,c***	10.2±1.1 a***,b**,c ^{ns}	64.3±1.7 a***,b***,c*	110.3±1 a***, b ^{ns} , c**
EC 400 before treatment	40.6±2.8	86.7±0.4	4.2±0.2	76.8±0.9	18.4±0.8	67.2±1.8	98.4±0.8
EC 400 after treatment	27.4±0.9 a***,b***,c*	124.7±3.1 a***,b*,c**	14.1±2.0 a**,b**,c**	60.8±2.2 a***,b*,c**	20.5±0.6 a**,b*,c*	53.8±1.3 a***,b***,c***	113.8±4.8 a***,b*,c*
EC 200 before treatment	42.7±0.9	65.6±0.8	4.3±0.4	66.2±1.1	16.5±1	65.6±1.2	107.3±0.9
EC 200 after treatment	31.6±0.9 a***,b***,c***	111.5±2.1 a***,b**,c***	14.04±2.2 a*,b*,c***	41.3±1.4 a***,b***,c***	18.5±1 a**,b ^{ns} ,c ^{ns}	72.8±1.4 a***,b***,c**	131.9±1.2 a***,b**,c***

Data are expressed as mean±SD, n=5. Data were analyzed by t-test: two sample assuming unequal variance. Values with letters: a = comparison with infected untreated control, b = comparison with PZQ (Praziquantel), c = comparison of before treatment with after treatment. Superscripts: * significant (P<0.05), ** highly significant (P<0.01) and *** very highly significant (P<0.001) and ^{ns} not significant

4.4.2 Effect of treatment with *A. indica* at 7 weeks p.i on Th2 cytokines

4.4.2.1 IL-4

Treatment with *A. indica* at a dose of 400mg/kg showed a 18% reduction in the mean serum level of IL-4 when pre-treatment and post-treatment levels were compared and this was a highly significant ($P<0.01$) difference. The mean serum level of IL-4 post treatment was 17% higher in this group when compared to the PZQ treated group and this was a very highly significant ($P<0.001$) difference. When compared to the infected untreated control group, there was a 21% reduction in mean serum level post treatment and this was a highly significant ($P<0.01$) difference.

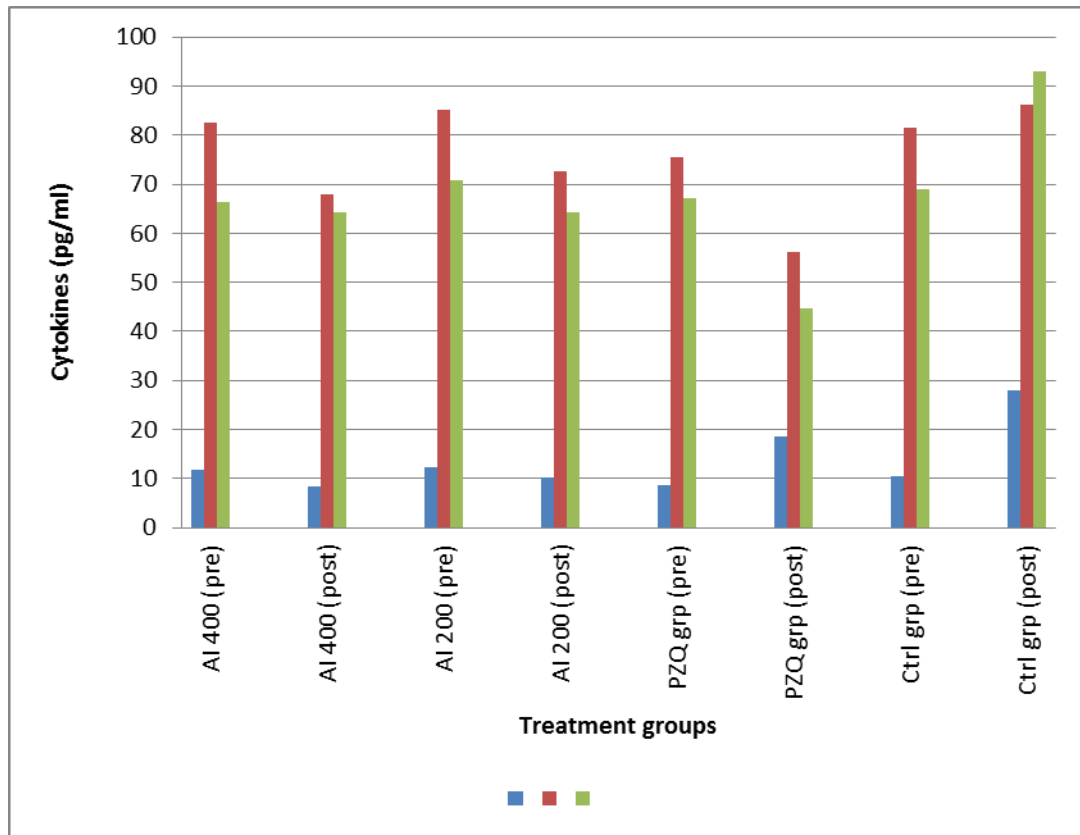
A. indica at 200mg/kg resulted in a 15% decrease in the mean serum level of IL-4 which was very highly significant ($P<0.001$) difference when pre-treatment level was compared with post-treatment level. The mean serum level of IL-4 post treatment was 23% higher in this group compared to the PZQ group and this difference was very highly significant ($P<0.001$). When compared with the infected untreated group, the mean serum level of IL-4 post-treatment was 16% lower in this group and this was a very highly significant ($P<0.001$) difference.

4.4.2.2 IL-6

Treatment with *A. indica* at a dose of 400mg/kg resulted in a 29% reduction in the mean serum level of IL-6 when pre-treatment level and post-treatment levels were compared. This reduction was highly significant ($P<0.01$). In comparison with PZQ group, the mean serum level of IL-6 was 55% higher in the PZQ group and this difference was very highly significant ($P<0.001$). In comparison with the infected untreated control group, mean serum level of IL-6 was 70% lower post treatment and this difference was very highly significant ($P<0.001$).

A. indica at 200mg/kg resulted in a 16% decrease in the mean serum level of IL-6 which was not significantly ($P>0.05$) different between pre-treatment and post-treatment. In comparison with PZQ group, the post-treatment level of IL-6 was 45% higher in PZQ group post treatment and this difference was highly significant ($P<0.01$). Treatment with *A. indica* at 200mg/kg showed a 64% decrease in the mean

serum level of IL-6 when compared with the infected untreated group and this was a very highly significant ($P < 0.001$) difference as shown on Table 4.4 and Figure 4.5.



Graph showing the mean serum levels of Th2 cytokines before and after treatment. $n=5$. PZQ was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post- refers to after treatment. A representative of 2 similar experiments.

Figure 4.5 Mean serum levels of Th2 cytokines after treatment with aqueous extracts of *A. indica* 7 weeks post infection.

4.4.2.3 IL-10

Treatment with *A. indica* at a dose of 400mg/kg resulted in a 3% reduction in the mean serum level of IL-10 when pre-treatment level was compared to post-treatment level and this difference was not significant ($P > 0.05$). When compared with PZQ group, the mean serum level of IL-10 was 30% higher following treatment with *A. indica* at 400mg/kg and the difference was highly significant ($P < 0.01$). The mean serum level of IL-10 was 31% lower post-treatment with *A. indica* at 400mg/kg when compared to the infected untreated control group. This difference was very highly significant ($P < 0.001$).

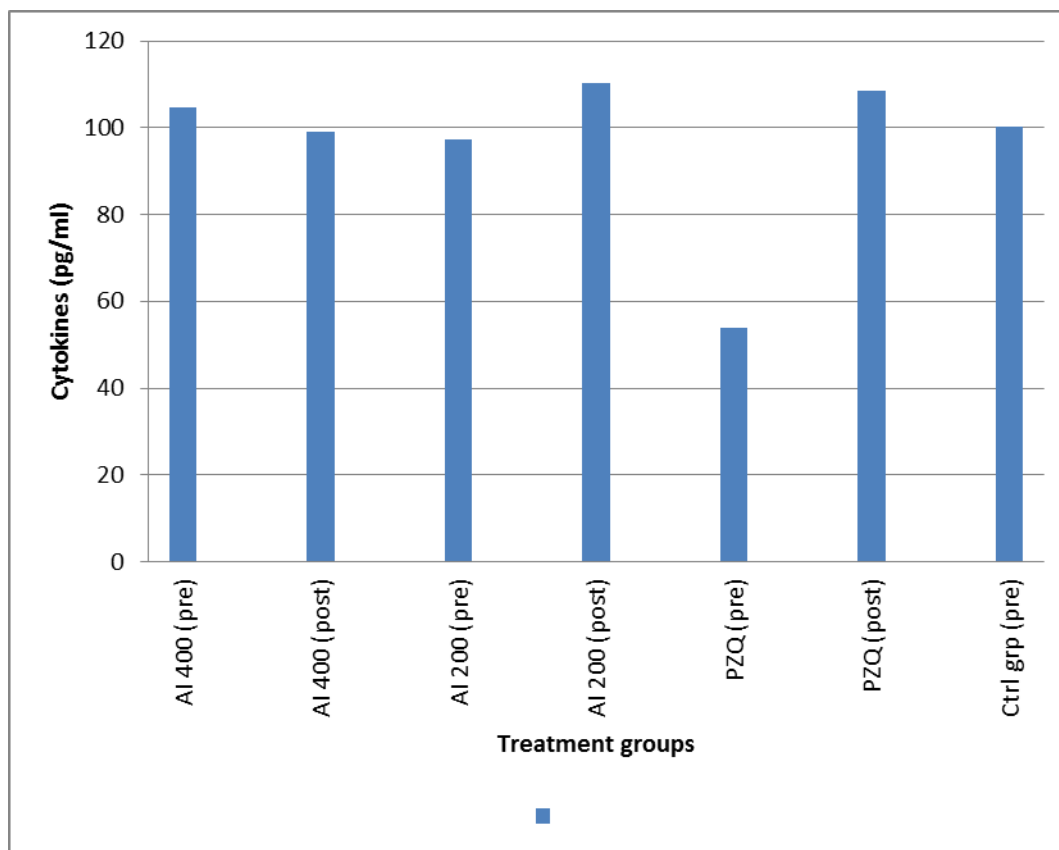
Treatment with *A. indica* at 200mg/kg reduced the mean serum level of IL-10 by 9% when pre-treatment and post-treatment levels were compared and this was a significant reduction a significant ($P<0.05$). In comparison with PZQ group, the serum levels of IL-10 in this group was 30% higher and this difference was very highly significant ($P<0.001$). The mean serum level of IL-10 was 31% lower in comparison with the infected untreated group and this difference was very highly significant ($P<0.001$) as shown on Table 4.4 and Figure 4.5.

4.4.3 Effect of treatment with *A. indica* at 7 weeks p.i on Th17 cytokines

4.4.3.1 IL-17 mean serum level of IL-17

Treatment with *A. indica* at a dose of 400mg/kg resulted in a 5% decrease in the mean serum level of IL-17 when pre-treatment and post-treatment levels were compared. This difference was not significant ($P>0.05$). In comparison with PZQ group, mean serum level of IL-17 was lower by 9% and this difference was significant ($P<0.05$). When compared to the infected untreated control group, the mean serum level of IL-17 was higher by 44% and the difference was very highly significant ($P<0.001$).

A. indica at 200mg/kg resulted in a 12% increase in the mean serum level of IL-17 when pre-treatment and post-treatment levels were compared and this difference was highly significant ($P<0.01$). In comparison with PZQ group, the post-treatment serum level of IL-17 was 2% higher and the difference was not significant ($P>0.05$). The mean serum level of IL-17 was higher by 49% when compared with the infected untreated group and this difference was very highly significant ($P<0.001$) as shown on Table 4.4 and Figure 4.6.



Graph showing the mean serum levels of Th17 cytokines before and after treatment. n=5. PZQ was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post- refers to after treatment. A representative of 2 similar experiments.

Figure 4.6 Mean serum levels of Th17 cytokines after treatment with aqueous extracts of *A. indica* 7 weeks post infection.

4.4.4 Effect of treatment with *E. capensis* at 7 weeks p.i on Th1 cytokines

4.4.4.1 TNF- α

Treatment with *E. capensis* at a dose of 400mg/kg caused a 33% decrease in the mean serum level of TNF- α post-treatment in comparison with pre-treatment that was significant ($P < 0.05$). The mean serum level of TNF- α in this group was 43% higher when compared to the PZQ group and this was a very highly significant ($P < 0.001$) difference. In comparison with the infected untreated control group, the mean serum level of TNF- α was lower by 34% and this was a very highly significant ($P < 0.001$) difference.

E. capensis at 200mg/kg resulted in a 26% reduction in the mean serum level of TNF- α when pre-treatment level was compared with post-treatment level and this was a very highly significant ($P<0.001$) difference. In comparison with PZQ group, the post-treatment mean serum level of TNF- α was higher by 51% and this was a very highly significant ($P<0.001$) difference. In comparison to the infected untreated control group, the mean serum level of TNF- α was 24% lower. This was a very highly significant ($P<0.001$) difference as shown on Table 4.4 and Figure 4.7.

4.4.4.2 IFN- γ

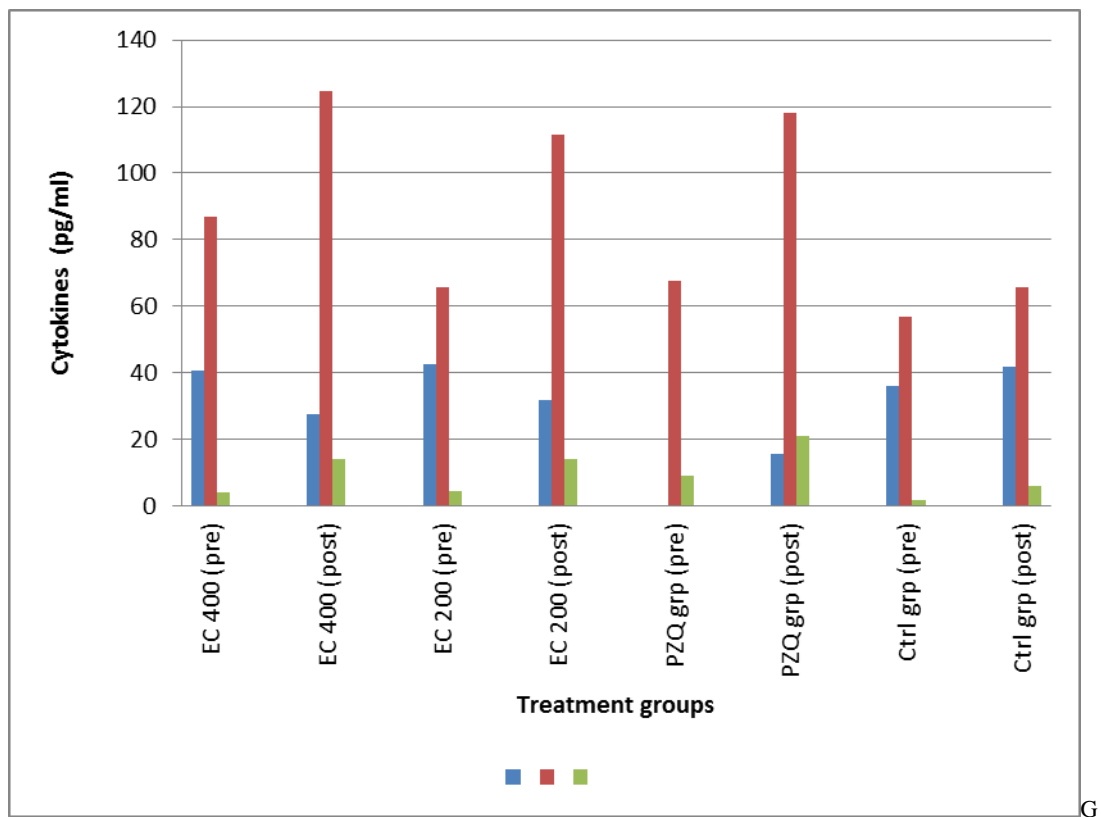
Treatment with *E. capensis* at a dose of 400mg/kg caused a 30% increase in the mean serum level of IFN- γ which was a highly significant ($P<0.01$) difference when pre-treatment level was compared to post treatment level. In comparison with PZQ group, the mean serum level of IFN- γ in this group was higher by 5% a difference that was significant ($P<0.05$). In comparison with the infected untreated control group, the mean serum level of IFN- γ increased by 47% and this was a very highly significant ($P<0.001$) difference.

