University of Nairobi

ANTISCHISTOSOMAL ACTIVITY, IMMUNOMODULATION, SAFETY AND PHYTOCHEMICAL COMPOSITION OF *BRIDELIA MICRANTHA*, *OCIMUM AMERICANUM* AND *CHENOPODIUM AMBROSOIDES* EXTRACTS

BY

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I819/90061/2013

PhD. APPLIED PARASITOLOGY

A thesis submitted in fulfillment of the requirements for the award of the Degree of Doctor of Philosophy in Applied Parasitology of the University of Nairobi

2018

Declaration

I declare that this thesis is my original work and has not been submitted elsewhere for examination or award of a degree. Where other people's work has been used, this has been properly acknowledged and referenced in according with the University of Nairobi's requirements.

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Dedication

To my late father Daniel Osebe, the best friend I ever had. For his commitment to my success and constant encouragement throughout my journey, reminding me in my low moments that I had never failed before. I still remember at the very end when he asked me for an update of this work and he was immensely happy, a celebratory kind of happiness, the kind that is reserved for a graduation ceremony, like one who was going to miss it.

To my mother Eunice Osebe, your kindness is immeasurable

To my dear wife Brenda Nyaundi Mwancha, for tolerating me on the long periods that I had to devote to this work. Your are my forever partner in crime!

To my siblings Cliff Kiage, Charles Ooko, Edna Kerubo, Samuel Mogaka and Joel Nyamweya. Aren't you just an awesome bunch!

Acknowledgement

It would not be possible to write this thesis without the help and support of the kind people around me. Above all, I would like to thank my supervisors Prof. Dorcas Yole, Dr. David Odongo and Prof. Horace Ochanda for many insightful conversations during development of ideas for this write up.

I thank Stanley Mathenge, Nicodemus Muia, Thimbo, Collins Kisara, Sammy Kisara, Dorcas Wachira and Joseph Nderitu for technical help during this work. I thank Mr. Tom Oduor of KEBS for the guidance with head space GCMS analysis and Dr. Geoffrey Otieno for guidance during the phytochemical analysis phase of this study. I also thank Prof. James Mbaria for guidance and many insights during the toxicology phase of this study

For hosting of this study, I thank University of Nairobi, National Museum of Kenya, Institute of Primate Research, Kemri, KEBS and Technical University of Kenya

Finally I thank Deutsche Akademischer Austausch Dienst (DAAD) and National Commission for Science Technology and Innovation for funding, without which this study would not have been possible.

ABSTRACT

Schistosomiasis is among the most common, debilitating disease of humans caused by several species of digenetic blood trematodes of the genus *Schistosoma.* It is estimated that 200 million people are infected with approximately 600 million at risk. The disease manifests itself as acute schistosomiasis which occurs relatively early in the infection and the more severe chronic schistosomiasis after a number of years following infection. Praziquantel which is effective against adult worm stages is the only commercially available antischistosomal drug after oxamniquine and metrifonate production was discontinued. Extracts of three plants shown in preliminary studies to have antischistosomal activity were initially validated in mice model followed by gross pathology, histopathology and immune reaction studies. Acute and sub-chronic toxicity of the extracts was determined and finally headspace GCMS analysis was performed to determine chemical compounds. Worm reduction efficacy percentages were 35% for *C. ambrosoides* methanol extract, 33% for *C. ambrosoides* water extract, 36% for *B. micrantha* water extract, 17% for *O. americanum* water extract, 31% for *O. americanum* hexane extract against the adult worm while for the juvenile worm it was 37%, 33%, 11%, 44%, and 39% respectively. Praziquantel which served as a positive control had efficacy of 81% and 77% against the adult and juvenile worm respectively. The extracts were also observed to have immunomodulation properties suggesting they play a role in disease pathology which is the more important aspect of schistosomiasis. Toxicity studies showed that *B. micrantha* water extract was toxic and was therefore excluded from subsequent studies, progressing with the relatively non-toxic *O. americanum* and *C. ambrosoides* extracts. Phytochemical compounds that were identified for *O. americanum* and *C. ambrosoides* water extracts were 49 and 43 respectively

confirming they were rich in antioxidants with potential antihelminth activity. Future work should include LCMS for identification of heavy molecules, druggability studies, determination of relevant antischistosomal molecules, determination of effective dose and finaly undertake downstream steps in drug development.

Key words: Schistosomiasis, Praziquantel, ELISA, Flow cytometry, Toxicology, Mass spectrometry

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Abbreviations

App	Approximately
^{o}C	degrees centigrade
ddH_2O	double distille
g	Grams
h	Hour
kg	Kilograms
kPa	Kilopascals
MPa	Megapascals
µg	Micrograms
µl	Microlite
µm	Micrometers
mg	Milligrams
ml	Milliliters
mm	Millimeters
Min	Minutes
Nm	Nanometers
pg	Pictograms
Rpm	revolutions

CHAPTER 1

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Human Schistosomiasis and its global distribution

Schistosomiasis is among the most common, debilitating disease of humans caused by several species of digenetic blood trematodes of the genus *Schistosoma* (Secor and Colley, 2005). It is estimated that 200 million people are infected of which 120 million are asymptomatic and 20 million exhibit severe disease. Approximately 600 million people are at risk of infection globally (Chitsulo *et al.,* 2000). It is estimated that annual mortality due to schistosomiasis in Africa alone is 280,000 and the global burden is estimated at 4.5 million DALYs (WHO, 2002; van der Werf *et al.,* 2003). Since it is typically a chronic disease, it is poorly recognized in early stages and becomes a threat to development as it disables people in their most productive ages (Engels *et al.,* 2002).

1.1.1 The life cycle of *Schistosoma* and their intermediate host

The species that infect humans are *Schistosoma haematobium*, *S. mansoni*, *S. japonicum*, *S. intercalatum* and *S. mekongi*. These species, their intermediate host and geographical distribution are shown in Table 1 (Fenwick and Utzinger, 2008).

Table 1. The intermediate host and distribution of human schistosomes (Fenwick and Utzinger, 2008)

Parasite intermediate host	
S. haematobium	*Bulinas* spp
S. mansoni	*Biomphalaria* spp
S. japonicum	*Oncomelania* spp
S. intercalatum	*Bulinus* spp
S. mekongi	*Neotricula aperta*
Parasite distribution	
S. haematobium	sub-Saharan Africa, Middle East, some islands in the Indian Ocean
S. mansoni	sub-Saharan Africa, parts of South America, some Caribbean islands
S. japonicum	China, Indonesia, the Philippines
S. intercalatum	Parts of Central and West Africa
S. mekongi	Cambodia, Laos

Schistosomes have a typical trematode vertebrate/invertebrate life cycle with human beings as the definitive host. Eggs are eliminated through urine or faeces depending on species and hatch in water to release miracidium. This larval stage penetrates a specific snail in which it undergoes two generations of sporocysts to produce cercariae. Upon release from the snail, cercariae penetrate human skin of people who are about their usual activities such as washing, swimming or bathing. This stage is characterized by the head penetrating the host, discarding the forked tail to form schistosomule. Schistosomule migrates through several tissues and stages to rest in veins. Adult worms rest in mesenteric veins in various places which apparently varying with species (Secor and Colley, 2005). For example, *S. japonicum* is commonly found in the superior mesenteric veins of the small intestines, *S. mansoni* in the superior mesenteric veins of the large intestines and *S. haematobium* in the venous plexus of the bladder (Secor and Colley, 2005). An illustration of the life cycle is shown in Figure 1.