E. capensis at 200mg/kg resulted in a 41% increase in the mean serum level of IFN- γ when pre-treatment and post-treatment level was compared. This was very highly significant ($P<0.001$) difference. There was a 6% increase in the mean serum level of IFN- γ in this treatment group in comparison to the PZQ group showing a significant ($P<0.05$) difference. The mean serum level of IFN- γ was 41% higher in this treatment group in comparison to the infected untreated group. This was a very highly significant ($P<0.001$) difference as shown on Table 4.4 and Figure 4.7.

4.4.4.3 IL-2

Treatment with *E. capensis* at a dose of 400mg/kg caused a 70% increase in the mean serum level of IL-2 in the post-treatment level in comparison to the pre-treatment level and this difference was highly significant ($P<0.01$). In comparison with PZQ group, the mean serum level of IL-2 was lower by 33% in this group and this was a highly significant ($P<0.01$) difference. The mean serum level of IL-2 in this group was 59% higher when compared to the infected untreated control group and this was a highly significant ($P<0.01$) difference.

Treatment with *E. capensis* at a dose of 200mg/kg resulted in a 69% increase in the mean serum level of IL-2 in the post-treatment level in comparison to the pre-treatment level and this difference was very highly significant ($P<0.001$). In comparison with PZQ group, the mean serum level of IL-2 was lower by 33% in this group and this was significant ($P<0.05$) difference. The mean serum level of IL-2 in this group was 59% higher in comparison with the infected untreated control group and this was a significant ($P<0.05$) difference as shown in on Table 4.4 and Figure 4.7.



Graph showing the mean serum levels of Th1 cytokines before and after treatment. $n=5$. PZQ was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post- refers to after treatment. A representative of 2 similar experiments.

Figure 4.7 Mean serum levels of Th1 cytokines after treatment with aqueous extracts of *E. capensis* 7 weeks post infection.

4.4.5 Effect of treatment with *E. capensis* at 7 weeks p.i on Th2 cytokines

4.4.5.1 IL-4

Treatment with *E. capensis* at a dose of 400mg/kg resulted in a 21% decrease in the mean serum level of IL-4 post-treatment in comparison to the pre-treatment level and this difference was highly significant ($P<0.01$). When compared with the PZQ treatment group, the mean serum level of IL-4 was 8% higher in this group a difference that was significant ($P<0.05$). When compared to the infected untreated group, the post-treatment level of IL-4 was 29% lower in this group a difference that was highly significant ($P<0.01$).

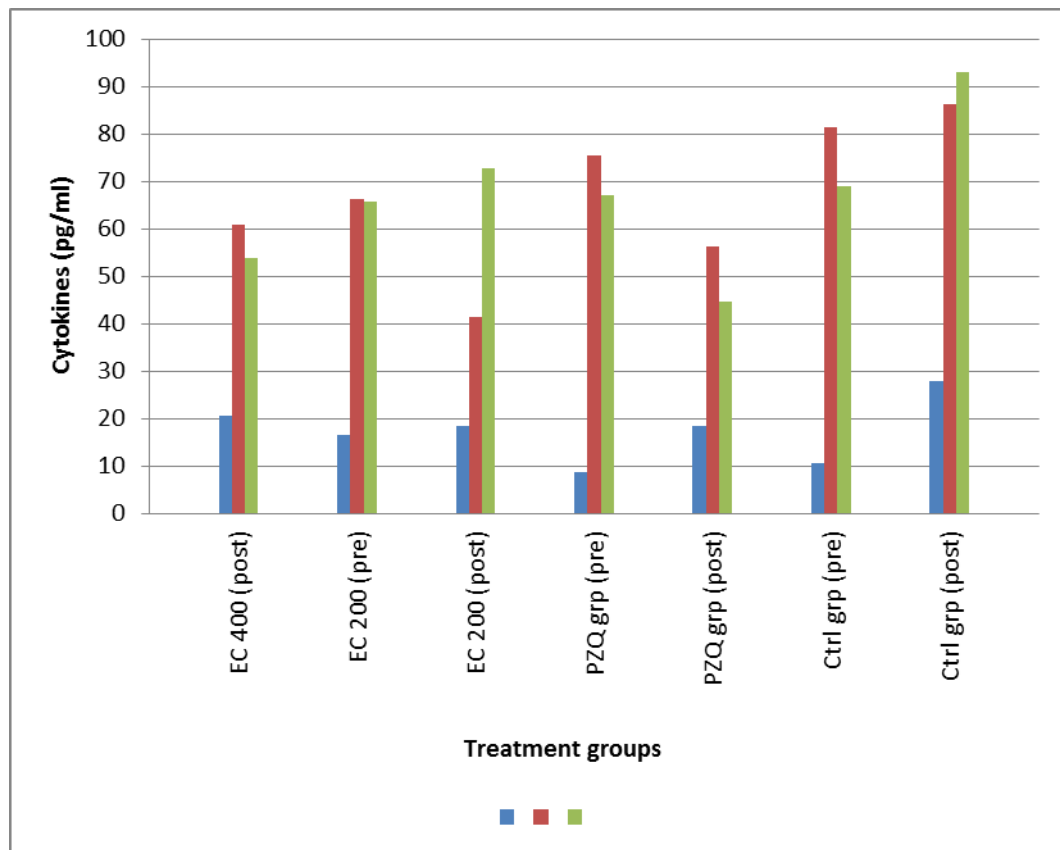
At 200mg/kg, *E. capensis* resulted in a 38% decrease in the mean serum level of IL-4 post-treatment in comparison to pre-treatment level and this difference was very highly significant ($P<0.001$). The mean serum level of IL-4 was lower by 27% in this group in comparison to the PZQ treatment group, a difference that was very highly significant ($P<0.001$). When compared to the infected untreated group, the mean serum level of IL-4 was lower by 52%, and this was very highly significant ($P<0.001$) difference as shown on Table 4.4 and Figure 4.8.

4.4.5.2 IL-6

Treatment with *E. capensis* at a dose of 400mg/kg showed a 10% increase in the mean serum level of IL-6 when pre-treatment level was compared to post treatment level and this was a significant ($P<0.05$) difference. When compared to PZQ group, the mean serum level of IL-6 was higher by 10% showing a significant ($P<0.05$) difference. When compared to the infected untreated group, the mean serum level of IL-6 was lower by 37% and this a highly significant ($P<0.01$) difference.

At 200mg/kg, *E. capensis* caused a 11% increase in in the mean serum level of IL-6 in the post-treatment level compared to the pre-treatment level and this was not a significant difference ($P>0.05$). In comparison with PZQ, there was no significant difference($P>0.05$) in the mean serum level of IL-6. When compared to the infected

untreated group, the post-treatment mean serum level of IL-6 was 34% lower a difference that was highly significant ($P < 0.01$) as shown on Table 4.4 and Figure 4.8.



Graph showing the mean serum levels of Th2 cytokines before and after treatment. $n=5$. PZQ was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post- refers to after treatment. A representative of 2 similar experiments.

Figure 4.8 Mean serum levels of Th2 cytokines after treatment with aqueous extracts of *E. capensis* 7 weeks post infection.

4.4.5.3 IL-10

Treatment with *E. capensis* at a dose of 400mg/kg showed a 20% decrease in the mean serum level of IL-10 post-treatment in comparison to pre-treatment level and this was a very highly significant ($P < 0.001$) difference. When compared to the PZQ treatment group, the post-treatment serum level was 17% higher showing a very highly significant ($P < 0.001$) difference. In comparison with the infected untreated control group, the mean serum level of IL-10 post-treatment was 42% lower a difference that was very highly significant ($P < 0.001$).

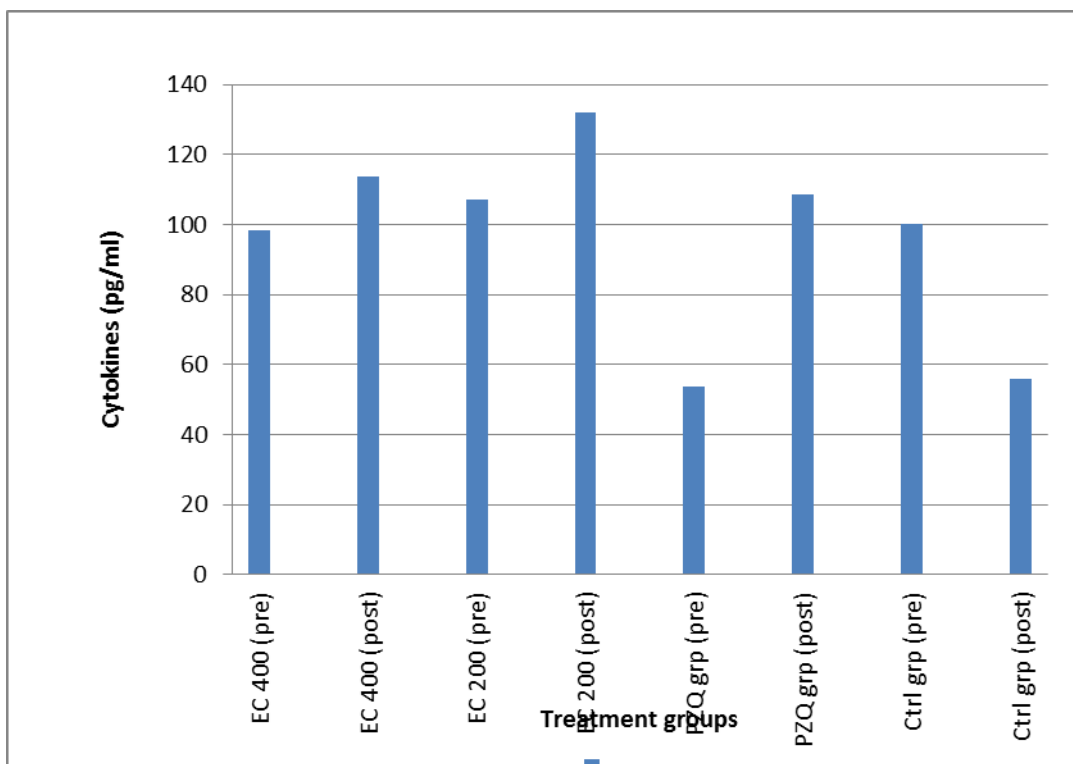
E. capensis at 200mg/kg resulted in a 10% increase in the mean serum level of IL-10 post-treatment which was highly significant ($P<0.01$) difference. In comparison with PZQ group, the post-treatment mean serum level of IL-10 was 38% higher indicating a very highly significant ($P<0.001$) difference. When compared to the infected untreated group, the post-treatment mean serum level of IL-10 was 22% lower in this group showing a very highly significant ($P<0.001$) difference as shown on Table 4.4 and Figure 4.8.

4.4.6 Effect of treatment with *E. capensis* at 7 weeks p.i on Th17 cytokines

4.4.6.1 IL-17

Treatment with *E. capensis* at a dose of 400mg/kg caused a 14% increase in the post-treatment mean serum level of IL-17 in comparison to the pre-treatment level and this was significant ($P<0.05$) difference. In comparison with PZQ group, the post-treatment mean serum level of IL-17 was 5% higher in this group a difference that was significant ($P<0.05$). In comparison with the infected untreated control group, the post-treatment mean serum level of IL-10 was 51% higher showing a very highly significant ($P<0.001$) difference.

E. capensis at a dose of 200mg/kg resulted in a 19% increase in the post-treatment level of IL-17 in comparison to the pre-treatment level indicating a very highly significant ($P<0.001$) difference. In comparison with PZQ group, the post-treatment mean serum level of IL-17 was 18% higher a difference that was highly significant ($P<0.01$). In comparison with the infected untreated control group, the post-treatment mean serum level of IL-10 was 56% higher showing a very highly significant ($P<0.001$) difference as shown on Table 4.4 and Figure 4.9.



Graph showing the mean serum levels of Th17 cytokines before and after treatment. n=5. PZQ was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post- refers to after treatment. A representative of 2 similar experiments.

Figure 4.9 Mean serum levels of Th17 cytokines after treatment with aqueous extracts of *E. capensis* 7 weeks post infection.