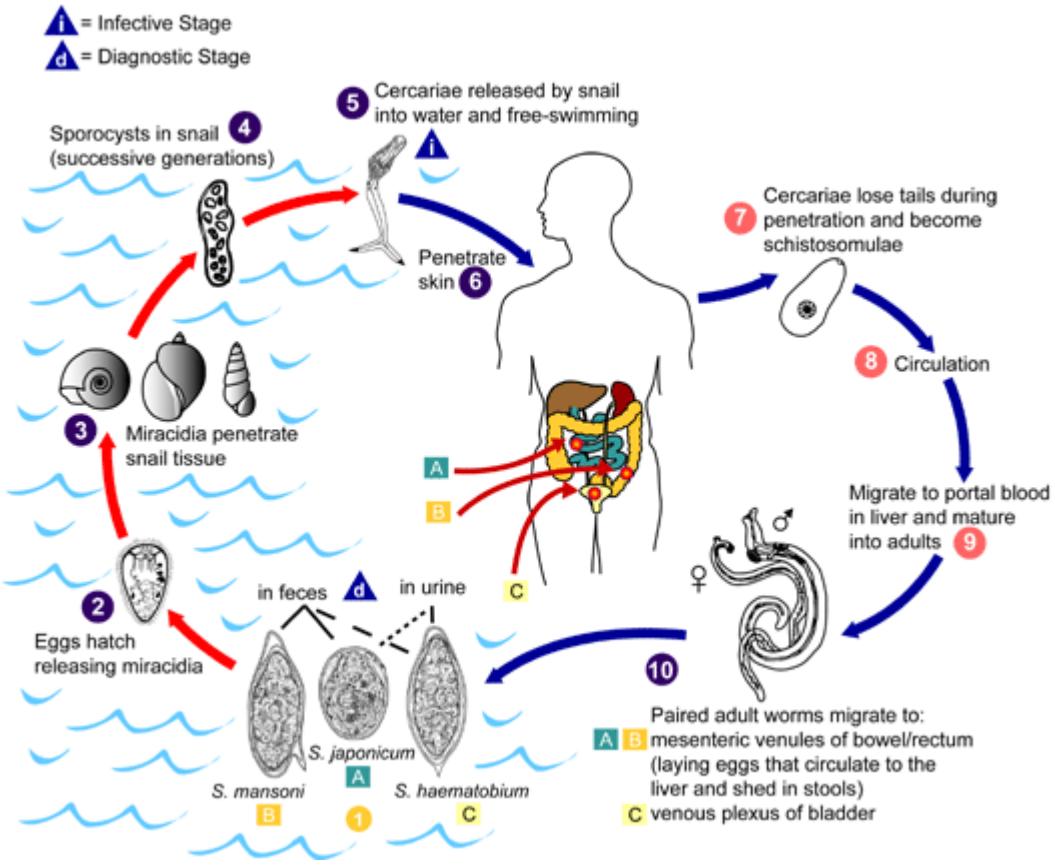


Figure 1: Life cycle of Schistosoma species (https://www.cdc.gov/parasites/schistosomiasis/biology.html), downloaded 27th April 2017.

1.1.2 The global distribution of schistosomiasis

Schistosomiasis is endemic in 76 countries although Japan and Montserrat have been removed from this list after successful eradication (Figure 2). *S. mansoni* occurs in 54 countries including sub-Saharan Africa, Egypt, Sudan, Libya, Arabian peninsula some Carribean islands, Suriname and Venezuela (WHO, 1993; Southgate, 1997; Urbani *et al.*, 1997). *S. intercalatum* has been reported in 10 countries in Africa (WHO, 1993) while *S. japonicum* is endemic in China, Indonesia, Philipines and Thailand. *S. mekongi*, also oriental, is found in Cambodia and Laos and

S. malayensis affects arborigins in a small jungle focus in Malaysia (Sornmani *et al.*, 1971; Sturrock, 1993, WHO, 1993). *S. haematobium* is endemic in 53 countries in the Middle East and most of Africa (Chitsulo *et al.*, 2000).

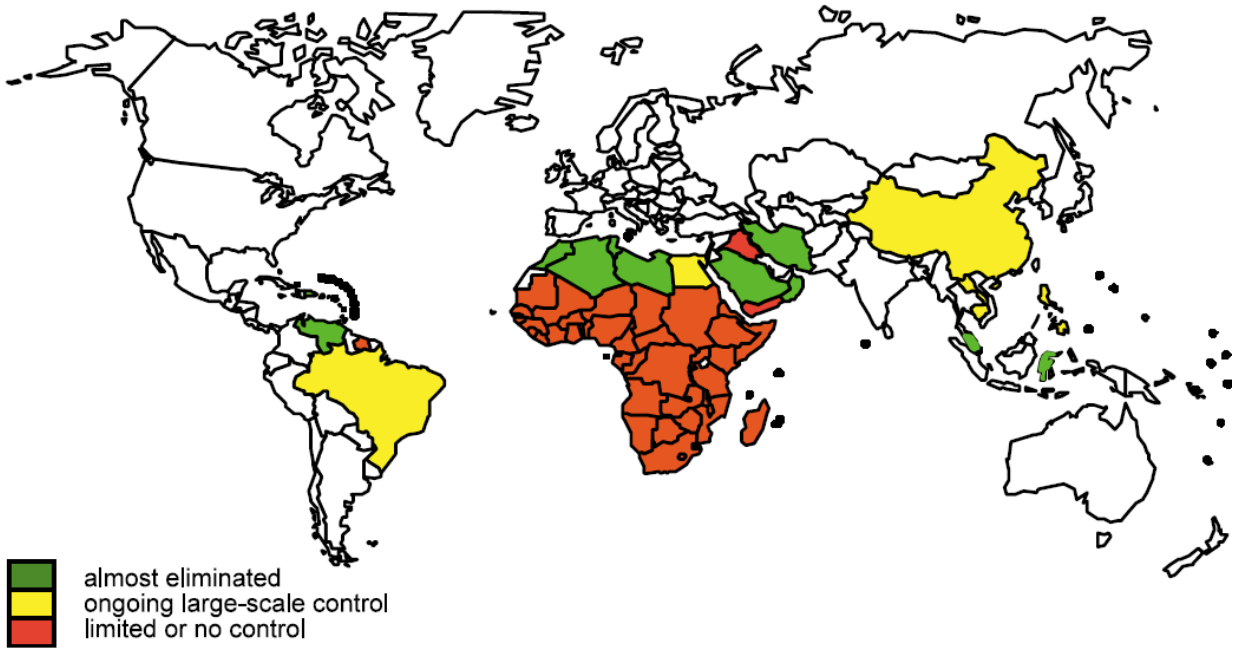


Figure 2: Current status of schistosomiasis control in the world (Engels *et al.*, 2002)

1.2 Pathogenesis of schistosomiasis

Infected individuals exposed to cercariae experience intense itching within 1 h of water contact. A skin rash consisting of rounded erythematous papules form and when healed may leave pigmented spots (Boros, 1989). Papules may get infected with bacteria causing pustules to appear (Barlow 1936; Amer, 1982). On primary exposure however, cercarial dermatitis (swimmers itch) causes a mild itch and appearance of tiny macules at the site of penetration occur (Boros, 1989).

1.2.1 Acute schistosomiasis

This occurs 4 to 10 weeks post infection coinciding with the migration stage of maturing schistosomules in the lungs and liver, maturation of male and female worms or early oviposition in the mesenteric veins (Boros, 1989). Symptoms are manifested by fever, malaise, hepatosplenomegaly, eosinophilia, diarrhoea and in some cases oedema, urticaria, lymphadenopathy and arthralgia (Diaz-Rivera, 1956; Warren, 1973; Nash *et al.*, 1982). The symptoms are however transient and spontaneously disappear with passage of infection to chronic stage (Boros, 1989).

1.2.2 Chronic schistosomiasis

This manifests a number of years after infection and may be mild and asymptomatic, hepatointestinal or the more severe hepatosplenic form (Boros, 1989). Hepatointestinal predominantly affects persons living in endemic areas and about 5-10% exhibit the hepatosplenic form, the genesis of portal hypertension leading to high morbidity and mortality (Strauss, 2002).

Mature female worms such as in the case of *S. mansoni*, lay hundreds of eggs in a day, many of which exit the body through the gut or urinary system for *S. haematobium*. A substantial number however gets trapped in host tissues such as intestinal walls, liver, lung, eyes, nervous, genital organs or systemic organs inducing a local immune reaction (Crump *et al.*, 2000; Hesse *et al.*, 2004). The formation of granulomas in intestinal, renal, pulmonary, liver and nervous tissues is a function of cellular immunity mediated by $CD4^+$ T cells (Andrade and Andrade 1970; Cheever 1992). T-helper 2 (Th2) associated cytokines such as IL-4, IL-5 and IL-13 all play important roles in the pathogenesis of schistosomiasis with IL-4 and IL-13 involved in granuloma

formation (Chiaramonte *et al.,* 1999a). IL-13 is primarily the stimulus for tissue fibrosis (Chiaramonte *et al.,* 1999b; Fallon *et al.,* 2000).

1.3 Control situation of schistosomiasis

The first step in schistosomiasis control is to deal with morbidity in the strict sense (Engels *et al.,* 2002). This would therefore require accurate detection, treatment and environmental manipulation to limit human/parasite interaction.