4.5 Effects of treatment with *A. indica* and *E. capensis* on cytokine profiles (2 weeks pi)

4.5.1 Effect of treatment with *A. indica* at 2 weeks p.i on Th1 cytokines

4.5.1.1 TNF- α

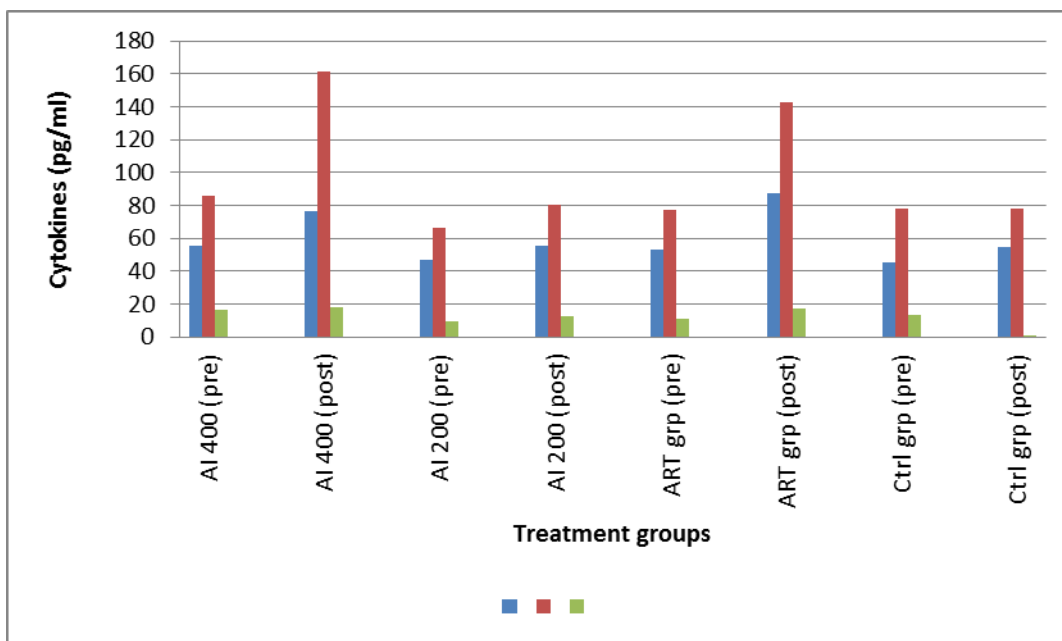
Treatment with *A. indica* at a dose of 400mg/kg resulted in a 28% increase in the mean serum level of TNF- α in the post-treatment serum level in comparison to the pre-treatment level and this was a very highly significant ($P < 0.001$) difference. In comparison with ART group, the mean serum level of TNF- α was 12% lower post-treatment a difference that was very highly significant ($P < 0.001$). In comparison with the infected untreated control group, the mean serum level of TNF- α post-treatment was 29% higher showing a very highly significant ($P < 0.001$) difference.

At a dose of 200mg/kg *A. indica* resulted in a 16% increase in the post-treatment mean serum level of TNF- α in comparison to the pre-treatment level, a difference that was highly significant ($P < 0.01$). In comparison with ART group, the mean serum level of TNF- α was 36% lower post-treatment a difference that was very highly significant ($P < 0.001$). In comparison with the infected untreated control group, the mean serum level of TNF- α post-treatment was 2% higher showing no significant ($P > 0.05$) difference as shown on Table 4.5 and Figure 4.10.

4.5.1.2 IFN- γ

Treatment with *A. indica* at a dose of 400mg/kg resulted in a 47% increase in the mean serum level of IFN- γ in the post-treatment serum level in comparison to the pre-treatment level and this was a very highly significant ($P < 0.001$) difference. In comparison with ART group, the mean serum level of IFN- γ was 12% higher post-treatment a difference that was very highly significant ($P < 0.001$). In comparison with the infected untreated control group, the mean serum level of IFN- γ post-treatment was 52% higher showing a very highly significant ($P < 0.001$) difference.

At a dose of 200mg/kg *A. indica* resulted in a 17% increase in the post-treatment mean serum level of TNF- α in comparison to the pre-treatment level, a difference that was very highly significant ($P < 0.001$). In comparison with ART group, the mean serum level of TNF- α was 44% lower post-treatment a difference that was very highly significant ($P < 0.001$). In comparison with the infected untreated control group, the mean serum level of TNF- α post-treatment was 3% higher showing no significant ($P > 0.05$) difference as shown on Table 4.5 and Figure 4.10.



Graph showing the mean serum levels of Th1 cytokines before and after treatment. n=5. ART (artemether) was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post- refers to after treatment. A representative of 2 similar experiments.

Figure 4.10 Mean serum levels of Th1 cytokines after treatment with aqueous extracts of *A. indica* 2 weeks post infection.

4.5.1.3 IL-2

Treatment with *A. indica* at a dose of 400mg/kg resulted in a 12% increase in the post-treatment mean serum level of IL-2 in comparison to the pre-treatment level and this was a highly significant ($P < 0.01$) difference. In comparison with ART group, the mean serum level of IL-2 increased by 8% a difference that was significant ($P < 0.05$). In comparison with the infected untreated control group, the mean serum level of IL-2 post-treatment was 94% higher and this was a very highly significant ($P < 0.001$) difference.

At a dose of 200mg/kg *A. indica* caused a 23% increase in the mean serum level of IL-2 post-treatment and this was a very highly significant ($P < 0.001$) difference. In comparison with the ART group, the mean serum level of IL-2 post-treatment was 28% lower, a very highly significant ($P < 0.001$) difference. When compared to the infected untreated group, the mean serum level of IL-2 post-treatment was higher by 92%, a very highly significant ($P < 0.001$) difference as shown on Table 4.5 and Figure 4.10.

Table 4.5. Mean serum levels of cytokines following treatment with *A. indica* and *E. capensis* 2 weeks p.i.

	Th1 TNF	IFN	IL-2	Th2 IL-4	IL-6	IL-10	Th17 IL-17
Uninfected untreated	22.3±1	59.3±1.4	0.2±0.1	43.7±0.7	0.71±0.7	29.51±0.1	35.75±0.3
Infected untreated	54.7±0.7	77.9±1.4	1.02±0.1	85.7±1.6	8.9±0.2	67.4±1.5	55.9±1.9
ART before treatment	53.1±0.5	76.9±0.2	10.8±0.2	55.2±0.5	8.9±0.1	66.2±0.5	51.9±0.4
ART after treatment	87.5±1.1 a ^{***} , c ^{***}	142.5±2.5 a ^{***} , c ^{***}	16.9±0.3 a ^{***} , c ^{**}	44.2±0.8 a ^{***} , c ^{**}	6.2±0.4 a ^{**} , c ^{**}	54.7±0.4 a ^{**} , c ^{**}	59.4±1.0 a ^{**} , c ^{**}
AI 400 before treatment	55.6±0.7	85.6±0.5	16.2±0.5	47.1±0.3	6.6±0.2	61.5±1.1	58.2±1.6
AI 400 after treatment	76.7±1.6 a ^{***} , b ^{***} , c ^{***}	161.2±1.3 a ^{***} , b ^{***} , c ^{***}	18.4±0.4 a ^{***} , b [*] , c ^{**}	33.5±1.1 a ^{***} , b ^{***} , c ^{***}	5.8±0.2 a ^{***} , b ^{ns} , c ^{**}	57.8±0.9 a ^{**} , b ^{**} , c ^{**}	86.8±1.5 a ^{**} , b ^{***} , c ^{***}
AI 200 before treatment	46.6±1.5	66.4±1.1	9.4±0.3	63.9±0.8	8.1±0.1	61.8±1.1	54.9±0.7
AI 200 after treatment	55.6±0.4 a ^{ns} , b ^{***} , c ^{**}	80.2±0.8 a ^{ns} , b ^{***} , c ^{***}	12.2±0.4 a ^{***} , b ^{***} , c ^{***}	56.1±0.3 a ^{***} , b ^{***} , c ^{***}	7.6±0.5 a ^{***} , b [*] , c ^{ns}	59.6±0.9 a ^{**} , b ^{**} , c [*]	54.4±0.7 a ^{ns} , b ^{**} , c ^{ns}
EC 400 before treatment	65.6±1.4	77±0.5	11.1±0.2	61.5±0.5	6.7±0.3	55.8±0.3	56.5±0.8
EC 400 after treatment	86.7±0.9 a ^{***} , b ^{ns} , c ^{***}	106.9±5.6 a ^{**} , b ^{**} , c ^{**}	15.9±0.1 a ^{***} , b [*] , c ^{***}	30.6±0.7 a ^{***} , b ^{***} , c ^{***}	6.1±0.2 a ^{***} , b ^{ns} , c ^{ns}	50.3±0.8 a ^{***} , b ^{**} , c ^{**}	62.4±0.9 a ^{**} , b ^{**} , c ^{***}
EC 200 before treatment	70.7±0.8	93.9±0.4	16.1±0.2	36.6±0.8	6.6±0.2	56.4±0.7	66.1±0.3
EC 200 after treatment	90.6±0.8 a ^{***} , b [*] , c ^{***}	119.7±0.9 a ^{***} , b ^{***} , c ^{***}	17.8±0.5 a ^{***} , b ^{ns} , c [*]	24.7±1.6 a ^{***} , b ^{***} , c ^{***}	5.08±1.8 a ^{***} , b [*] , c ^{**}	50.3±1.0 a ^{***} , b [*] , c ^{***}	83.9±0.5 a ^{***} , b ^{***} , c ^{***}

Data are expressed as mean±SD, n=5. Data were analyzed by t-test: two sample assuming unequal variance. Values with letters: a = comparison with infected untreated control, b = comparison with ART (Artemether), c = comparison of before treatment with after treatment. Superscripts: * significant (P<0.05), ** highly significant (P<0.01) and *** very highly significant (P<0.001) and ^{ns} not significant.

4.5.2 Effect of treatment with *A. indica* at 2 weeks p.i on Th2 cytokines

4.5.2.1 IL-4

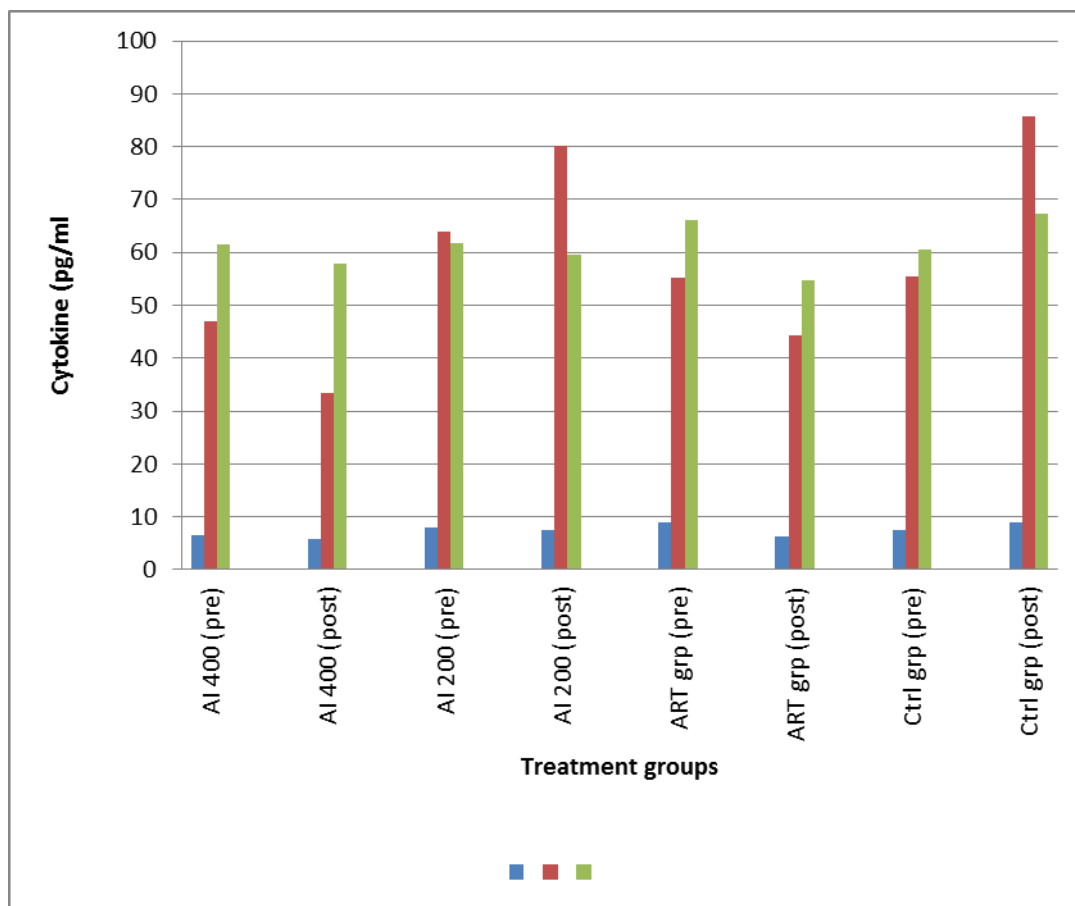
Treatment with *A. indica* at a dose of 400mg/kg caused a 29% decrease in the post-treatment mean serum level of IL-4, a very highly significant ($P<0.001$) difference. When compared to the ART group the post-treatment mean serum level of IL-4 was 24% lower and this was a very highly significant ($P<0.001$) difference. In comparison with the infected untreated control group, the post-treatment mean serum level of IL-4 was 61% lower, a very highly significant ($P<0.001$) difference.

Treatment with *A. indica* at a dose of 200mg/kg caused a 12% decrease in the post-treatment mean serum level of IL-4, a very highly significant ($P<0.001$) difference. When compared to the ART group the post-treatment mean serum level of IL-4 was 21% higher and this was a very highly significant ($P<0.001$) difference. In comparison with the infected untreated control group, the post-treatment mean serum level of IL-4 was 35% lower, a very highly significant ($P<0.001$) difference as shown on Table 4.5 and Figure 4.11.

4.5.2.2 IL-6

Treatment with *A. indica* at a dose of 400mg/kg caused a 12% decrease in the post-treatment mean serum level of IL-6, a high significant ($P<0.01$) difference. In comparison with ART group, the post-treatment mean serum level of IL-6 was 6% lower and this showed no significant ($P>0.05$) difference. In comparison with the infected untreated control group, the post-treatment mean serum level of IL-6 was 35% lower, a very highly significant ($P<0.001$) difference.

A. indica at 200mg/kg resulted in a 6% reduction in the post-treatment mean serum level of IL-6 in comparison to the pre-treatment level showing no significant ($P>0.05$) difference. When compared to the ART group, the post-treatment mean serum level of IL-6 was 18% higher, a significant ($P<0.05$) difference. When compared to the infected untreated group the post-treatment mean serum level of IL-6 was 15% lower indicating a very highly significant ($P<0.001$) difference as shown on Table 4.5 and Figure 4.11.



Graph showing the mean serum levels of Th2 cytokines before and after treatment. n=5. ART (artemether) was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post-refers to after treatment. A representative of 2 similar experiments.

Figure 4.11 Mean serum levels of Th2 cytokines after treatment with aqueous extracts of *A. indica* 2 weeks post infection.

4.5.2.3 IL-10

Treatment with *A. indica* at a dose of 400mg/kg showed a 6% decrease in the post-treatment mean serum level of IL-10 and this was a highly significant ($P < 0.01$) difference. In comparison with ART group, the post-treatment mean serum level of IL-10 was 5% higher, a high significant ($P < 0.01$) difference. When compared with the infected untreated control group, the post-treatment mean serum level of IL-10 was 14% lower, a highly significant ($P < 0.01$) difference.