1.3.1 Reliable diagnosis

Identification of eggs in stool or urine samples by microscopy is the most practical method. A commonly used method of urinary schistosomiasis detection is filtration of urine collected between 10am and 2pm for eggs (Plouvier *et al.,* 1975; Mott *et al.,* 1982). The Kato-Katz technique originally developed in the 1950s (Kato and Miura, 1954), and later modified (Katz *et al*., 1972), is a thick smear technique used in combination with microscopy for diagnosis of a stool sample (Utzinger *et al.,* 2010). Other stool diagnostic techniques are formal-ether concentration method (Ridley and Hagwood, 1956; Allen and Ridley, 1970) and the relatively inferior zinc sulphate floatation method (Ritchie *et al.,* 1953).

Stool or urine techniques may however fail to detect infection, especially when the parasite load is low. For such cases, highly sensitive immunological techniques which involve the detection of schistosome antigens such as circulating anodic antigens (CAA) and circulating cathodic antigens (CCA) (van Lieshout *et al.,* 2000) or *S. mansoni* soluble egg antigen (SEA) (Chand *et al.,* 2010) in blood or urine using enzyme-linked immunosorbent assay (ELISA) are useful. Since ELISA is time consuming, requiring skilled technicians, rapid diagnostic assays have been

developed (Utzinger *et al.,* 2010). Molecular techniques such as polymerase chain reaction (PCR) have also been found to be much more sensitive than the microscope dependent techniques (Pontes *et al.,* 2002)

1.3.2 Intermediate host control

The control of the snail intermediate host is an important aspect of the integrated control of schistosomiasis. Despite research initiatives aimed at discovery of more molluscicidal chemical compounds, niclosamide (Bayluside®) remains virtually the only chemical in use (McCullough *et al.,* 1980, McCullough, 1992). Yurimine which was in use in Japan was stopped due to toxicity to non-target species, and Frescon was stopped after not living up to its expectation (McCullough, 1992; Souza, 1995).

Potential biological control by employing micropathogens such as virus, fungi, protozoa and bacteria or natural predators, may offer an alternative to using chemical molluscicides (WHO, 1984; Hofkin *et al.,* 1991a,b; Loker *et al.,* 1993; Mkoji *et al.,* 1995; Madsen, 1995).

1.3.3 Immunological control

Since the worms do not multiply in a host, vaccine acquired immunity could go a long way in decreasing human pathology and disease transmission. Immune response in schistosomiasis is T-cell dependent. T-helper 1 (Th1) responses directed against the worm is predominant in the early stages and is succeeded by the egg induced Th2 responses. T-cell mediated immunity is therefore fundamental to acquired resistance to schistosomes. Some anti-*S. mansoni/S. haematobium* candidate vaccine antigens include Sm-p80, Sm14, Sm-TSP2, Sh28GST, Sm29, Sm100.3, SmCB1, Sm-CB, Sm32, Sm-LAMP, DLC 12, DLC 13, SmSynt, radiation-attenuated cercariae,

copper/zinc superoxide dismutase, signal peptide containing superoxide dismutase (SOD) and glutathione peroxidise enzymes (GPX) (Tebeje *et al*., 2016). Anti- *S. japonicum* candidate vaccine antigens include Paramyosin (Sj97), Sj26GST, SjTPI, SjTHRβ, SjFABP, Sj23, SjPDI, Ad-SjIAP, SjAR, Sj-F1 and other cocktail preparations such as SjGP-3 which is a combination of Sj26GST and a fragment of paramomyosin (Tebeje *et al*., 2016). Two schistosome antigens are currently under clinical trials, the fatty acid-binding protein of 14 kDa from *S. mansoni*, Sm14, and the glutathione-*S*-transferase of 28 kDa from *S. haematobium*, Sh28GST. (Ricciardi and Ndao, 2015). The *S. mansoni* tetraspanin 2 protein, TSP-2, has now been produced under good manufacture practices (GMP) to soon be evaluated in Phase I clinical trial (Curti *et al*., 2013).

1.3.4 Parasite chemotherapy

Successful treatment of patients infected with *S. haematobium* was first reported from Sudan in 1918 after 4 injections with an antimony compound resulting in cure. Subsequently, new antischistosomal drugs developed were niridazole, hycanthone, metrifonate and oxamniquine. Treatment with niridazole and hycanthone were stopped because of adverse effects while the 3 dose treatment regime with metrifonate was logistically challenging. Oxamniquine has been used in Brasil against *S. mansoni* but is less efficacious for the African isolates. Since it is limited to *S. mansoni*, drug resistance and the emergence of the cheap praziquantel has cast oxamniquine future manufacture in doubt making it redundant (Fenwick and Utzinger, 2008).

Praziquantel is presently the only commercially available antischistosomal drug with a broad spectrum activity and a good safety profile (Fenwick *et al.*, 2003). It is produced and administered as a chiral mixture of 1:1 ratio with only one chiral molecule being active against

the parasite (El-Subbagh and Al-Badr, 1998). Since expiry of the patent, the drug originally produced by Bayer, has become relatively cheap leading to a price reduction from $1 to below $ 0.10 for a 600mg tablet (Appleton and Mbaye, 2001; Fenwick and Utzinger, 2008) making it easily available in peripheral areas.

Resistance to praziquantel has been reported in Egypt where some individuals could not be cured for schistosomiasis leading to identification of resistant isolates (Ismail *et al.,* 1999). Reports from Senegal in 1997 suggested praziquantel was observed to be ineffective but this could be attributed to the fact that it is refractory against immature stages resulting in high infection (Danso-Appiah, 2002; Utzinger and Keiser, 2004). Moreover, exposing the parasite to drug pressure during control initiatives is thought to be unlikely since 100% coverage is never achieved, since only high risk groups being targeted (Fenwick *et al.,* 2003).

It is not satisfactory to have only one effective treatment that is commercially available since the ideal strategy of avoiding resistance is to alternate treatments. Artemether, a derivative of malaria drug artemisinine was shown to be active against schisosomes, exhibiting higher activity for juvenile stages (Xiao *et al.,* 2002). A praziquantel/artemether combination treatment was found to cover the parasite lifespan thus opening an avenue for a more effective treatment option (Xiao *et al.,* 2002). In Egypt, it was reported that the oleo-gum resin from the stem of *Commiphora molmol* (myrrh) exhibits antischistosomal properties (Sheir *et al.,* 2001). An Egyptian pharmaceutical company markets it as a gelatin capsule (Mirazid) containing 300mg of purified extract. Further tests carried out in Egypt however reported very low cure rates suggesting original published claims to be difficult to replicate (Fenwick *et al.,* 2003).

1.3.5 Natural products as a source of new drugs

The use of natural products with therapeutic properties is as ancient as human civilisation with use of mineral, plant and animal products acting as main sources (De Pasquale, 1984). However, the pharmaceutical industry's productivity continues to be dismal, arguably due to diminished interest in natural products in the face of new technologies particularly combination chemistry (Newman and Cragg, 2007). The interest in the use of natural products as sources of new chemical entities (NCE) as drug leads is now increasing with combination chemistry failing to live up to expectations, and the relative higher success rate of natural products (Paterson and Anderson, 2005, Chin *et al.*, 2006). Moreover, advances in separation technologies followed by speed and sensitivity of structure elucidation of natural products such as reported by Bilia *et al* (2002) has led to overcoming difficulties associated with natural products drug discovery.

Inspection of NCEs for the period 1981-2006 showed that natural products contributed approximately 50% despite the low attention from pharmaceutical companies (Newman and Cragg, 2007). Scrutinizing source of compounds of medicinal indications demonstrated that drugs used to treat 87% of the categorized human diseases are of natural products origin (Newman *et al.*, 2003). A total of 23 new drugs from natural sources were launched between 2000 and 2005 alone with more NCEs still undergoing trials (Chin *et al.*, 2006). This indicates the rich potential of natural products, contributing a significant number of new drugs and NCEs at a time when most pharmaceuticals have discontinued their discovery programs from natural products (Chin *et al.*, 2006). Plants are important sources contributing 25% of drugs prescribed worldwide and 11% of drugs considered by World Health Organisation (WHO) as essential (Rates, 2001).

Plants have been traditionally used in Africa and Asia for the treatment of many diseases including schistosomiasis (Moraes, 2012). Studies have shown that *Abrus precatorius, Pterocarpus angolensis* and *Ozoroa insignis* which are all plants used as traditional remedies against schistosomiasis in Zimbabwe, were efficacious against the adult worm (Ndamba *et al*., 1994). Other studies have shown that 5 of the 10 selected extracts of plants used in traditional medicine in Zimbabwe exhibited antischistosomal activity (Molgaard *et al.,* 2001). *Vernonia amygdalina* extract which is rich in sesquiterpene lactones has been shown to have antischistosomal and antiplasmodial activity (Ohigashi *et al*., 1994). *Eurycoma longifolia* which is used for traditional medicine in Asia has been shown to have longilactone which has antischistosomal activity (Jiwajinda *et al*., 2002). Other plant derivatives that have been found to be active against *Schistosoma* sp include pumpkin seeds, artemether, artesunate, Chinese herb *Hemerocallis thumbregii* Baker, *Milletia thonningii* among others (Lyddiard, Whitfield and Bartlett, 2002; Xiao *et al.,* 2010). A more exhaustive list of plants found to have *in vitro* antischistosomal activity has been published by Moraes (2012).

Other plants whose extracts have been shown to be antiparasitic including against helminths include *Moringa oleifera*, *Flemingia vestita*, *Khaya senegalensis*, *Piliostigma reticulatum*, *Securidaca longepedunculata* and *Terminalia avicennoides* among others (Caceres *et al*., 1991; Tandon *et al*., 1997; Rahman *et al*., 1999; Atawodi *et al*., 2003; Temjenmongla *et al*., 2005; Muregi *et al*., 2007; Steinmetz *et al*., 2009).

1.4 Phytochemical screening

An important step in evaluation of plant extracts for drugs is in the isolation of pharmacologically active constituents. Thin layer chromatography (TLC) is the cheapest and

easiest method of detecting plant constituents since the method is easy to run and is reproducible (Marston *et al.,* 1997). However, for efficient separation, good selectivity, good sensitivity together with the capability of providing structural information, hyphenated, high performance liquid chromatographic (HPLC), high performance thin layer chromatography (HPTLC) or gas chromatography coupled with mass spectroscopy are preferred (Hostettmann *et al.,* 1997; Reich and Schibli, 2007; Adams, 2007).

1.5 Justification and significance

Praziquantel is the only commercially available antischistosomal drug after metrifonate and oxamniquine were discontinued. With the prices now standing at below $0.10, this drug has become easily accessible leading to wide distribution and is usually applied in mass treatment programs. This has brought to fore the risk of resistance development due to drug pressure which leads to mutation without an alternative. A good way of avoiding resistance would be the classical approach of alternating treatments with other antischistosomal options thereby making the need to discover new drugs important. Other than being ineffective against juvenile worms, praziquantel has been reported to induce hemorrhage in the lung tissue of the host as well as abdominal pain and diarrhoea which further makes the case for new studies to develop alternative therapies (Sabah *et al.,* 1986; Flisser and McLaren. 1989; Kabatereine *et al*., 2003) In this study, extracts from 3 plants shown in preliminary studies to have antischistosomal activity (Moilo *et al*., 2014; Waiganjo *et al*., 2014), were tested in mice models against juvenile and adult schistosomes, followed by toxicological analysis, then finally phytochemical analysis of the plant extracts.

1.6 Hypothesis

B. micrantha, O. americanum and *C. ambrosoides* extracts are effective against *S. mansoni* infection, they are non-toxic and can be analysed for the identification of constituent phytochemicals.

1.7 Objective

To determine antischistosomal activity, immunomodulation, safety and phytochemical composition of *B. Micrantha, O. americanum* and *C. ambrosoides.*

1.7.1 Specific objectives

1. To assess the anti-schistosomal activity of the plant extracts against juvenile and adult worms relative to standard treatment based on worm reduction
2. To analyze and compare the pathological manifestation and immune responses following infection and treatment relative to conventional treatment
3. To determine the safety of the *B. micrantha, O. americanum* and *C. ambrosoides* extracts
4. To perform phytochemical analysis of the plant extracts for the identification of phytochemicals

Each of the above objectives were tackled in subsequent sections of this write up, each comprising a chapter. In chapter 2, the plant extracts were tested in mice models for the determination of the extracts' antischistosomal activity against both the juvenile and adult worm. In chapter 3, the pathological manifestations of the disease and immune responses following infection was analyzed, followed by chapter 4 where the safety and toxicity of the extracts was

determined. Phytochemical analysis of the plant extracts was handled in chapter 5 and finally a general discussion follows in chapter 6.

Ethical approval for use of the animals was obtained from Institute of Primate Research's Institutional Scientific and Ethics Review Committee (ISERC), reference number IRC/02/12.

CHAPTER 2

2 Anti-schistosomal activity of *B. micrantha, O. americanum* and *C. ambrosoides* plant extracts against juvenile and adult worms of *S. mansoni*

2.1 Introduction

A significant number of people living in Sub-Sahara African countries die regularly from preventable and curable diseases due to inadequate primary healthcare system (Sofowora, 1982). The primary healthcare system can be complemented with traditional medicine which is standardized in India and China unlike in Africa, despite of disease pressure and abundance of medicinal plants in the continent (Sofowora, 1982). Policy makers may even find it useful to add such plants either as crude preparations or as pure active compounds to the national list of drugs to complement or replace the drugs that need importing (Farnsworth *et al*., 1985). This study builds on the work initially reported by Moilo *et al*., 2014 and Waiganjo *et al*., 2014. They studied the antischistosomal efficacy of various extracts of the wormseed plant (*Chenopodium ambrosoides*), *Bridelia micrantha*, *Croton megalocarpus*, *Ocimum americanum*, *Aloe secundiflora* and *Sonchus luxurians* determining the effective extracts as well as the optimal treatment dose.

B. micrantha is a plant used in traditional medicine as a remedy for stomach ache and diarrhea. Studies have confirmed that it has antihelminthic and antidiarrhoeal properties (Lin *et al*., 2002; Waterman *et al*., 2010). *C. ambrosoides*, is a herbal wormicidal plant which is also commonly used for treatment of intestinal worms (Kliks, 1985). Its essential oil has been found to have nematocidal properties arguably due to its high level of ascaridole (MacDonald *et al*., 2004). *Ocimum* species are also medically important plants with known antihelminthic and

antidiarhoeal activity (Fakae *et al*., 2000; Ezekwesili, Obiora and Ugwu, 2004; Taur *et al*., 2009; Buchineni, Pathapati and Kandati, 2015).

This study tested the antischistosomal activity of the three plants extracts against both adult and juvenile *S. mansoni* in mice models. The findings of this study may inform subsequent steps in a potential drug discovery project (Hughes *et al*., 2011).

2.2 Materials and methods

2.2.1 Data mining, plant collection and taxonomic identification

Data generated in pilot studies undertaken at Institute of Primate Research (IPR) was examined for selection of 3 promising antischistosomal plant candidates for onward study. Care was taken to further determine the relevant plant parts to inform field plant collection exercise. *B. micrantha* bark was collected from Nyeri, *C. ambrosoides* leaves and fruits from Umoja area of Nairobi and *O. americanum* whole plant from Machakos area of Kenya. Subsequent taxonomic identification was undertaken at University of Nairobi's School of Biological Sciences (SBS) and National Museums of Kenya Herbariums. All the collected plants were thereafter dried under shade at room temperature for 2 months with periodic turning to prevent molding. Representative pictures of the three plants are shown in Figures 3, 4 and 5 below.