At the dose of 200mg/kg, the post treatment mean serum level of IL-10 decreased by 4% showing a significant ($P < 0.05$) difference. When the post-treatment mean serum level of IL-10 was compared to the ART level it was higher by 8% indicating a high

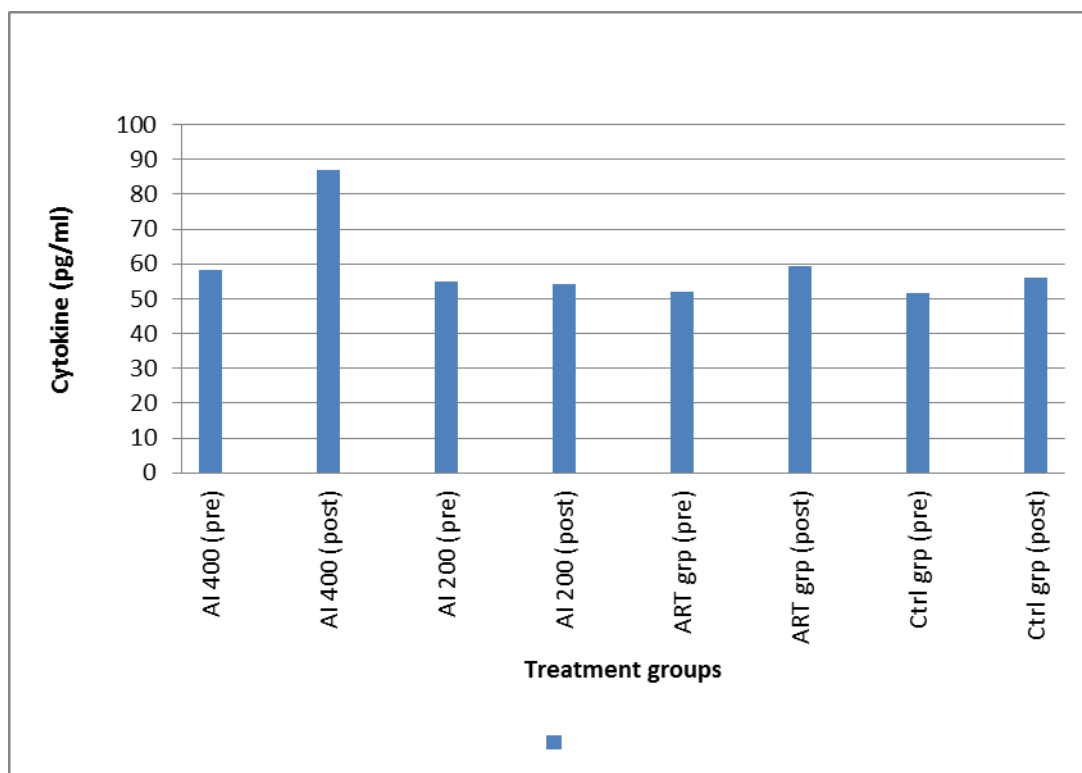
significant ($P < 0.01$) difference. In comparison with the infected untreated control group, the post treatment mean serum level of IL-10 was 8% lower and this was a highly significant ($P < 0.01$) difference as shown on Table 4.5 and Figure 4.11.

4.5.3 Effect of treatment with *A. indica* at 2 weeks p.i Th17 cytokines

4.5.3.1 IL-17

Treatment with *A. indica* at a dose of 400mg/kg resulted in a 34% increase in post-treatment mean serum level of IL-17 in comparison to its pre-treatment level, a very highly significant ($P < 0.001$) difference. In comparison with ART group, the post-treatment mean serum level of IL-17 was 32% higher showing a very highly significant ($P < 0.001$) difference. When compared to the infected untreated control group, the post-treatment mean serum level of IL-17 was higher by 36%, a highly significant ($P < 0.01$) difference.

At a dose of 200mg/kg *A. indica* resulted in a 0.9% decrease in the post-treatment mean serum level of IL-17 and this was not a significant ($P > 0.05$) difference. Compared to the ART treatment group, the post-treatment mean serum level of IL-17 was 8% lower indicating a high significant ($P < 0.01$) difference. When the post-treatment mean serum level of IL-17 was compared to the infected untreated group, it was 3% lower thus no significant ($P > 0.05$) difference as shown on Table 4.5 and Figure 4.12.



Graph showing the mean serum levels of Th17 cytokines before and after treatment. n=5. ART (artemether) was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post-refers to after treatment. A representative of 2 similar experiments.

Figure 4.12 Mean serum levels of Th17 cytokines after treatment with aqueous extracts of *A. indica* 2 weeks post infection.

4.5.4 Effect of treatment with *E. capensis* at 2 weeks p.i on Th1 cytokines

4.5.4.1 TNF- α

Treatment with *E. capensis* at a dose of 400mg/kg resulted in a 24% increase in the mean serum level of TNF- α in the post-treatment serum level in comparison to the pre-treatment level and this was a very highly significant ($P < 0.001$) difference. In comparison with ART group, the mean serum level of TNF- α was 1% lower post-treatment a difference that was not significant ($P > 0.05$). In comparison with the infected untreated control group, the mean serum level of TNF- α post-treatment was 37% higher showing a very highly significant ($P < 0.001$) difference.

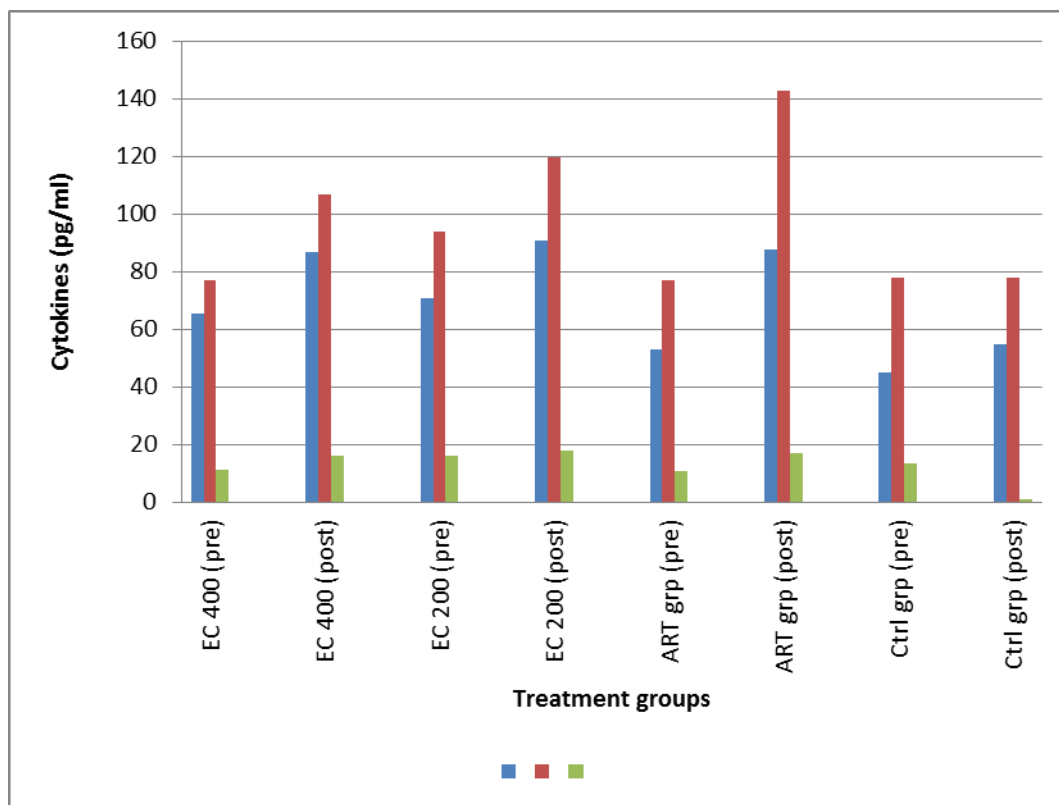
At a dose of 200mg/kg *E. capensis* resulted in a 22% increase in the post-treatment mean serum level of TNF- α in comparison to the pre-treatment level, a difference that was a very highly significant ($P < 0.001$). In comparison with ART group, the

mean serum level of TNF- α was 3.4% higher post-treatment a difference that was significant (P<0.05). In comparison with the infected untreated control group, the mean serum level of TNF- α post-treatment was 40% higher showing a very highly significant (P<0.001) difference as shown on Table 4.5 and Figure 4.13.

4.5.4.2 IFN- γ

Treatment with *E. capensis* at a dose of 400mg/kg resulted in a 28% increase in the mean serum level of IFN- γ in the post-treatment serum level in comparison to the pre-treatment level and this was a highly significant (P<0.01) difference. In comparison with ART group, the mean serum level of IFN- γ was 25% lower post-treatment a difference that was highly significant (P<0.01). In comparison with the infected untreated control group, the mean serum level of IFN- γ post-treatment was 27% higher showing a highly significant (P<0.01) difference.

At a dose of 200mg/kg *E. capensis* resulted in a 22% increase in the post-treatment mean serum level of TNF- α in comparison to the pre-treatment level, a difference that was very highly significant (P<0.001). In comparison with ART group, the mean serum level of TNF- α was 16% lower post-treatment a difference that was very highly significant (P<0.001). In comparison with the infected untreated control group, the mean serum level of TNF- α post-treatment was 35% higher showing a very highly significant (P<0.001) difference as shown on Table 4.5 and Figure 4.13.



Graph showing the mean serum levels of Th1 cytokines before and after treatment. n=5. ART (artemether) was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post-refers to after treatment. A representative of 2 similar experiment.

Figure 4.13 Mean serum levels of Th1 cytokines after treatment with aqueous extracts of *E. capensis* 2 weeks post infection.

4.5.4.3 IL-2

Treatment with *E. capensis* at a dose of 400mg/kg caused a 30% increase in the mean serum level of IL-2 in the post-treatment level in comparison to the pre-treatment level and this difference was a very highly significant ($P<0.001$). In comparison with ART group, the mean serum level of IL-2 was lower by 6% in this group and this was a significant ($P<0.05$) difference. The mean serum level of IL-2 increased by 94% when comparison with the infected untreated control group and this was a very highly significant ($P<0.01$) difference.

Treatment with *E. capensis* at a dose of 200mg/kg resulted in a 10% increase in the mean serum level of IL-2 in the post-treatment level in comparison to the pre-treatment level and this difference was a significant ($P<0.05$). In comparison with ART group, the mean serum level of IL-2 was higher by 5% in this group and this

was not a significant ($P>0.05$) difference. The mean serum level of IL-2 was higher by 94% when compared with the infected untreated control group and this was a very highly significant ($P<0.001$) difference as shown on Table 4.5 and Figure 4.13.

4.5.5 Effect of treatment with *E. capensis* at 2 weeks p.i on Th2 cytokines

4.5.5.1 IL-4

Treatment with *E. capensis* at a dose of 400mg/kg resulted in a 50% decrease in the mean serum level of IL-4 post-treatment in comparison to the pre-treatment level and this difference was a very highly significant ($P<0.001$). When compared with the ART treatment group, the mean serum level of IL-4 was 31% lower in this group a difference that was very highly significant ($P<0.001$). When compared to the infected untreated group, the post-treatment level of IL-4 was 64% lower in this group a difference that was very highly significant ($P<0.001$).

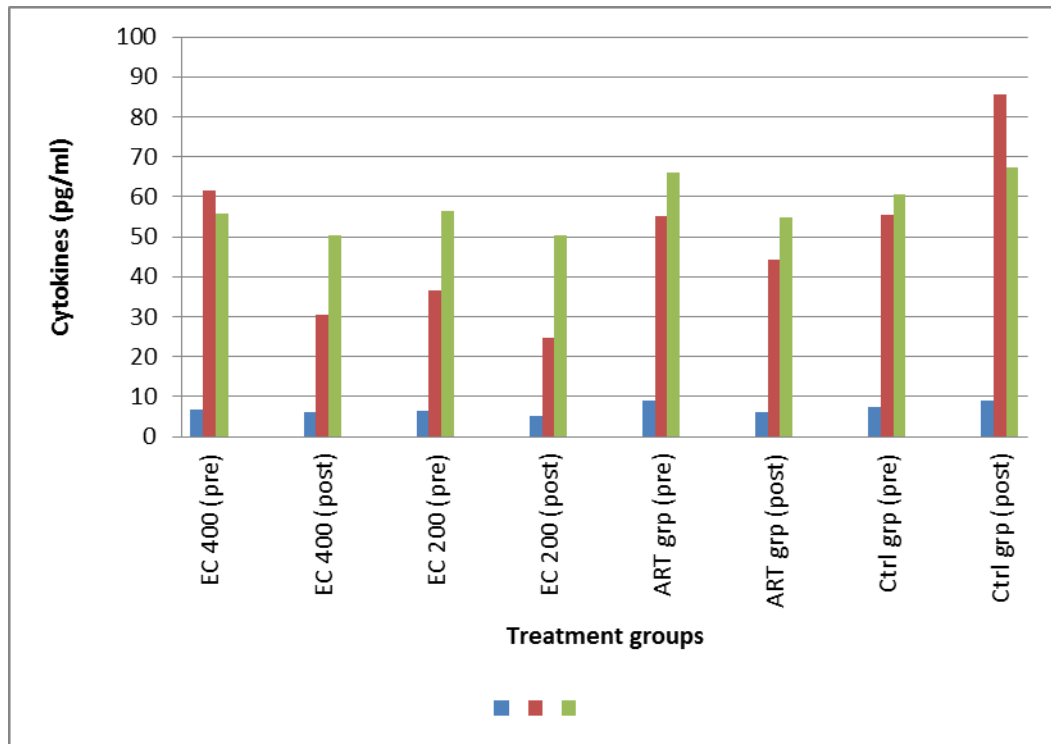
At 200mg/kg, *E. capensis* resulted in a 33% decrease in the mean serum level of IL-4 post-treatment in comparison to pre-treatment level and this difference was very highly significant ($P<0.001$). The mean serum level of IL-4 was lower by 44% in this group in comparison to the ART treatment group, a difference that was very highly significant ($P<0.001$). When compared to the infected untreated group, the mean serum level of IL-4 was lower by 71%, and this was very highly significant ($P<0.001$) difference as shown on Table 4.5 and Figure 4.14.

4.5.5.2 IL-6

Treatment with *E. capensis* at a dose of 400mg/kg showed a 6% decrease in the mean serum level of IL-6 when pre-treatment level was compared to post treatment level and this was not a significant ($P>0.05$) difference. When compared to ART group, the mean serum level of IL-6 was lower by 1.6% showing a non-significant ($P>0.05$) difference. When compared to the infected untreated group, the mean serum level of IL-6 was lower by 31% and this was a very highly significant ($P<0.01$) difference.

At 200mg/kg, *E. capensis* caused a 23% decrease in in the mean serum level of IL-6 in the post-treatment level compared to the pre-treatment level and this was highly

significant difference ($P < 0.01$). In comparison with ART, there was a significant difference ($P < 0.05$) in the mean serum level of IL-6. When compared to the infected untreated group, the post-treatment mean serum level of IL-6 was 43% lower a difference that was very highly significant ($P < 0.001$) as shown on Table 4.5 and Figure 4.14.



Graph showing the mean serum levels of Th2 cytokines before and after treatment. $n=5$. ART (artemether) was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post- refers to after treatment. A representative of 2 similar experiments.