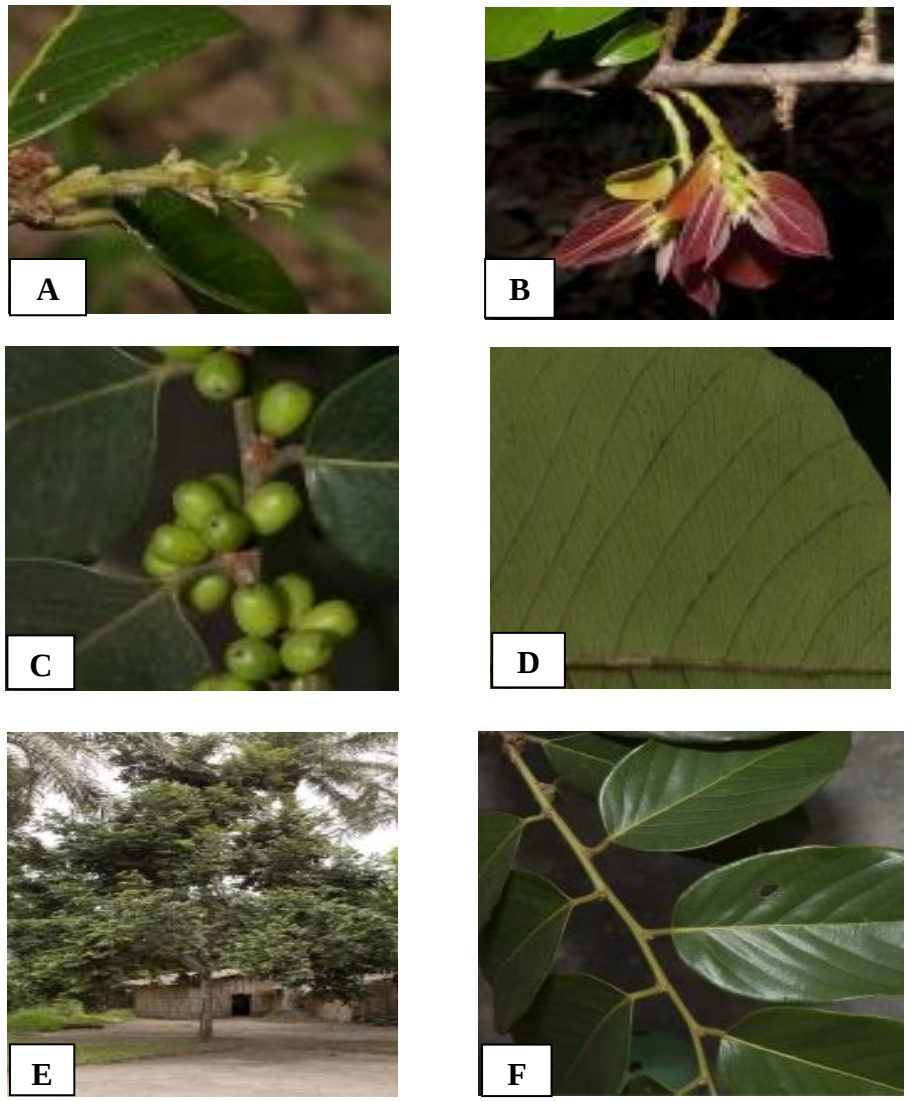


Figure 3. Pictures of *B. micrantha*

(http://congotrees.rbge.org.uk/species/details/bridelia-micrantha downloaded on 15th August 2017)

Key: A. Terminal shoot with stipules, B. Flushing young leaves, C. Immature fruit, D. Midrib and venation, lower surface, E. Tree, F. Leafy branch respectively

Figure 4. Picture of *C. ambrosoides*

(http://www.ville-ge.ch/musinfo/bd/cjb/africa/details.php?langue=an&id=26531 downloaded on 15th August 2017)

Figure 5. Picture of *O. americanum*

(http://www.mpbd.info/plants/ocimum-americanum.php downloaded on 15th August 2017)

2.2.2 Recovery of crude extracts from collected plants

The dried plants were ground to fine powder using a grinding machine from Sihra Engineering Works (Nairobi, Kenya) and passed through 0.5mm mesh to standardize particle size. Approximately 2kg of the powder was kept in 6 litre jars for each plant where water extract was required, topped up with double distilled water (ddH_2O) and soaked for 72 h. Another 2kg of *Ocimum americanum* powder was soaked in analytical grade hexane from Unilabs (Nairobi, Kenya) and *Chenopodium ambrosoides* fruit powder was soaked in analytical grade methanol from Pancreac Quimica (Barcelona, Spain) also for 72 h.

Filtering was initially done through cotton wool to remove coarse debris and subsequently through Whatman® qualitative filter paper, grade 1 from Sigma-Aldrich (Taufkirchen, Germany). The filtrate was stored at 4^{o}C in labelled jars waiting further processing.

Freeze drying was done using a freeze dryer from Chemlabs Instruments (United Kingdom) following standard protocol. Briefly, each water extract was frozen to -10^{o}C for 12 h and ice block inserted in vacuum chamber of the freeze dryer adjusted to -100^{o}C and 1 torr. Freeze drying was carried out until only solid extract was retained in vacuum chamber.

To recover extracts in hexane and methanol solutions, a rotor evaporator from Joh, Achelis and Johne (Bremen, Germany) was used at 70^{o}C. The pasty extracts were transferred into small jars and further dried in fume chambers at room temperature for approximately 2 weeks.

2.2.3 Snail collection, screening and maintenance

Biomphalaria pfeifferi snails were collected from Mwea district by scooping using pole attached sieves, individually picked from sieves using forceps and then transfered into plastic basins. Sand and gravel was also collected from the same ecosystem for later use in snail tanks. The snails were then transferred in plastic containers lined with moist cotton wool to the IPR Malacology lab. To obtain naive snails, all collected snails were screened by being exposed to 100 watts light bulb for duration of 2 h once a week for 5 weeks where cercariae shedding snails were separated. Naive snails were retained and stored in snail tanks in a temperature controlled room at 25-27^{o}C. The snail tanks were prepared by adding chlorine free water and layering with sand and gravel referred to above, which had been sterilized by heating at 150^{o}C for 12 h. The snails were thus maintained with weekly change of water, *Daphnia* aeration and fed on lightly steamed and oven dried lettuce.

2.2.4 Hatching of *S. mansoni* miracidia for snail infection

S. mansoni eggs were recovered from chronically infected olive baboon (*Papio anubis*) for use in this study. Briefly, faecal matter collected from baboon at IPR was suspended in 1 litre water in a plastic jar by stirring with a wooden spatula. The slurry was sequentially passed through standard test sieve (Arthur Thomas Co. USA) first with 600 µm and then 250 µm pore size into a collection tray. The sieved product was transferred into urine jars, suspended in water and allowed to stand in the dark for 30 min. The supernatant was discarded and pellet re-suspended in water as before. This process was repeated until supernatant was clear (approx. 3 times). The pellet was then dispensed in a petridish with water and then exposed to 100 watts light for 1 h for miracidia to emerge from eggs.

2.2.5 Infection of snails with *S. mansoni* miracidia and recovery of cercariae

A 24 well culture plate was used for this step of the study. Using a Pasteur pipette, 3-5 miracidia were picked under a dissecting microscope and transferred into each well. Snails were then retrieved from the snail tank and left to stand for 30 min at room temperature for penetration. The snails were then transferred to freshly prepared snail tanks and after 4 weeks, covered with black cloth so as not to induce cercariae shedding.

Five weeks after infection, the snails were carefully picked using forceps and a number of them put in a clean 80ml beaker. The snail laden beakers were then exposed to 100 watts light for 3h for cercariae to emerge. The cercariae suspension was pooled in a beaker and the process of determining volume that would contain approx. 250 of them followed. Briefly, several 200µl aliquots of cercariae suspension were put on a clean petridish and stained with iodine. The average number of cercariae in each aliquot was determined as 129 hence adopting 400µl as the volume that would contain approx. 250 cercariae.

2.2.6 Infection of mice with *S. mansoni* cercariae

Seven week old BALB/c mice bred at IPR's rodent house were each shaved on the belly area in readiness for infection then injected subcutaneously with 0.025ml ketamine preparation (Agar, Holland) as a general anaesthesia. The mice were then affixed on a rack, the shaven area wetted by moist cotton wool and a ring placed on the area. To each ring, 400µl of cercariae suspension was added and allowed to stand for 30 min as per Smithers and Terry (1965), verified by absence of free swimming cercariae. The infected mice were then kept in cages, fed on food pellets (Unga Group LTD, Kenya) and given water *ad libitum*. They were maintained in the IPR rodent house awaiting treatment.

2.2.7 Treatment of infected mice with crude plant extracts

The treatment groups are as shown below in Table 2 using dosage as recommended by Moilo *et al* (2014) and Waiganjo *et al* (2014). One other group was included as infected control (IC) and some naive mice were also retained for serum preparation. A group treated with praziquantel (Bayer, Germany) was included as a positive control.

Table 2. Experimental groups used in this study

Plant and drug treatment	Extract	Dosage
B. micrantha	Water	150mg/kg
C. ambrosoides	Methanol	150mg/kg
C. ambrosoides	Water	150mg/kg
O. americanum	Water	150mg/kg
O. americanum	Hexane	150mg/kg
Praziquantel		900mg/kg

Using a dosing syringe, mice were treated by administering the treatment dose directly on the oesophagus. Treatment was on week 3 post infection and a second dose administered 2 days later to target juvenile *S. mansoni*. To determine efficacy against adult worms, groups of mice were treated on week 4 in a similar manner. The treatment groups were distinguished from each other by marking with pictric acid on a unique part of the body. The administered high dose of praziquantel at 900 mg/kg has been shown to be highly effective in mice while at the same time being within toxicity limits (Frohberg, 1984; Muchirah *et al*., 2012).