Figure 4.14 Mean serum levels of Th2 cytokines after treatment with aqueous extracts of *E. capensis* 2 weeks post infection.

4.5.5.3 IL-10

Treatment with *E. capensis* at a dose of 400mg/kg showed a 10% decrease in the mean serum level of IL-10 post-treatment in comparison to pre-treatment level and this was a highly significant ($P < 0.01$) difference. When compared to the ART treatment group, the post-treatment serum level was 8% lower showing a highly significant ($P < 0.01$) difference. In comparison with the infected untreated control

group, the mean serum level of IL-10 post-treatment was 25% lower a difference that was very highly significant ($P<0.001$).

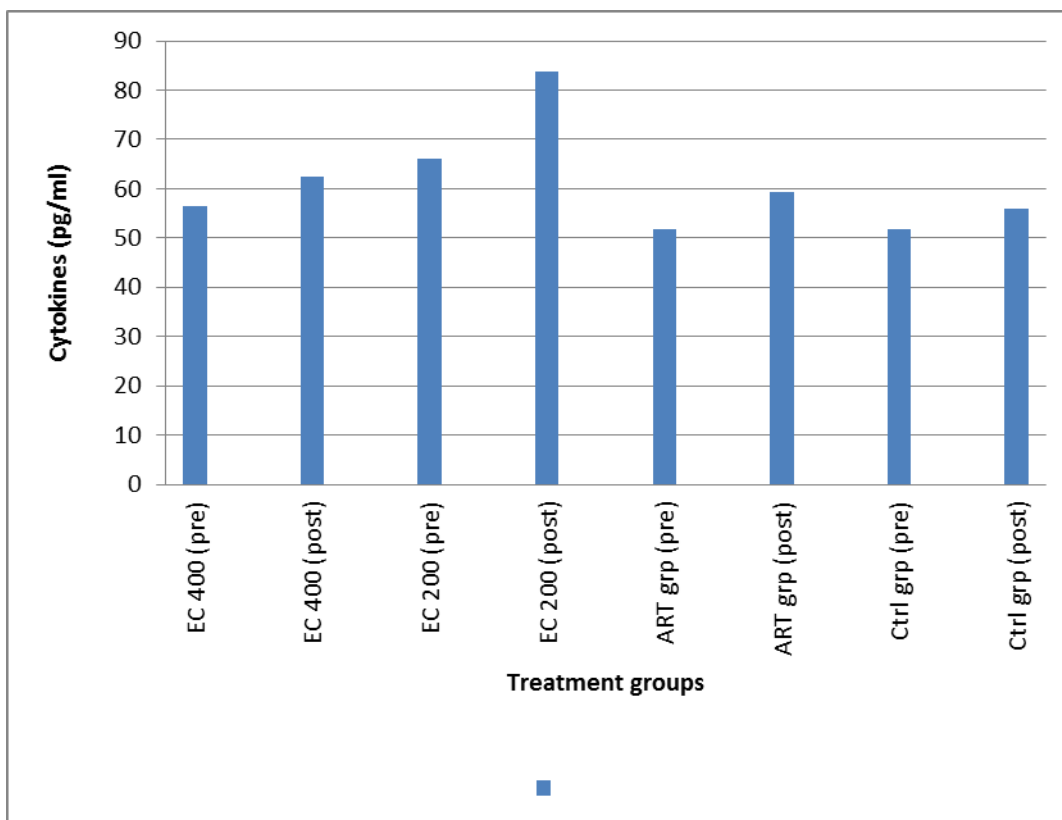
E. capensis at 200mg/kg resulted in a 11% decrease in the mean serum level of IL-10 post-treatment which was very highly significant ($P<0.001$) difference. In comparison with ART group, the post-treatment mean serum level of IL-10 was 8% lower indicating a significant ($P<0.05$) difference. When compared to the infected untreated group, the post-treatment mean serum level of IL-10 was 25% lower showing a very highly significant ($P<0.001$) difference as shown on Table 4.5 and Figure 4.14.

4.5.6 Effect of treatment with *E. capensis* at 2 weeks p.i on Th17 cytokines

4.5.6.1 IL-17

Treatment with *E. capensis* at a dose of 400mg/kg caused a 9% increase in the post-treatment mean serum level of IL-17 in comparison to the pre-treatment level and this was significant ($P<0.05$) difference. In comparison with ART group, the post-treatment mean serum level of IL-17 was 5% higher a difference that was highly significant ($P<0.01$). In comparison with the infected untreated control group, the post-treatment mean serum level of IL-10 was 10% higher showing a highly significant ($P<0.01$) difference.

E. capensis at a dose of 200mg/kg resulted in a 21% increase in the post-treatment level of IL-17 in comparison to the pre-treatment level indicating a very highly significant ($P<0.001$) difference. In comparison with ART group, the post-treatment mean serum level of IL-17 was 29% higher a difference that was very highly significant ($P<0.001$). In comparison with the infected untreated control group, the post-treatment mean serum level of IL-10 was 33% higher showing a very highly significant ($P<0.001$) difference as shown on Table 4.5 and Figure 4.15.



Graph showing the mean serum levels of Th17 cytokines before and after treatment. n=5. ART (artemether) was used as a control drug while the Ctrl group in infected untreated group. Pre- refers to before treatment while post- refers to after treatment. A representative of 2 similar experiment.

Figure 4.15 Mean serum levels of Th17 cytokines after treatment with aqueous extracts of *E. capensis* 2 weeks post infection.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

One of the earliest reported treatments against schistosomiasis in wide clinical use was antimonial compounds which began in 1918 and over the next 50 years. The antimonials were administered intravenously and caused numerous side effects such as nausea, vomiting, diarrhea, anorexia cardiovascular, hepatic and dermatological disturbances. Lethality from cardiac syncope and anaphylactic shock was also reported (Cioli *et al.*, 1995). These side effects led to the search for metal-free drugs as alternatives (Katz & Coelho, 2008). In the 1960s there was a breakthrough in the treatment of schistosomiasis with the advent of metrifonate, nitrofurans, lucanthone, niridazole, hyacanthone and finally oxamniquine. In 1970s, several antischistosomal drugs emerged such as tubercidin, amoscanate, PZQ and its benzodiazepine derivative Ro11-3128 and oltipraz. The therapeutic doses of most of these drugs were found to cause major side effects (Cioli *et al.*, 1995; Fenwich & Webster, 2006). Currently there are few drugs in use for the treatment of schistosomiasis. These are PZQ and oxamniquine and under special circumstances metrifonate and artemether. (Doenhoff, Cioli & Kimani, 2000). Of these, PZQ is the drug of choice as it is effective against all human infecting species of schistosomiasis and has the ability to irreversibly cause damage to adult worms and eggs lodged in host organs (Giboda & Smith, 1994). Never the less, PZQ has its limitations in that: it is less effective against developing worms necessitating multiple drug treatments (Utzing, Keiser, Shuhua, Tanner & Singer, 2003). At the recommended dosage, it achieves at best a 70% - 90% worm reduction and its efficacy is lowest in heavily infected individuals (Raso *et al.*, 2004). There are reported cases of schistosome isolates with reduced susceptibility to PZQ in the field in endemic areas and under laboratory conditions (You-Sheng *et al.*, 2001) which could see emergence and spread of PZQ resistant strains.

Medicinal plants may offer alternative remedies in the management of schistosomiasis and indeed there are reports of several plant species with

antischistosomal properties (Sparg *et al.*, 2000 and Molgaard *et al.*, 2001). Amongst these is the Chinese herb *Artemisia annua* which is effective against juvenile but not adult schistosomes (Utzinger *et al.*, 2001). The plants *A. indica* and *E. capensis* have been documented as having been used to treat a myriad of diseases that afflict man and domestic animals including tuberculosis and other bacteria, viral infections, malaria, and helminth infections (Elavasaru *et al.*, 2012; Muregi *et al.*, 2004; Lall *et al.*, 1999 and Jamnah, Khadijah, & Vincent, 2006). It is well effective against insect and tick vectors of medical importance (Abakala, Oyewole & Kolawole, 2012).

The fruit, seeds, oil, leaves, roots, bark and almost every part of the neem tree are bitter and contain compounds with proven antiviral, antiretroviral, anti-inflammatory, anti-ulcer, antifungal, anti-bacterial, anti-plasmodial, anti-septic, anti-pyretic and anti-diabetic properties (Subaprya & Nagini, 2005); El-Hawary *et al.*, 2013); Pandey *et al.*, 2012). Many bioactive compounds have been identified and isolated from various parts of the plant and these include azadiractin, nimbin and meliantriol. The leaves of this plant contain compounds like saponins, tannins and glycosides. The efficacy of neem as a broad-spectrum pesticide, non-toxic to humans has important implications for its application to parasitic diseases. The bark, seeds (Neem Foundation), leaf juice and roots are all traditionally used for the treatment of worms. Tender leaves are used in combination with *Piper nigrum* for intestinal helminthiasis (Chatterjee & Pakrashi, 1994). A decoction of leaves is used as a head wash to treat lice and scabies (Cole, 2002) and has shown satisfactory results in clinical trials among school children with headlice. Neem has immunostimulatory, anti-inflammatory and immunomodulatory effects as it enhances the humoral immune system (Willcox and Chamberlain, 2003). Intraperitoneal injection of neem seed oil enhances cell mediated immunity by increasing leucocyte counts and enhancing phagocytosis by macrophages (Upadhyay *et al.*, 1992).

Leaf macerations of *E. capensis* have been used to treat headache, fever, cough and skin diseases, while the Agĩkũyũ community treats diarrhea with its stem bark (Gachathi, 2007; Okello *et al.*, 2010). Pharmacological studies have indicated antiplasmodial, antiinflammatory, hypotensive, uterotonic and antituberculous activities of the crude extracts of this plant (Sewram *et al.*, 2000; Kamadyaapa *et al.*, 2009; Lall and Meyer, 1999; Mulaudzi *et al.*, 2013; Muregi *et al.*, 2004), providing

scientific support for its indigenous use. *E. capensis* extract was among the plants that showed anti plasmodial activity and may be used as a source of novel anti-plasmodial compounds (Muregi *et al.*, 2004). Uterotonic properties of extracts from this tree were also evaluated in an *in vivo* study where the extract yielded compounds of which two viz, oleonic acid and 3-epioleonic acid displayed uterotonic activity. Phytochemical investigations of its stem bark led to isolation of triterpenoids, steroids and flavonoids (Sewram *et al.*, 2000; Murata *et al.*, 2008; Nishiyama *et al.*, 1996). The stem bark of *E. capensis* was isolated and resulted in 10 new triterpenoid compounds, ekeberins A (1), B (2), C1 (3), C2 (4), C3 (5), D1 (6), D2 (7), D3 (8), D4 (9), and D5 (10), together with 17 known compounds. Several of these compounds were screened *in vitro* against both chloroquine (CQ)-sensitive and -resistant *Plasmodium falciparum* isolates and were found to exhibit moderate anti-plasmodial activity (Murata *et al.*, 2008). However, there are no documented reports of evaluation of these plants for their potential anti-schistosome properties. This study aimed at determining the antischistosomal and immune enhancing effect of aqueous extracts of *A. indica* and *E. capensis* extracts in murine infection.

5.1.1 *In vivo* effect of *E. capensis* and *A. indica* on adult *S. mansoni* worms

Mice treated with *E. capensis* at 7 weeks p.i showed a high percentage of worm reduction of 50%, 74% and 85% at the doses of 100mg/kg, 200mg/kg and 400mg/kg respectively in comparison to the infected untreated control group. The liver egg load reduction was 59%, 61%, 68% and 74% at doses of 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively in comparison to the infected untreated control group. Intestinal egg load reduction of 62%, 63%, 67% and 73% at 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively was observed relative to the infected untreated group. The group treated with PZQ (1000mg/kg) as the positive control recorded 94% worm reduction, 99% egg reduction in the livers and 99% egg reduction in the intestines relative to the infected untreated control group. This dose of PZQ was used because it has been reported to cause parasite reduction of at least 95% in mice (Gönnert & Andrews, 1977). Percentage worm and egg reduction in both liver and the intestines in the groups treated with high doses of the two plant extracts at 200mg/kg and 400mg/kg was almost comparable to the reductions observed in PZQ

control group. Treatment with *A. indica* at 7 week p.i resulted in high reduction of worms of 49%, 51% and 55% at the doses of 100mg/kg, 200mg/kg and 400mg/kg respectively in comparison to the infected untreated control group. Percentage egg reduction in the liver was 60%, 62%, 64% and 67% at 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively in comparison to the infected untreated control group. Percentage egg reduction in intestines was high in the doses 60%, 60%, 63%, 65% and 68% at 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively in comparison to the infected untreated control group. Although *A. indica* showed appreciable antischistosomal activity, it was lower than that of *E. capensis* and was not comparable to that of PZQ.

These results have shown that both plant extracts have antischistosomal activity with *E. capensis* showing more activity than *A. indica* in adult worm reduction and egg load reduction in livers and intestines. *E. capensis* administered at a dose of 400mg/kg showed activity against adult parasite worms

which was comparable to that of PZQ administered at a murine curative dose of 1000mg/kg body weight. A similar effect was observed in tissue egg load reduction. *A. indica* at the highest dose tested (400mg/kg) had lower activity against adult worms in comparison to PZQ.

5.1.2 *In vivo* effects of *E. capensis* and *A. indica* on juvenile *S. mansoni* worms

Mice treated with *E. capensis* at 2 weeks p.i recorded significant worm load reduction of 57%, 67% and 76% at the doses of 100mg/kg, 200mg/kg and 400mg/kg respectively in comparison to the infected untreated control group. Highly significant to very highly significant liver egg load reduction of 78% and 83% was recorded at doses of 200mg/kg and 400mg/kg respectively in comparison to the infected untreated control group. Intestinal egg load reduction was 76% and 89% at doses of 200mg/kg and 400mg/kg respectively in comparison to the infected untreated control group. *A. indica* treatment resulted in high worm load reduction of 46% and 51% at doses of 200mg/kg and 400mg/kg respectively in comparison to the infected untreated control group. Liver egg load reduction was high at 49% and 51% at doses of 200mg/kg and 400mg/kg respectively. High egg load reductions in the intestines

was 47% and 58% at doses of 200mg/kg and 400mg/kg respectively in comparison to the infected untreated control group.