2.2.8 Blood collection, perfusion and tissue collection for histopathology

Six weeks post-infection, the mice were retrieved and anaesthetized by injection with 0.05ml ketamine preparation. An incision was made with a pair of scissors transversely in the abdomen area then skin peeled upwards and downwards. The abdominal wall was then cut carefully, cutting upwards through the diaphragm and ribcage to expose the heart. Care was exercised when cutting both sides of the xiphoid cartilage and rib cage to avoid major blood vessels. Blood was collected by drawing from the right ventricle of the heart using a 1ml syringe and transferred into 1ml microfuge tubes for subsequent production of serum.

Perfusion was done using a modified method initially reported by Smithers and Terry (1965). Briefly, the hepatic portal vein was cut and perfusion fluid (0.85g/100ml sodium chloride and 1.5g/100ml sodium citrate) injected at high pressure into the left ventricle using a perfusion pump (Manostat®, division of Barant Company, England) set at 5-6 rpm until liver was pale in colour. Adult *S. mansoni* were drained into 6 inch petridish and then emptied into 1 litre beaker. The mice were left immersed in perfusion fluid in petridishes for 1 h for the recovery of any remnant worms. The perfusate initially transferred into beakers was topped up to 1 litre with perfusion fluid, allowed to stand for sedimentation and the supernatant discarded. This was repeated thrice and the worms were transferred into labelled petridishes with some perfusion fluid.

The liver and mesenteric lymph nodes were removed and stored in 10% formaldehyde pending a detailed histopathology study. Serum was prepared by spinning clotted blood in a micro-

centrifuge at 3000rpm for 15min. The clear supernatant which is the serum was then transferred into clean microfuge tubes and then stored at -20^{o}C pending analysis.

2.2.9 Adult worm count and analysis

The worms recovered in section 2.2.8 above were manually counted and the numbers analysed as outlined below;

- Worm maturation = Number of worms in infected control (IC) ÷ Initial number of cercariae X 100
- Worm recovery = Mean worms in group ÷ Mean worms in IC X 100
- Worm reduction = (Mean IC – Mean Group) ÷ Mean IC X 100

Test of significance was performed between the groups using ANOVA at 95% significance in MS Excel 2007.

2.3 Results

2.3.1 Efficacy of plant extracts against juvenile and adult *S. mansoni*

The efficacy of the three plant extracts were determined based on worm reduction (Tables 3 and 4). The adult efficacy study results showed highest worm reduction at 81% for praziquantel, *B. micrantha* water extract 36%, *C. ambrosoides* methanol extract 35%, *C. ambrosoides* water extract 33% and *O. americanum* hexane extract 31%. *O. americanum* water extract had the lowest efficacy against adult stage *S. mansoni* at 17%. Comparing the 4 treatment groups excluding praziquantel and *O. americanum* water extract using ANOVA were found not to be statistically different ($P>0.05$).

Juvenile efficacy study results on the other hand showed praziquantel worm reduction was 77%, *O. americanum* water extract 44%, *O. americanum* hexane extract 39%, *C. ambrosoides* methanol extract 37% and *C. ambrosoides* water 33%. *B. micrantha* water extract was found not to be significantly different from IC but all other treatment groups were significantly better. All treatment groups were not as effective as praziquantel.

Comparing efficacy against adult and juvenile worms, *B. micrantha* water extract was significantly different being more effective against adult worms. Although *O. americanum* water extract efficacy was found to be statistically insignificant between the 2 studies, it had the highest variance being much more effective against juvenile worms. All other treatments were as effective against juvenile as adult worms.

Table 3: Results of adult *S. mansoni* treatment with selected plant extracts relative to standard treatment

MEAN NUMBER OF WORMS ± SE					
Treatment	Males	Females	Total	% Worm Recovery	% Worm Reduction
Praziquantel	10.11±1.49	3.00±0.53	13.11±1.39	19%	81%
C. ambrosoides(methanol)	29.29±3.34	16.29±2.01	45.57±4.93	65%	35%
C. ambrosoides(water)	30.5±3.5	16±4	46.5±0.5	67%	33%
B. micrantha(water)	28.25±4.14	16.13±2.75	44.38±6.62	64%	36%
O. americanum(water)	38.14±3.89	19.57±3.16	57.71±6.91	83%	17%
O. americanum(hexane)	30.33±3.91	17.5±3.4	47.83±6.79	69%	31%
Infected Control	46.67±2.96	23±2.51	69.67±4.98		

Table 4: Results of juvenile *S. mansoni* treatment with selected plant extracts relative to standard treatment

	MEAN NUMBER OF WORMS ± SE				
Treatment	Males	Females	Total	% Worm Recovery	% Worm Reduction
Praziquantel	11±3.39	5±1.41	16±4.49	23%	77%
C. ambrosoides(methanol)	28.75±3.09	15.25±2.95	44±5.94	63%	37%
C. ambrosoides(water)	31.88±3.44	15±3.22	46.88±6.12	67%	33%
B. micrantha(water)	41.57±4.89	20.71±2.43	62.29±7	89%	11%
O. americanum(water)	25.83±4.9	13.17±3.63	39±8.49	56%	44%
O. americanum(hexane)	27.4±6.65	14.8±4.72	42.2±10.82	61%	39%
Infected Control	46.67±2.96	23±2.51	69.67±4.98		

2.4 Discussion

Generally, all treatments were more effective than the negative control suggesting they contain phytochemicals that have activity against *S. mansoni*. Treatment with praziquantel which acted as positive control was however observed to be more effective than the plant extracts. The antischistosomal activity of the extracts is potentially due to phytochemicals which occur in wide variety in plants in various quatities. For example, it is suggested that plants produce approx 12000 different alkaloids (Ziegler and Facchini, 2008). Arguably therefore, it is expected that in crude plant extracts such as the ones used in this study, the specific active ingredients effective against *S. mansoni* may not be present in optimal amounts and worm reduction may not be as high as praziquantel where the worm is exposed to a specific active molecule in a determined dose.

It was interesting to observe that *O. americanum* water extract was more effective against juvenile worms while *B. micrantha* water extract more effective against adult worms. However, it will be important to also analyze histopathological and immunological data to determine if they correlate with this observation. By week three post infection, the majority of the worms are already in the portal blood stream (Doenhoff *et al.,* 1978), where they remain in adult stage. Potentially, the reason could be attributed to changes in gene expressions with the stages in the life cycle of the worm (Franco *et al.,* 2000; Jolly *et al.,* 2007).

An important aspect of this study was to determine if the plant extracts were effective against juvenile stages of the worm relative to praziquantel. The findings however do not agree with studies performed by Sabah *et al* (1986), since praziquantel was observed to be as effective on 3

week old juvenile worms as 4 week old adult worms. This may be due to differences in experimental design where the mice were exposed to 900mg/kg dose compared to 250mg/kg in Sabah's study. There may also be some differences between the Kenyan strain of *S. mansoni* when compared to the Puerto Rican one. This therefore suggests that the plant extracts used in this study could be important in offering alternatives or offer cocktail constituents with praziquantel thus minimizing risk of resistance.

CHAPTER 3

3 Pathological manifestations and immune responses following treatment of *S. mansoni* with *B. micrantha, C. ambrosoides* and *O. americanum* extracts

3.1 Introduction

The pathology of schistosomiasis is essentially associated with immune responses against the worm and its eggs. Host immune system evasion involves schistosome excretory/secretory (ES) products in early infection and soluble egg antigen (SEA) in later stage infection which have imunomodulatory activity (Nayak and Kishore, 2013). Acute schistosomiasis which occurs 4-10 weeks post-infection coincides with the migration of maturing schistosomules in the lungs and liver, maturation of male and female worms or early oviposition in the mesenteric veins (Boros, 1989). This stage is characterized by T-helper 1 (Th1) responses toward the worm causing symptoms such as fever, malaise, hepatosplenomegaly eosinophilia, diarrhoea and in some cases oedema, urticaria, lymphadenopathy and arthralgia (Diaz-Rivera, 1956; Warren, 1973; Nash *et al.,* 1982). The egg laying stage of the infection on the other hand is characterized by Th2 responses (McManus and Loukas, 2008).

The high number of eggs laid by the female worm as the disease progresses to the chronic stage exit the body through the gut for *S. mansoni* or the urinary system for *S. haematobium*. However, a significant number of eggs become trapped in tissues such as genital organs, nervous tissues, eyes, lungs, intestinal walls, liver or various systemic organs inducing a localised immune response (Crump *et al.,* 2000; Hesse *et al.,* 2004). This leads to the formation of granulomas in

the said tissues which is a function of $CD4^+$ T cells (Andrade *et al* 1970; Cheever 1992). Th2 associated cytokines IL-4, IL-5 and IL-13 all play significant roles in the pathogenesis of schistosomiasis with IL-4 and IL-13 promoting granuloma formation (Chiaramonte *et al.,* 1999a). IL-13 is primarily the stimulus for tissue fibrosis (Chiaramonte *et al.,* 1999b; Fallon *et al.,* 2000).

The fact that treatment with praziquantel elicits protective immune responses has been well researched (Mutapi *et al.,* 2005; Tweyongyere *et al.,* 2011). In this chapter, the pathology and immune profiles of BALB/c mice treated groups between praziquantel and plant extracts relative to control group was compared.

3.2 Materials and methods

3.2.1 Gross pathology

To perform gross pathology analysis, the mice livers were observed for adhesion between liver lobes, inflammation and presence of granuloma. For granuloma presence, each liver was subjectively categorized depending on number of granuloma per lobe- ranging from None, Few (1-3), Moderate (4-10) to severe (more than 10).

3.2.2 Histopathology

The livers were recovered from the mice and stored in 10% formaldehyde (Hopwood, 1996; Carson, 2007; Carson and Christa, 2009). The tissues were then processed using automatic tissue processor at IPR pathology laboratory where sequential dehydration of the tussues was performed by immersion under increasing concentrations of ethanol of 50%, 80%, 90% and 96%

respectively at 1 h intervals. The dehydrated tissues then underwent clearing using two changes of xylene (clearing agent) each for a duration of 1 min which served to replace the ethanol in the tissues . Wax infiltration was done for 3 h in paraffin wax oven set at 2^{o}C below the melting point. Finally, embedding was done in a mould with molten wax and allowed to solidify. The embedded tissues were sectioned at 0.7 µm thickness using a microtome then floated in warm water to spread out and finally mounted on slides. The slides were placed in hot oven for 15 min and dewaxing performed using xylene then rehydrated and stained with haematoxylin and eosin dyes. The slides were examined under microscope and granuloma sizes were measured to determine mean size (Farah *et al.,* 2000).

3.2.3 Immune reaction of treatment groups

3.2.3.1 Reagents and Kits

3.2.3.2 ELISA and Flow cytometry reagents and kits

ELISA 96 well plates (Nunc-Immuno ^{TM} plate marxi sorp ^{TM}) and goat anti-mouse IgG were obtained from Sigma-Aldrich, USA while the substrate (SureBlue^{TM}, TMB microwell peroxidase substrate -1 component) was procured from KPL, Gaithersburg USA.

All materials used in flow cytometry were obtained from BD Biosciences (Califonia, USA). BD cytometric bead array (CBA) mouse Th1/Th2 cytokine kit contained all the necessary reagents and capture antibodies specific for IL-2, IL-4, IL-5, IFN-γ and TNF proteins. BD FACSCalibur^{TM} flow cytometer, BD CellQuest^{TM} Pro version 5.2.1 and FCap Array software

version 1 were used in the analysis of cytokine profiles. BD Falcon^{TM} 12 x 75mm sample acquisition tubes for the flow cytometer and 15ml conical propylene tubes were also used.

3.2.4 Determination of IgG profiles of the different treatments using ELISA

Soluble egg antigen (SEA), schistosomular (0-3 hr) and soluble adult worm antigen preparation (SWAP) antigens were diluted in 1 X PBS at a concentration of 10 µg /ml and 50µl of each added to ELISA plates wells. This was allowed to stand overnight at 4^{o}C then washed three times using wash buffer (PBS 0.05% v/v Tween 20) with ELISA washer system (Dynex Technologies LTD, Guernsey channel Great Britain).

After draining off all the wash buffer, 100µl blocking buffer (3% BSA) was added to each well and allowed to stand at 37^{o}C for 1 h, then washed three times using wash buffer. Fifty microlitres of antibody mixture prepared by mixing mice serum with 0.5% BSA at a ratio of 0.0025:1 was added to each well in duplicate and allowed to stand at 37^{o}C for 2 h. This was washed six times and 50µl conjugate goat anti-mouse IgG in a dilution of 0.0005:1 of 1% BSA added then incubated at 37^{o}C for 1.5 h. The plates were then washed six times and 50µl of substrate added. This plates were put in the dark for 30 min and reading done using ELISA reader (Dynatech Laboratories, USA) at OD 450nm.

3.2.5 Determination of cytokine profiles of the different treatments using flow cytometry

3.2.5.1 Preparation of serum for immunological analysis

Mouse serum for immunological analysis was prepared as described in section 2.2.8.

3.2.5.2 Reconstitution and serial dilution of the standards

Reconstitution and serial dilution was done according to manufacturer instruction. Briefly, the lyophilized standard spheres were transferred into a 15ml conical propylene tube and labeled "Top standard". Two mililitres of Assay Diluent was added and allowed to stand at room temperature for 15 min then gently mixed using a pipette. Eight acquisition tubes were labeled in the order of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and 300µl assay diluent was added to each tube. To perform the serial dilutions, 300µl of top standard was added to tube marked 1:2 and mixed using pipette, another 300µl of 1:2 mixture to tube marked 1:4 and so on until tube marked 1:256. One acquisition tube was reserved containing only assay diluent as a negative control.

3.2.5.3 Mixing of mouse Th1/Th2 cytokine capture beads and cytokine assay

Mixing of mouse Th1/Th2 cytokine capture beads was done as per manufacturer instruction. Briefly, 70 microfuge tubes containing serum for cytokine profile analysis were selected and kept at 4^{o}C. Each of the capture bead suspension was individually vortexed and 10µl times 70 aliquot of each pooled in a single tube labeled "Mixed Capture Beads" and vortexed thoroughly followed by adding 50µl of the mixed capture beads to labeled assay tubes.

Fifty microlitres of the Th1/Th2 cytokine standard solutions were added to control tubes as listed in Table 5 below.

Table 5. Serial dilutions of Th1/Th2 cytokine standard solutions

Tube label	Concentration (pg/ml)	Cytokine standard solution
1	0	Negative control (Assay Diluent only)
2	20	1:256
3	40	1:128
4	80	1:64
5	156	1:32
6	312.5	1:16
7	625	1:8
8	1250	1:4
9	2500	1:2
10	5000	Top Standard

Fifty microlitres of serum was added to labeled assay tubes, 50µl of mouse Th1/Th2 PE detection reagent added to each and incubated in the dark for 2 h. One mililitre of wash buffer was added to each assay tube and centrifuged at 200g for 5 min. After discarding supernatant from each assay tube, 300µl of wash buffer was added to each tube to resuspend the bead pellet and suspension loaded on BD FACSCalibur™ flow cytometer. The data was acquired using BD CellQuest™ Pro version 5.2.1 and analyzed using FCap Array software version 1.

3.3 Results

3.3.1 Result of liver gross pathology after treatment with *C. ambrosoides* water and methanol extracts

Adhesion between liver lobes was observed in all mice livers both for adult and juvenile worm treatment experiments. Both extracts were relatively effective in preventing liver inflammation with no more than 14% of the mice presenting with liver inflammation. *C. ambrosoides* water extract was more effective in inhibition of granuloma formation in adult than in juvenile worm treatment group but methanol extract was more effective in juvenile worm treatment. When compared to praziquantel, the water extract was a better inhibiter of granuloma formation in adult worm treatment and both extracts were slightly worse in juvenile treatment relative to praziquantel. Pathology results of these extracts relative to the other extracts, the standard treatment praziquantel and infected control are summarized in Tables 6 and 7.

3.3.2 Result of liver gross pathology after treatment with *O. americanum* hexane and water extracts

Adhesion between liver lobes was observed in all mice livers and inflammation was generally worse than in *C. ambrosoides* treatment highlighted in above section. *O. americanum* water extract was better at preventing inflammation in juvenile worm treatment with 0% of the livers presenting with inflammation relative to 57% in adult worm treatment. The hexane extract was also better at preventing inflammation in juvenile worm treatment than in adult group with 33% of the livers presenting with inflammation relative to 43% respectively. *O. americanum* extracts

juvenile worm treatment was more effective than for the adult worm. This is inferred by adult worm treatment groups having some livers with severe granuloma formation compared to none in juvenile worm treatment. The extracts were however worse inhibitors of granuloma formation than *C. ambrosoides* extracts. Neither of the extracts was similar to nor more effective in granuloma inhibition formation when compared to praziquantel. Further information on effectiveness of these *O. americanum* extracts against other extracts, the standard treatment praziquantel and infected control is in Tables 6 and 7.