Treatment at 4 weeks pi with *E. capensis* recorded 52%, 77% and 82% worm load reduction at the doses of 100mg/kg, 200mg/kg and 400mg/kg respectively relative to the infected untreated control group. In liver egg load reduction recorded was 73%, 89% and 91% at the doses of 100mg/kg, 200mg/kg and 400mg/kg respectively. Intestinal egg load reduction was 51%, 67%, 87% and 91% at doses of 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively. *A. indica* at 4 weeks p.i recorded high worm load reduction of 46% and 70% at the doses of 200mg/kg and 400mg/kg respectively in comparison to the infected untreated control group. Liver egg load reduction was at 79%, 87% and 91% at doses of 100mg/kg, 200mg/kg and 400mg/kg respectively. The egg load reduction in the intestines was at 68%, 75%, 80% and 89% at doses of 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg respectively. Both doses of 200mg/kg and 400mg/kg of *E. capensis* and *A. indica* showed high percentage juvenile *S. mansoni* worm and egg reduction in both liver and intestines. This reduction was comparable to that observed in the ART group that was used as positive control drug for the juvenile stages of *S. mansoni* infection (88% in worm reduction, 85% in liver egg load reduction and 89% in intestine egg reduction). These results on effect of ART are supported by Madbouly, Shalash, El Deeb & El Amir, 2015 and El-Beshbishi *et al.*, 2013 who have recorded a 90% to 92% juvenile worm reduction. This may be attributed to the ultrastructural toxic effect of ART on schistosome worms as ART was proved to induce tegumental damage, muscular paralysis and even sustained shrinkage, atrophy and degeneration of the worm's reproductive glands which include the testis in males, the ovary and vitelline gland in females (Pearce., 2005).

Both aqueous extracts showed appreciable antischistosomal activity but *E. capensis* was more potent with high activity on all the parasite stages. The high worm load and tissue egg load reduction observed after treatment with the plant extracts on juvenile worms is a clear indication that treatment resulted in early killing of worms thus preventing worm pairing and egg laying by the female worms. The results from this study are supported by other studies which have reported on plants that possess antischistosomal activity. Oliveira *et al* (2014) reported that *Baccharis trimera* Less

DC "Carqueja-amarga" exhibited *in vivo* schistosomicidal effect against immature, adult worms of *S. mansoni* and also significant reduction in egg load due to reduction in worm load. The crushed seeds of the plant *Nigella sativa* have also been found to have antischistosomal activity against cercariae and juvenile worms of *S. mansoni in vitro* (Mahmoud, El Abhar & Sales, 2002). These results are also in harmony with schistosomicidal activity of crude aqueous extract of ginger against *S. mansoni* reported by Mostafa, Eid & Adly (2011). He observed that parasite load and egg density in the liver and feces of mice treated with ginger were smaller than those in untreated cases. Male worms recovered from mice treated with ginger lost their normal surface architecture, extended erosion beyond the tegument, besides numerous bubbles around tubers. Mahmoud *et al* (2002) reported that treatment of mice infected with *S. mansoni* parasite using black seed oil, was effective in reducing egg count in both liver and intestines.

5.1.3 The *in vivo* effect of *E. capensis* and *A. indica* on cytokines.

Effect of treatment with the *E. capensis* and *A. indica* on cytokine profiles was analyzed for the two doses (200mg/kg and 400mg/kg) that recorded very high percentage reductions in worms and tissue egg load. The analysis was done using BD-CBA mouse Th1/Th2/Th17 cytokine kit. At 7 weeks p.i, treatment with *E. capensis* at 200mg/kg and 400mg/kg resulted in a reduction in TNF- α but an increase in IFN- γ and IL-2 (Th1 cytokines). Effect on Th2 cytokines resulted in a reduction in IL-4, an increase in IL-6 and a reduction in IL-10 levels. There was however an increase in IL-17 at both doses. The effect of treatment with the two doses of *A. indica* resulted in a reduction in TNF- α , an increase in IFN- γ and IL-2 which are Th1 cytokines. In the Th2 cytokines, there was a reduction in IL-4, an increase in IL-6 and a decrease in IL-10 levels. At 400mg/kg there was no significant effect on the level of IL-17 but there was an increase at 200mg/kg. This was comparable to the observation in the PZQ treatment group. The effect of these two plant extracts on juvenile *S. mansoni* infection (2 weeks p.i) resulted in an increase in TNF- α , IFN- γ and IL-2 cytokines. There was a reduction in Th2 cytokines IL-4, IL-6 and IL-10 and an increase in IL-17 (Th17) cytokine.

Both plants were found to modulate the course of schistosome infection at 7 weeks and 2 weeks p.i with a trend that showed an increase in Th1, a decrease in Th2 and an increase in Th17 cytokines. The increase of Th1 cytokines observed in this study could be as a result of helminthotoxic effect of these medicinal plant extracts to schistosomulae thus preventing the development of egg laying adult worm pairs (2 weeks p.i). The reduction in worm burden (as seen in treatment at 7 weeks p.i) could have resulted in down regulation of egg induced Th2 response and maintenance of Th1 predominant cytokine profile characterized by high IFN- γ and low IL-4. IFN- γ has been shown to be involved in protective immunity to schistosomiasis in murine models (Hewitson, Hamblin & Mountford, 2005). Maintenance of high levels of Th1 and Th17 cytokines and low levels of Th2 cytokines is also indicative of a failure of a switch from Th1 to Th2 due to the death of worms after treatment and therefore fewer eggs being laid by the surviving worms.

Murine schistosomiasis is characterized by Th1 reaction (with a predominant secretion of IFN- γ , minimal level of IL-4 and IL-5) occurring during prepatency and then shifting to a Th2-based profile which develops after the onset of oviposition and persists throughout the acute phase of infection (with high IL-4 and IL-5, but low IFN- γ) (Davies *et al*, 2004). Ironically, egg induced Th2 responses are an immunologic double-edged sword, participating in protection of host tissues from egg-induced injury (Brunet *et al.*,1997) and in the development of the egg- induced pathology and fibrosis associated with chronic schistosome infection (Wynn and MacDonald, 2004). The natural and induced forms of severe schistosomiasis correlates with high levels of pro-inflammatory cytokines IFN- γ and IL-17 (Rutitzky *et al.*, 2008). This is indicative of the Th1 and Th17 subpopulations of CD4 T lymphocytes. This can be related to the observation in this study. The shift from Th2 to Th1-like immune response (as observed in this study) is essential for the down modulation of granuloma reaction and disease control. Th1 cytokine profile results in the development of smaller granulomas (Brunet *et al*, 1998). PZQ activity has been shown to be dependent on T cell mediated immunity (Ammann *et al.*, 2004). IFN- γ is involved in protective immunity to schistosomiasis in murine models. Immunomodulatory effects of the two medicinal plant extracts was observed in this study as indicated by an increase or a decrease in serum levels of cytokines which

was dose dependent. The results from this study are supported by studies that have been carried out on *A. indica* showing that it has immunomodulation ability. The aqueous extracts of neem leaves have been shown to have immunomodulatory response to live Newcastle disease vaccine (Garbaa *et al*, 2013). Neem leaf preparation enhances Th1 immune response and anti-tumour immunity against breast tumour associated antigen (Mandal-Ghosh, Chattopadhyay & Baral, 2007). There is no evidence of previous studies on the immunomodulatory effect of *E. capensis*, thus this is the first one.

5.2 Conclusions

Both plants demonstrated significant *in vivo* antischistosomal activity against adult, immature and juvenile infection which was dose dependent with *E. capensis* being more potent with high activity on all the parasite stages. *E. capensis* showed greater ability in worm burden reduction and also tissue egg load reduction in both adult and juvenile *S. mansoni* infection compared to *A. indica*. *Azadirachta indica* showed a high activity on juvenile worms (4 weeks p.i) at the highest dose (400mg/kg) and this was also comparable to artemether. The high reduction of worm load in the groups treated with *E. capensis* and *A. indica* at 2 weeks and 4 weeks p.i and a consequent reduction in tissue egg load (in both livers and intestines) is an indication that treatment with these plant extracts prevented worm pairing which in turn resulted in low eggs laid by surviving worms.

Treatment with both plant extracts also had an effect on the immune system. This was evident from the increase and decrease in the levels of cytokines before and after treatment at the two different points during the course of the infection. The results from this study indicate a switch over from Th2 to Th1 immune response in the 7 weeks p.i group due to the helminthotoxic effects of the two plant extracts. At 2 weeks p.i, a Th1 immune response was maintained due to death of larval worms after treatment and therefore fewer eggs being laid by the surviving worms. Both test plant extracts showed immunomodulatory effects as they caused an increase or a decrease in serum levels of cytokines which was dose dependent.

5.3 Recommendations

The results from this study show that *E. capensis* and *A. indica* have appreciable antischistosomal and immunomodulating effect. Further studies should be done to determine if the extracts from these plants can be used singly or in combination with PZQ in the management of schistosomiasis since this could become a strategy for transmission control which can reduce the morbidity due to schistosomiasis in endemic regions. Isolation and characterization of the active compound(s) of these plants and determination of their mechanism(s) of action is also recommended in search for novel antischistosomal agents. These medicinal plants should also be tested for antiparasitic effect against other species of schistosomes and other helminthic infections.

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APPENDICES

Appendix 1: Ethical Review Committee approval letter.


 

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **July 8, 2013**

TO: **RAEL MUSILI,**
PRINCIPAL INVESTIGATOR

THRO': **DR. KIMANI GACHUHI,**  **THE DIRECTOR, CBRD,**
NAIROBI 10/7/13

RE: **SSC PROTOCOL NO. 2526 (RE-SUBMISSION): ANTI-SCHISTOSOMAL AND IMMUNE ENHANCING POTENTIAL OF AZADIRACTA INDICA AND EKEBERGIA CAPENSIS IN MICE INFECTED WITH SCHISTOSOMA MANSONI**

Reference is made to your letter dated June 7th, 2013. The ERC Secretariat acknowledges receipt of the Revised Study Protocol –Version 1.2, dated 7th June 2013 on 27th May 2013.

The Committee notes that the all the issues raised have been properly addressed and the study is hereby **granted approval** for implementation effective this **8th day of July 2013**.

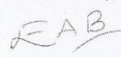
Please note that authorization to conduct this study will automatically expire on **7th day of July, 2014**.

If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **May 26th, 2014**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study

Yours faithfully,



DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix II: Scientific Steering Committee approval letter.



KENYA MEDICAL RESEARCH INSTITUTE

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ESACIPAC/SSC/100489

12th March, 2013

Rael Musili

Thro'

Director, CBRD
NAIROBI

[Handwritten signature] 12/3/13

REF: SSC No. 2526 (Revised) – Antischistosomal and immune enhancing potential of *Azadiracta indica* and *Ekebergia capensis* in mice infected with *Schistosoma mansoni*.

Thank you for your letter dated 11th March, 2013 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval.

[Handwritten signature]

Sammy Njenga, PhD
SECRETARY, SSC

Appendix III: Animal Care and Use Committee approval letter.



KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O. Box 54628 - 00200 NAIROBI - Kenya
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KEMRI/ACUC/ 01.04.13

5th April 2013

Rael Mueni Musili,
TM 305-0004 / 2008

RE: Animal use approval for SSC 2526 "Antischistosomal and immune enhancing potential of *Azadiracta Indica* and *Ekebergia Capensis* in mice infected with *Schistosoma Mansoni*" protocol

The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that all the issues raised earlier have been addressed appropriately.

The committee grants you the approval to use laboratory mice in your study but recommends that you proceed with the study after obtaining all the other necessary approvals that may be needed.

The committee also expects you to adhere to all the animal handling and experimental procedures as described in the protocol.

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

In Search of Better Health

Appendix IV: Publication



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Antischistosomal Activity of *Azadirachta indica* and *Ekebergia capensis* in Mice Infected with *Schistosoma mansoni*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RM and GK designed the study. Authors RM, JM, DM, LM, TK, AM, SC and JT participated in carrying out the study. Author RM performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors RM, FM, ZN and GK managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Schistosomiasis is a parasitic disease of great socio-economic and public health importance in tropical and sub-tropical countries. Praziquantel (PZQ) is the drug of choice for treatment of schistosomiasis since it is effective against all species of schistosomes. However, PZQ is less efficacious against larval stages of the parasite and there are recent concerns that long term mass drug treatment could lead to development of drug resistant strains thus prompting the need for alternative antischistosomal drugs. Plants have over the years provided a rich source of novel

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drugs for a wide range of diseases afflicting man and domestic animals.

Study Design: Swiss albino mice were infected and randomized into groups of five for plant extract treated groups, positive control groups treated with conventional drugs PZQ and artemether, as well as infected but untreated (negative control) groups.

Place and Duration of Study: The study was done at the Animal Facility in the Centre for Biotechnology Research and Development, Kenya Medical Research Institute from July 2013 to July 2014.

Methodology: Swiss albino mice were infected with 90 cercariae each and treated orally with varying doses of aqueous extracts of *Ekebergia capensis* and *Azadirachta indica* at doses of 25, 50, 100, 200 and 400 mg/kg at 2 weeks (juvenile worms), 4 weeks (immature worms) and 7 weeks (adult worms) post infection. PZQ and artemether were used as positive controls while infected untreated group was used as negative controls. Total reduction of worm load as well as egg load in the liver and intestine was used as an indicator of drug activity, relative to the infected but untreated control groups.

Results: Both *E. capensis* and *A. indica* showed significant dose-dependent percentage worm load reduction ($P < 0.05$) at different doses ranging from 100 mg/kg to 400 mg/kg. These extracts also significantly reduced tissues (liver and intestine) egg load counts at doses ranging from 50 mg/kg to 400 mg/kg which was also dose-dependent.

Conclusion: The antischistosomal activity of the two plant extracts was dose dependent with *E. capensis* being more potent in reducing both the worm burden at all the stages and tissue egg load. These findings validate the potential use of medicinal plants in the management of schistosomiasis and provide a basis for exploring medicinal plants as sources for new antischistosomal agents.

Keywords: Schistosomiasis; antischistosomal agents; *Schistosoma mansoni*; *Azadirachta indica*; *Ekebergia capensis*.

1. INTRODUCTION

Schistosomiasis (bilharziasis or snail fever) is second only to malaria in terms of diseases that are of socio-economic and public health importance in tropical and subtropical areas [1]. The disease is endemic in 74 countries infecting more than 207 million people worldwide with 20000 deaths associated with the severe consequences of infection [2]. More than 85% of those infected live in Africa [2]. In Kenya, the infection is wide spread around Mwea irrigation scheme in Kirinyaga County, Machakos, Kitui, Taita Taveta and Nyanza [3].

The drugs that have been used in the recent past to treat schistosomiasis include metrifonate which is effective against *S. haematobium*, Oxamniquine which is effective against *S. mansoni* and artemisinin which exhibits highest activity against 1-3 week old liver stages of the parasite. Praziquantel (PZQ) has been the drug of choice for treatment of all species of schistosomes because of its efficacy, ease of administration, safety, and cost [4]. However, PZQ is only effective against adult worms and ova of schistosomes but is ineffective on immature worms that are present in recently acquired infections and this leads to reduced cure rates [5-8]. PZQ has been in use for more

than two decades and possible existence of tolerant *S. mansoni* isolates have been reported in Senegal [9]. In regions of Egypt and Kenya where there has been heavy exposure to PZQ, there are reports of *S. mansoni* and *S. haematobium* resistance to treatment [10]. The effectiveness of PZQ is dependent on an intact immune system [11] and its mechanisms of action are poorly understood [7]. There is, therefore, need for development of alternatives to PZQ and herbal plants are a potential source of new drugs.

Herbal plants have been used traditionally to treat or manage schistosomiasis in Eastern and Southern Africa [12-14]. However, limited information is available on the beneficial effects of herbal preparations in the treatment and management of the disease and few attempts have been made to verify scientifically, the antischistosomal properties of such preparations. Reports of availability of plant derived concoctions for treatment of schistosomiasis from traditional practitioners in Kenya are numerous but these claims have not been sufficiently validated. Kokwaro (1993) reported that at least 19 plant species occurring in East Africa region and belonging to 10 families and 16 genera have been used traditionally as remedies for schistosomiasis. Examination of potential

of plants in management of schistosomiasis by various investigators has revealed several plants with appreciable antischistosomal activities [13-15].

Artemether, a semi-synthetic derivative of artemisinin, which is a bioactive compound derived from the Chinese herb *Artemisia annua*, has been investigated for its activity on *S. mansoni*. The drug demonstrated efficacy on early stages of the infection with 75.3-82.0% worm reduction which was boosted to 97.2-100% when the animals were subjected to various schedules of repeated treatment [16]. More recently, artemisinin derivatives, either alone or in combination with PZQ, have been shown to be effective against immature stages of *S. mansoni*, *S. haematobium*, and *S. japonicum* in laboratory studies and limited field studies. However, the use of oral artemisinin based monotherapy is discouraged in schistosomiasis and malaria co-endemic areas as this could lead to development of resistance to artemisinin-based drugs by *Plasmodium* parasites.

In the present study, we investigate the antischistosomal activity of aqueous extracts of *A. indica* (family: Meliaceae, genus: *Azadirachta*) and *E. capensis* (family: Meliaceae, genus: *Ekebergia*) against juvenile, immature adult and adult worms of *S. mansoni* in infected mice. These plants have been reported to be used traditionally for treatment of parasitic infections and abdominal complications [12].

2. MATERIALS AND METHODS

2.1 Life Cycle Maintenance of *S. mansoni*

The livers of mice were collected from *S. mansoni* infected mice; eggs were extracted from them and were hatched into miracidia. Freshly hatched miracidia (less than 1 hour old) were used to infect 30 *Biomphalaria pfeifferi* snails whose diameter was 4 mm using the routine optimized technique used for schistosome cycle maintenance at the facility. Briefly, the livers were crushed through sieves to collect eggs that were exposed to water and light in a large glass petridish to hatch into miracidia. The miracidia were picked using a Pasteur pipette into wells of a 24-well plate. Each well of the plate had 5 miracidia. The 4mm diameter snails were placed into each well and the plate was covered and left for 2 hours to give time for the miracidia to infect the snails. The infected snails were transferred into a freshly prepared and well-labelled, aerated

aquarium and fed on lettuce and bone meal for 28 days at a temperature of 28-30°C.

2.2 Infection of Experimental Mice with *S. mansoni* Cercariae

Mice used in this study were Swiss albino, males, aged 6 weeks old and weighing 20-25 grams. The infected snails were placed under a lamp for illumination to enhance cercariae shedding. Freshly shed cercariae (not more than 1 hour old) were used. Each mouse was anaesthetized using sodium pentobarbitone and infected with 90 cercariae using the abdominal ring method [17]. The infected mice were randomized into cages in groups of 5 and maintained with pellets and water *ad libitum*.

2.3 Plant Materials and Extraction

The test plants *E. capensis* and *A. indica* were collected from central Kenya (Mount Kenya Forest) and Southern Rift Valley (Nguruman Escarpment in Magadi) respectively. The plants were identified by a taxonomic botanist from East African Herbarium in Nairobi, where they were catalogued and voucher specimens deposited (*E. capensis*: Stem bark (Ec-SB/04) 26 and *A. indica*: Leaf (Ai-L/04) 10). The plant samples were then air dried at room temperature under shade and ground to powder using an electric mill. The powder was packed into one kilogram packs and stored in a dry and well ventilated room until use. The plant parts used were bark for *E. capensis* and leaves for *A. indica*. The dried chaff of the plant parts to be used (50 g of each) was weighed and soaked in 500 ml of water. This was followed by thorough mixing on a shaker and soaking for 12 hours. The mixture was then filtered and freeze dried to give at least 2g of dry solid material.

2.4 Drug Treatment of *S. mansoni* Infected Mice

Infected mice were randomized into groups of 5 and placed in separate well labelled cages. To target different developmental stages, treatments with the plant extracts were done at 2, 4 and 7 weeks post infection (p.i.) to represent juvenile, immature adults and adult worms respectively. The plant extracts were administered at doses of 25, 50, 100, 200 and 400 mg/kg. Both plants extracts were administered orally by gastric gavage once a day for 3 consecutive days using an oral volume of 0.2 ml per mouse [18]. A positive control group (for 2 and 4 weeks p.i) was

treated with artemether at 200 mg/kg, administered orally by gastric gavage once a day for 3 consecutive days using an oral volume of 0.2 ml per mouse [18]. Since Praziquantel (PZQ) is known to target adult worms, another positive control group (for treatment at 7 weeks p.i) was included, where mice were orally treated with PZQ at a dose of 200 mg/kg body weight per day using a dose volume of 0.05 ml for 5 consecutive days to a total dosage of 1000 mg/kg [5]. Infected untreated negative control groups were also included that received 0.2 ml of distilled water for 3 consecutive days.

2.5 Assessment of the Effects of Treatment

2.5.1 Worm load counts

All the groups of mice were sacrificed 3 weeks post treatment by injecting them with 0.25 ml of heparinized sodium pentobarbitone intraperitoneally to euthanize them and the worms recovered through perfusion of liver and mesenteric veins [17]. Livers and guts were recovered from the perfused mice, wrapped in foil paper and stored in a freezer at -20°C until used. The worms were collected in petri-dishes and enumerated under a dissecting microscope.

2.5.2 Tissue egg load count

The stored liver and guts were processed for tissue egg counts by trypsin digestion. Briefly, the livers and intestines were removed from the freezer and their individual weights recorded. Each tissue was chopped using a scalpel blade and the pieces placed in 200 ml beakers. Trypsin enzyme (Merck) was used to digest the tissues at 0.01 mg per gram of tissue. Phosphate buffered saline (PBS) (200 ml) was added into the beaker and the mixture homogenized using a kitchen blender into a fine emulsion. The emulsion was poured into beakers, covered with Para film and incubated at 37°C for 2 hours with occasional shaking. After incubation, the emulsion was transferred into 50 ml centrifuge tubes and spun for 10 minutes at 2000 rpm. The supernatant was poured off leaving an egg pellet. PBS was added to help dissolve the egg pellet and the volume of the egg suspension was noted. Aliquots of 50 µl were transferred onto microscope slides using a micropipette and glass cover slips placed on top of sample. Eggs were counted under a microscope using 10X magnification. A mean was calculated and this was multiplied by 20 to estimate the number of

eggs per ml. This was multiplied by the volume of egg suspension to derive the number of eggs in the whole tissue.

2.6 Statistical Analysis

The number of worms recovered from the experimental groups was expressed as mean±SD. The efficacy of the drug regimens was calculated as the mean number of recovered worms from each group relative to the control group [16]. The mean number of worms and eggs recovered from the different groups were subjected to Student's t-test using Microsoft Excel® to determine their statistical significance in comparison with the control groups. Statistical significance was set as $P < 0.05$.

3. RESULTS

3.1 Treatment at 2 Weeks Post Infection

When *S. mansoni* infected mice were treated at 2 weeks p.i using *E. capensis* at a dose range of 100mg/kg to 400 mg/kg, the percentage worm load reduction as well as egg load reduction was above 50% (Table 1 and Fig. 1). The extract at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg showed worm load reduction of 57%, 67% and 76%, respectively. Similarly, egg load reduction in livers of these mice was recorded as 55%, 78% and 83% at doses of 100 mg/kg, 200 mg/kg and 400mg/kg respectively (Fig. 1). On the other hand, intestinal egg load reduction from mice for the same doses was 53%, 76% and 89% respectively. When mice were treated using *A. indica*, only a dose of 400 mg/kg gave worm and egg load reduction of >50%, which was recorded as 51% worm load reduction, with 51% and 58% egg load reduction in the liver and intestine, respectively (Fig. 1). The positive control drug, artemether at a dose of 200 mg/kg gave 88% worm load reduction, with 85% and 89% egg load reduction in the livers and intestines respectively (Fig. 1).

T-test: two sample assuming unequal variances was performed (excel data analysis tool for windows 7) to compare the statistical differences in mean worm and egg load recovered from livers and intestines between treatment groups and control groups. In the mice treated with *E. capensis* extract, there was significant difference in means of total worms recovered from all the treatment groups (25-400 mg/kg) and also in eggs recovered from the liver and intestine tissues (50-400 mg/kg) relative to the untreated

infected (negative) control group ($P < 0.05$) groups when compared to the positive control (Table 1). There was no significant difference (artemether treated) group. ($P > 0.05$) in the *E. capensis* extract treated

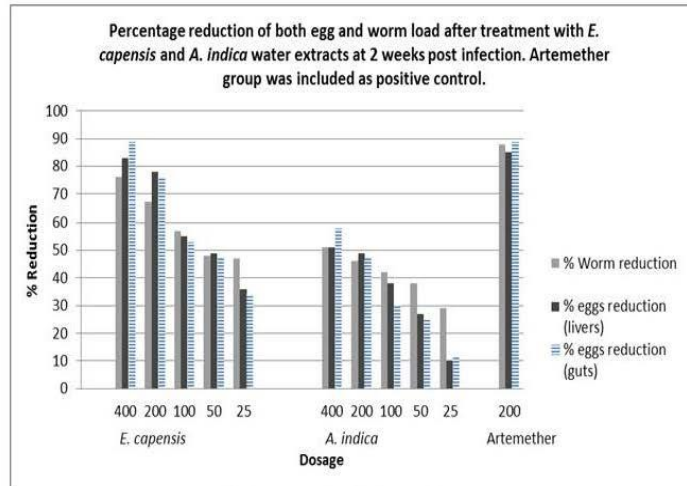


Fig. 1. Percentage worm and egg reduction after treatment with aqueous extracts of *A. indica* and *E. capensis*

Table 1. Mean number of worms and eggs recovered from livers and intestines following treatment with *A. indica* and *E. capensis* at 2 weeks post infection

Drug	Number of male worms (Mean ±SD)	Number of female worms (Mean ±SD)	Total number of worms (Mean ±SD)	Number of eggs (livers) (Mean ±SD)	Number of eggs (intestines) (Mean ±SD)
<i>E. capensis</i>					
400mg/kg	6.6±2.7	4.6±3	10.6±5.3 ^a	2580±753 ^a	1640±474 ^a
200mg/kg	8.4±1.1	6±2.1	14.4±3 ^a	3370±744 ^a	3735±1545 ^a
100mg/kg	11.2±2.3	8±2.5	19.2±4.3 ^a	6900±897 ^a	7284±406 ^a
50mg/kg	12±3.4	11±4.8	23±7.8 ^a	7772±586 ^a	8176±1368
25mg/kg	14±1.6	9.6±1.1	23.6±2.6 ^a	9752±934	10330±556
<i>A. indica</i>					
400mg/kg	10.4±2.1	11.2±3.3	21.6±4.1 ^a	7350±1199 ^a	6572±724 ^a
200mg/kg	11.4±2.1	12.4±4	23.8±3.8 ^a	7784±811 ^a	8190±1097 ^a
100mg/kg	12.6±2.7	13±2.1	25.6±3.8 ^a	9440±854	10895±1998
50mg/kg	13.8±2.8	13.6±2.9	27.4±3.4 ^a	11128±859	11697±1103
25mg/kg	15.6±3	16±2	31.6±4.8 ^a	15172±1302	13709±581
Artemether	9.8±2.4	8±3.1	17.8±5.3 ^a	2300±245 ^a	1638±385 ^a
Control	29±3.5	25±4.3	54±6.9	15307±549	15572±1342

Data are expressed as mean±SD, n=4 or 5. Data were analyzed by T-test: two sample assuming unequal variance. Values with superscript letters are significant ($P < 0.05$), relative to untreated controls. ^a Statistically significant ($P < 0.05$) compared to negative (untreated) controls

Similarly, mice treated with *A. indica* extract showed significant difference ($P<0.05$) in worm load reduction in all treated groups when compared to the untreated infected (negative) control group while significant difference ($P<0.05$) in egg load reduction both in the liver and intestine tissues was observed at 200 mg/kg and 400 mg/kg (Table 1). When comparison was done with the artemether-treated group, there was no significant difference that was observed ($P>0.05$).

Artemether treated group showed significant worm load and tissues egg load reduction ($P<0.05$) relative to the untreated controls (Table 1).

3.2 Treatment at 4 Weeks Post Infection

When *S. mansoni* infected mice were treated at 4 weeks p.i at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg using *E. capensis* extract, more than 50% worm reduction was observed as 57%, 77% and 82% respectively (Table 2 and Fig. 2). Egg load reduction at doses of 50mg/kg, 100 mg/kg, 200 mg/kg and 400 mg/kg in livers of these mice was recorded as 54%, 73%, 89% and 91%, respectively while corresponding egg load reduction from intestines of the mice at the same doses was 51%, 67%, 87% and 91% (Fig. 2). When treated using *A. indica* extract, 70% worm reduction was observed at a dose of 400 mg/kg. At doses of 50 mg/kg, 100 mg/kg, 200 mg/kg and 400mg/kg, the extract gave percentage egg reduction from the liver by 68%, 75%, 80% and

89% respectively (Fig. 2). The corresponding percentage egg load reduction from the intestines of the same mice at the same doses was 68%, 75%, 80% and 89% respectively. Artemether treated group had 60% worm load reduction, with 88% and 89% egg load reduction from the liver and intestine respectively (Fig. 2).

T-test two sample assuming unequal variance was performed (using excel data analysis tool for windows 7) to compare the statistical differences in mean worms and eggs recovered from livers and intestines between treated groups and control groups. When treated with *E. capensis* extract, all the treatment groups had significant difference ($P<0.05$) when compared to the untreated (negative) control in worm and egg load in both liver and intestine tissues except in mean total worm load at 25mg/kg (Table 2). When compared to the positive control (artemether) group, there was significant difference ($P<0.05$) in all the groups for mean worm and egg load in liver and intestine tissues except in mean worm load at 200 and 400mg/kg (Table 2).

When treated with *A. indica* extract, there was statistical difference ($P<0.05$) in all the groups tissues in mean worm and egg load reduction in both liver and intestine tissues relative to untreated (negative) control except for mean worm load reduction at 25 mg/kg dose group (Table 2).

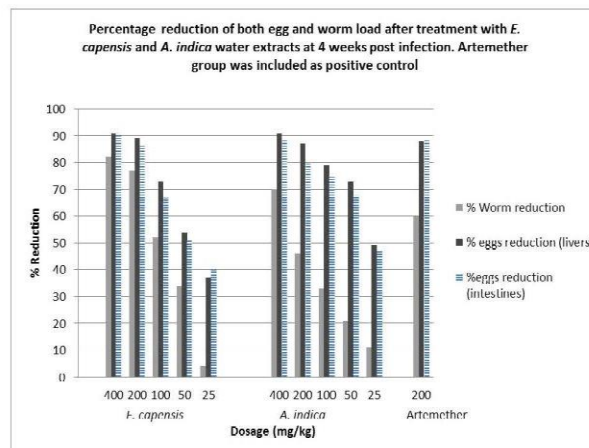


Fig. 2. Percentage worm and egg reduction after treatment with aqueous extracts of *A. indica* and *E. capensis*

Table 2. Mean number of worms and eggs recovered from livers and intestines following treatment with *A. indica* and *E. capensis* at 4 weeks post infection

Drug	Number of male worms (Mean \pm SD)	Number of female worms (Mean \pm SD)	Total number of worms (Mean \pm SD)	Number of eggs (livers) (Mean \pm SD)	Number of eggs (intestines) (Mean \pm SD)
<i>E. capensis</i>					
400mg/kg	6.8 \pm 1.9	4.6 \pm 1.8	11.4 \pm 3.6 ^a	2334 \pm 568 ^a	2823 \pm 878 ^a
200mg/kg	8.2 \pm 3.3	6.4 \pm 3	14.6 \pm 6.2 ^a	3097 \pm 1058 ^a	4001 \pm 1053 ^a
100mg/kg	16.8 \pm 1.9	13 \pm 2.5	29.8 \pm 4.2 ^a	7434 \pm 1150 ^a	9562 \pm 871 ^a
50mg/kg	22.2 \pm 3.8	19.2 \pm 6.9	41.4 \pm 10.5 ^a	12552 \pm 3279 ^a	15232 \pm 3680 ^a
25mg/kg	30.2 \pm 2.6	30.2 \pm 2.4	60.4 \pm 3.2	17320 \pm 3439 ^a	22760 \pm 7670
<i>A. indica</i>					
400mg/kg	9.2 \pm 5.3	9.4 \pm 5.8	18.6 \pm 10.6 ^a	2475 \pm 2412 ^a	3383 \pm 2404 ^a
200mg/kg	18.6 \pm 1.5	15 \pm 2.2	33.6 \pm 3.5 ^a	3666 \pm 1196 ^a	6186 \pm 1289 ^a
100mg/kg	22 \pm 2	20.2 \pm 2.58	42.2 \pm 4.2 ^a	5763 \pm 1170 ^a	7770 \pm 1126 ^a
50mg/kg	25.4 \pm 2.6	24.8 \pm 1.3	50.2 \pm 2.8 ^a	7398 \pm 1528 ^a	9751 \pm 1842 ^a
25mg/kg	29 \pm 3.2	26.8 \pm 4.9	55.8 \pm 6.5 ^a	14007 \pm 3324 ^a	16402 \pm 4265 ^a
Artemether	4.6 \pm 3.4	2.2 \pm 1.5	6.8 \pm 4.8 ^a	247 \pm 212 ^a	446 \pm 293 ^a
Control	33.4 \pm 6.9	29.2 \pm 3.7	62.6 \pm 9.1	27445 \pm 6148	30785 \pm 3654

Data are expressed as mean \pm SD, n=4 or 5. Data were analyzed by t-test (two sample assuming unequal variance). Values with superscript letters are significant (P<0.05). ^a Statistically significant values (P<0.05) relative to negative (untreated controls)

When the positive control (artemether) was compared with the negative control group, there was significant difference (P<0.05) in mean worm load reduction as well as liver and intestines egg load reduction as shown in Table 2.

3.3 Treatment at 7 Weeks Post Infection

When *S. mansoni* infected mice were treated with *E. capensis* extract at 7 weeks p.i at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg (Table 3), percentage worm load reduction was observed as 50%, 74% and 85% respectively (Fig. 3). Percentage egg load reduction at doses of 50 mg/kg, 100 mg/kg, 200 mg/kg and 400 mg/kg in the liver was recorded as 59%, 61%, 68% and 74% respectively, while corresponding percentage intestinal egg load reduction at the same doses was recorded as 62%, 63%, 67% and 73% (Fig. 3). When mice were treated using *A. indica* extract at doses of 200 mg/kg and 400 mg/kg (Table 3), the percentage worm reduction was observed as 51% and 55% respectively. Mice treated with *A. indica* extract at doses of 50 mg/kg, 100 mg/kg, 200 mg/kg and 400 mg/kg had percentage egg load reduction from liver tissue of 60%, 62%, 64% and 67 while corresponding intestinal egg load reduction at the same doses was recorded as 60%, 60%, 63%, 65% and 68% (Fig. 3). The results from the

PZQ treatment group were 94% worm reduction, and 99% egg load reduction for both the liver and intestinal tissues (Fig. 3).

T-test: two sample assuming unequal variances was performed (using excel data analysis tool for windows 7) to compare the statistical differences in mean worms and eggs recovered from livers and intestines between treatment groups and control groups. When treatment was done at 7 weeks post infection using *E. capensis*, all the treatment groups had significant difference (P<0.05) for both mean worm load as well as egg load reduction in liver and intestinal tissues relative to the untreated (negative) controls. There was no statistical difference that was observed in mean worm load reduction at 400 mg/kg of the plant extract relative to the positive control (PZQ treated) group (P>0.05).

When treated with *A. indica*, there was significant difference (P<0.05) in mean worm load as well as liver and intestine tissues egg load in all the treatment groups relative to infected untreated control groups.

When the positive (PZQ) group was compared to the negative (untreated) control group, significant difference (P<0.05) was observed in mean worm and egg load reduction in both liver and intestinal tissues (Table 3), an expected outcome.

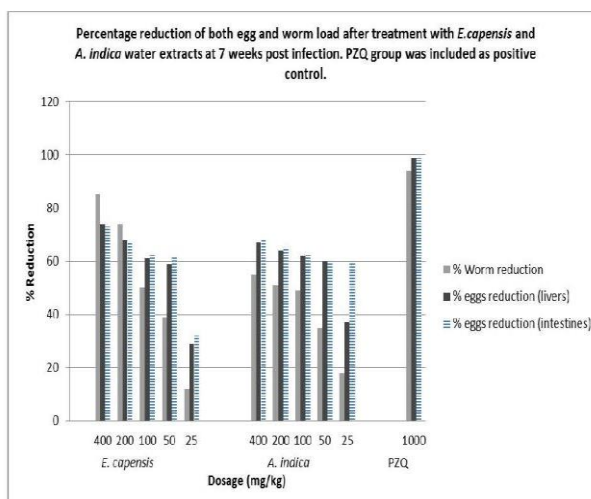


Fig. 3. Percentage worm and egg reduction after treatment with aqueous extracts of *A. indica* and *E. capensis*

Table 3. Mean number of worms and eggs recovered from livers and intestines following treatment with *E. capensis* and *A. indica* at 7 weeks post infection

Drug	Number of Male worms (Mean \pm SD)	Number of Female worms (Mean \pm SD)	Total Number of worms (Mean \pm SD)	Number of eggs in the livers (Mean \pm SD)	Number of eggs in the intestines (Mean \pm SD)
<i>E. capensis</i>					
400mg/kg	5.8 \pm 3.7	4.8 \pm 3.3	10.6 \pm 6.9 ^a	10292 \pm 1101 ^{a,b}	11744 \pm 1604 ^{a,b}
200mg/kg	10.4 \pm 1.1	8 \pm 1.6	18.4 \pm 2.7 ^{a,b}	12450 \pm 1766 ^{a,b}	13428 \pm 1858 ^{a,b}
100mg/kg	18.8 \pm 4.1	16.4 \pm 4.4	35.2 \pm 8.2 ^{a,b}	15158 \pm 1970 ^{a,b}	15694 \pm 1323 ^{a,b}
50mg/kg	22.2 \pm 2.3	21.4 \pm 1.5	43.6 \pm 3.5 ^{a,b}	15772 \pm 1371 ^{a,b}	16110 \pm 3079 ^{a,b}
25mg/kg	29.4 \pm 3.1	33 \pm 1.6	62.4 \pm 4.1 ^{a,b}	27858 \pm 5442 ^{a,b}	29298 \pm 5725 ^{a,b}
<i>A. indica</i>					
400mg/kg	17 \pm 4.6	14.6 \pm 2.8	31.6 \pm 4 ^{a,b}	12876 \pm 2643 ^{a,b}	13603 \pm 2819 ^{a,b}
200mg/kg	17.4 \pm 4.4	17.4 \pm 4.1	34.8 \pm 8.4 ^{a,b}	14005 \pm 2666 ^{a,b}	14872 \pm 3046 ^{a,b}
100mg/kg	19 \pm 5.3	16.6 \pm 6.3	35.6 \pm 11.3	14932 \pm 3546 ^{a,b}	15974 \pm 5229 ^{a,b}
50mg/kg	23.8 \pm 2.4	22.4 \pm 3.6	46.2 \pm 5.4	15577 \pm 1448 ^{a,b}	17222 \pm 1759 ^{a,b}
25mg/kg	30.8 \pm 2.1	27.2 \pm 3.7	58 \pm 5.6 ^{a,b}	24545 \pm 3326 ^{a,b}	27254 \pm 3181 ^{a,b}
PZQ	1.8 \pm 1.7	1.8 \pm 1.6	3.6 \pm 3.3 ^a	278 \pm 288 ^a	408 \pm 314 ^a
Control	36.4 \pm 4.1	34.6 \pm 2.6	71 \pm 6.4	39142 \pm 4963	42790 \pm 4827

Data are expressed as mean \pm SD, n=4 or 5. Data were analyzed by t-test: two sample assuming unequal variance. Values with superscript letters are significant ($P < 0.05$).^a Statistically significant ($P < 0.05$) compared to negative (untreated controls); ^b Statistically significant ($P < 0.05$) compared to positive control (PZQ).

4. DISCUSSION

Currently there are few drugs available for the treatment of schistosomiasis these being limited to PZQ and oxamniquine and under special circumstances metrifonate and artemether may

be used [19]. Of these, PZQ is the drug of choice as it is effective against all human infecting species of schistosomiasis, has the ability to irreversibly cause damage to adult worms and eggs lodged in host organ [20]. Never the less, PZQ has its limitations in that: it is less effective

against developing worms necessitating multiple drug treatments [21]; at the recommended dosage it achieves at best a 70% - 90% worm reduction and its efficacy is lowest in heavily infected individuals [22]; there are reported cases of schistosome isolates with reduced susceptibility to PZQ in the field in endemic areas and under laboratory conditions [23] which could see emergence and spread of PZQ resistant strains.

Medicinal plants may offer alternative remedies in the management of schistosomiasis and indeed there are reports of several plant species with anti-schistosomal properties [15] and [24]. Amongst these is the Chinese herb *Artemisia annua* which is effective against juvenile but not adult schistosomes [25]. The plants *A. indica* and *E. capensis* have been documented as having been used to treat a myriad of diseases that afflict man and domestic animals including tuberculosis and other bacteria, viral infections, malaria, and helminth infections [26-30], as well as being effective against insect and tick vectors of medical importance [31]. However, there are no documented reports of evaluation of these plants for their potential anti-schistosome properties.

This study aimed at determining the antischistosomal effect of aqueous extracts of *A. indica* and *E. capensis* extracts. We have demonstrated that both of these plants have appreciable antischistosomal activity. *E. capensis* administered at a dose of 400mg/kg showed activity on both adult and juvenile worms of the parasite which was comparable to both positive control drugs (PZQ and artemether). The same was also observed in tissue egg load reduction. On the other hand, *A. indica* had low activity on adult worms which was not comparable to the activity of PZQ. It however showed a high activity on juvenile worms and this was comparable to artemether. Both aqueous extracts showed appreciable antischistosomal activity but *E. capensis* was more potent with high activity on all the parasite stages. To the best of our knowledge, there is no previous reported use of *E. capensis* and *A. indica* as antischistosomal agents.

A reduction in egg load could be due to induction of separation of males and females which in turn reduces or even arrests the release of eggs, which is a relevant factor in the hepatic pathology and the transmission of the disease. A reduction in worm load especially in female worms results

in a reduction in egg load in the tissues with subsequent reduction in pathology. Plant extracts with such activity have the potential of being used as transmission control tools and for intervention to ameliorate adverse effects due to disease pathology. The antischistosomal effects of these extracts on juvenile worms indicate that plant extracts may be used as complimentary drugs or in combination with conventional drugs such as PZQ for effective management of the disease.

The results from this study are supported by other studies which have reported on plants that possess antischistosomal activity. Oliveira et al. [32] reported that *Baccharis trimera* Less DC "Carqueja-amarga" exhibited schistosomicidal effect *in vivo* against immature, adult worms of *S. mansoni* and also significant reduction in egg load due to reduction in worm load. The crushed seeds of the plant *Nigella sativa* have also been found to have antischistosomal activity against different stages (cercariae and juvenile worms) of *S. mansoni* *in vitro* [33]. These results are also in harmony with schistosomicidal activity of crude aqueous extract of ginger against *S. mansoni* reported by Mostafa et al. [34] who observed that parasite load and egg density in the liver and faeces of mice treated with ginger were smaller than their counterparts. Male worms recovered from mice treated with ginger lost their normal surface architecture, extended erosion beyond the tegument, besides numerous bubbles around tubers. Mahmoud et al. [33] reported that treatment of mice infected with *S. mansoni* parasite using black seed oil, was effective in reducing egg count in both liver and intestine.

5. CONCLUSION

In conclusion, *E. capensis* and *A. indica* aqueous extracts demonstrated reduction of *S. mansoni* infection at different developmental stages. *E. capensis* showed greater ability in worm burden reduction and also tissue egg load reduction. Further studies should be done to determine if the extracts from the plants can be used singly or in combination with PZQ in the management of schistosomiasis since this could become a strategy for transmission control which can reduce the morbidity of schistosomiasis in endemic regions. Isolation and characterization of the active compound(s) of these plants and determination of their mechanism(s) of action is also recommended in search for novel antischistosomal agents.

CONSENT

Not applicable.

ETHICAL APPROVAL

The mice used in this study were maintained according to international accepted procedures for animal care and management as recommended by the Kenya Medical Research Institute Animal Care and Use Committee (ACUC). All the mice in this experiment were maintained in clean well labelled cages and their beddings changed twice a week. These mice were maintained on water and mice pellets *ad libitum*. These mice were handled humanely at all times by anaesthetizing them during infection and perfusion using pentobarbitone sodium. After perfusion, the mice were disposed in the incinerator in biohazard plastic bags at the end of the study.

All authors hereby declare that "principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee. The approval for conducting this study was obtained from the Scientific Steering Committee (SSC) and the Ethical Review Committee (ERC) protocol number SSC 2526 from Kenya Medical Research Institute.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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