3.3.3 Result of liver gross pathology after treatment with *B. micrantha* water extract

Adhesion was observed in all mice liver lobes with 0% inflammation in adult worm treatment and 43% inflammation in juvenile worm treatment. Granuloma formation inhibition was better than *O. americanum, C. ambrosoides* and praziquantel in adult worm treatment. Inhibition of granuloma formation after treatment with *B. micrantha* water extract was better than those of *O. americanum, C. ambrosoides* and praziquantel in adult worms.

Table 6. Liver gross pathology analysis in juvenile worm treatment groups

		Adhesion	Inflamation		Granuloma categorisation			
Treatment	**No. of mice**	**Yes**	**Yes**	**No**	**Severe**	**Moderate**	**Few**	**None**
C. ambrosoides (water extract)	8	100%	0%	100%	0%	0%	50%	50%
C. ambrosoides (methanol extract)	7	100%	14%	86%	0%	0%	43%	57%
B. micrantha (water extract)	7	100%	43%	57%	0%	14%	71%	14%
O. americanum (hexane extract)	6	100%	33%	67%	0%	17%	50%	33%
O. americanum (water extract)	6	100%	0%	100%	0%	0%	33%	67%
Praziquantel	4	100%	0%	100%	0%	0%	25%	75%
Infected Control	6	100%	83%	17%	0%	67%	33%	0%

Granuloma classification Key:

None -No granuloma observed per lobe; Few- 1 to 3 granuloma per lobe; Moderate- 4 to 10 granuloma per lobe; Severe- more than 10 granuloma per lobe

Table 7. Liver gross pathology analysis in adult worm treatment groups

		Adhesion	Inflamation		Granuloma categorisation			
Treatment	**No. of mice**	**Yes**	**Yes**	**No**	**Severe**	**Moderate**	**Few**	**None**
C. ambrosoides (water extract)	7	100%	14%	86%	14%	0%	29%	57%
C. ambrosoides (methanol extract)	7	100%	14%	86%	0%	14%	57%	29%
B. micrantha (water extract)	8	100%	0%	100%	0%	0%	13%	88%
O. americanum (hexane extract)	7	100%	43%	57%	14%	29%	14%	43%
O. americanum (water extract)	7	100%	57%	43%	17%	50%	17%	17%
Praziquantel	9	100%	0%	100%	0%	0%	89%	11%
Infected Control	6	100%	83%	17%	0%	67%	33%	0%

Granuloma classification Key:

None -No granuloma observed per lobe; Few- 1 to 3 granuloma per lobe; Moderate- 4 to 10 granuloma per lobe; Severe- more than 10 granuloma per lobe

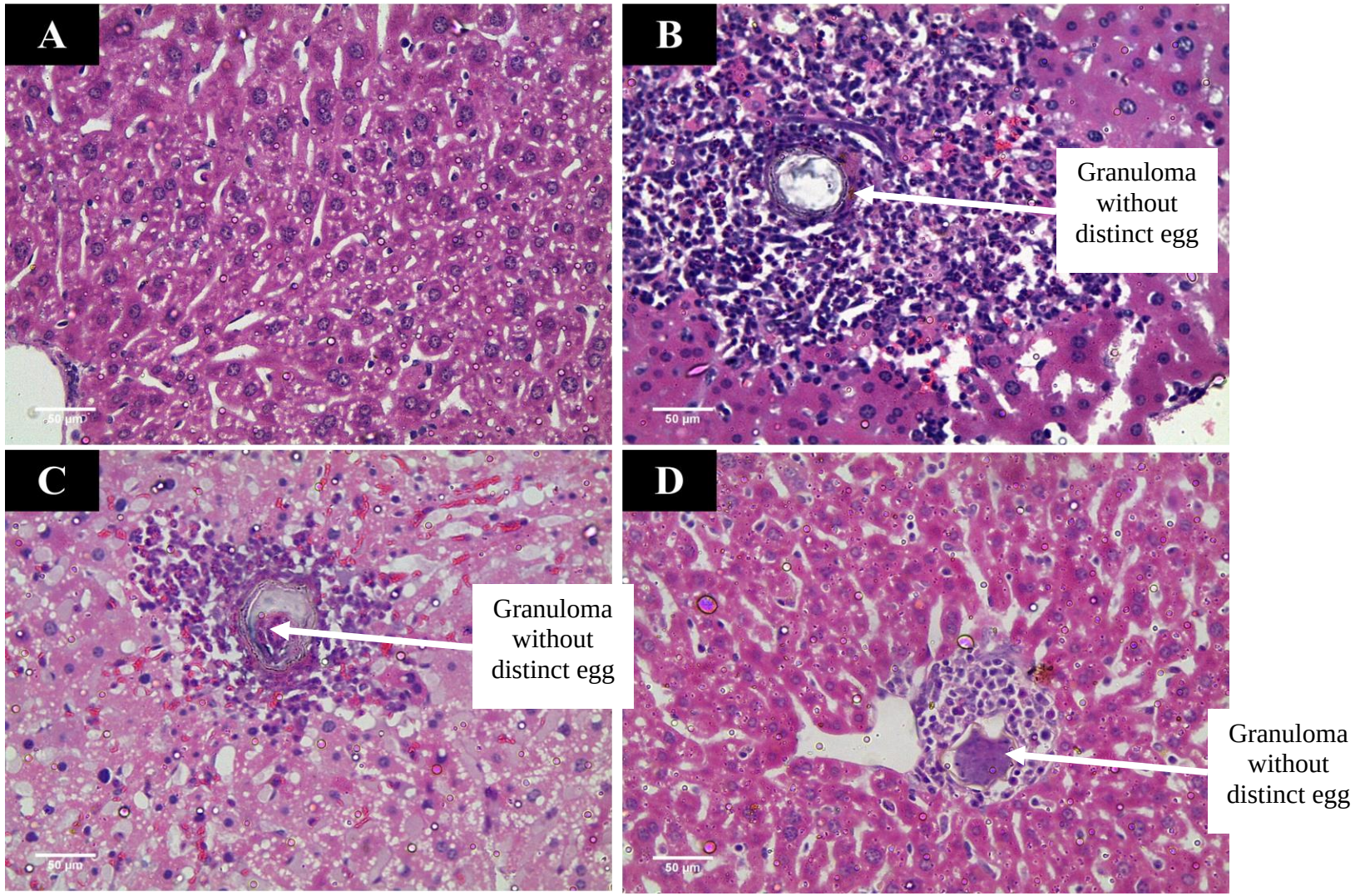
3.3.4 Histopathology of mice liver biopsies

Under an ocular micrometer, 10 granulomas from each animal's liver were measured on their vertical and horizontal dimensions, the average of which was assumed to be the granuloma diameter (Farah *et al*, 2000). Using one way ANOVA ($\acute{\alpha} = 0.05$) the means were found to be significantly different although the post hoc Tukey HSD did not detect any differences between paired means. Graphical representation of granuloma sizes are shown in Table 8 below.

Table 8. Mean hepatic granuloma diameter of BALB/c mice groups treated with plant extracts, praziquantel and infected control where treatment was withheld.

Treatment groups	Mean ± SE granuloma size
C. ambrosoides (water extract)	70±5
C. ambrosoides (methanol extract)	171.11±23.61
B. micrantha (water extract)	153.18±20.8
O. americanum (hexane extract)	170±19
O. americanum (water extract)	234.62±22.37
Praziquantel	160±15
Infected Control	240.93±17.24

Histopathology changes which were determined mainly by extent of lymphocytic cellular infiltration of liver from group treated with praziquantel was mild showing few areas of necrosis and granuloma. Other than for *B. micrantha* group where histopathology changes were moderate, the other groups showed mild to severe histopathology changes. Infected control group had highest number of granuloma per slide and none was observed to be resolving. The other treatment groups however had granulomas which were resolving without a distinct central egg. The photomicrographs of the liver sections are shown in Figure 6 below.


A
B
Granuloma without distinct egg
C
Granuloma without distinct egg
D
Granuloma without distinct egg
50 µm

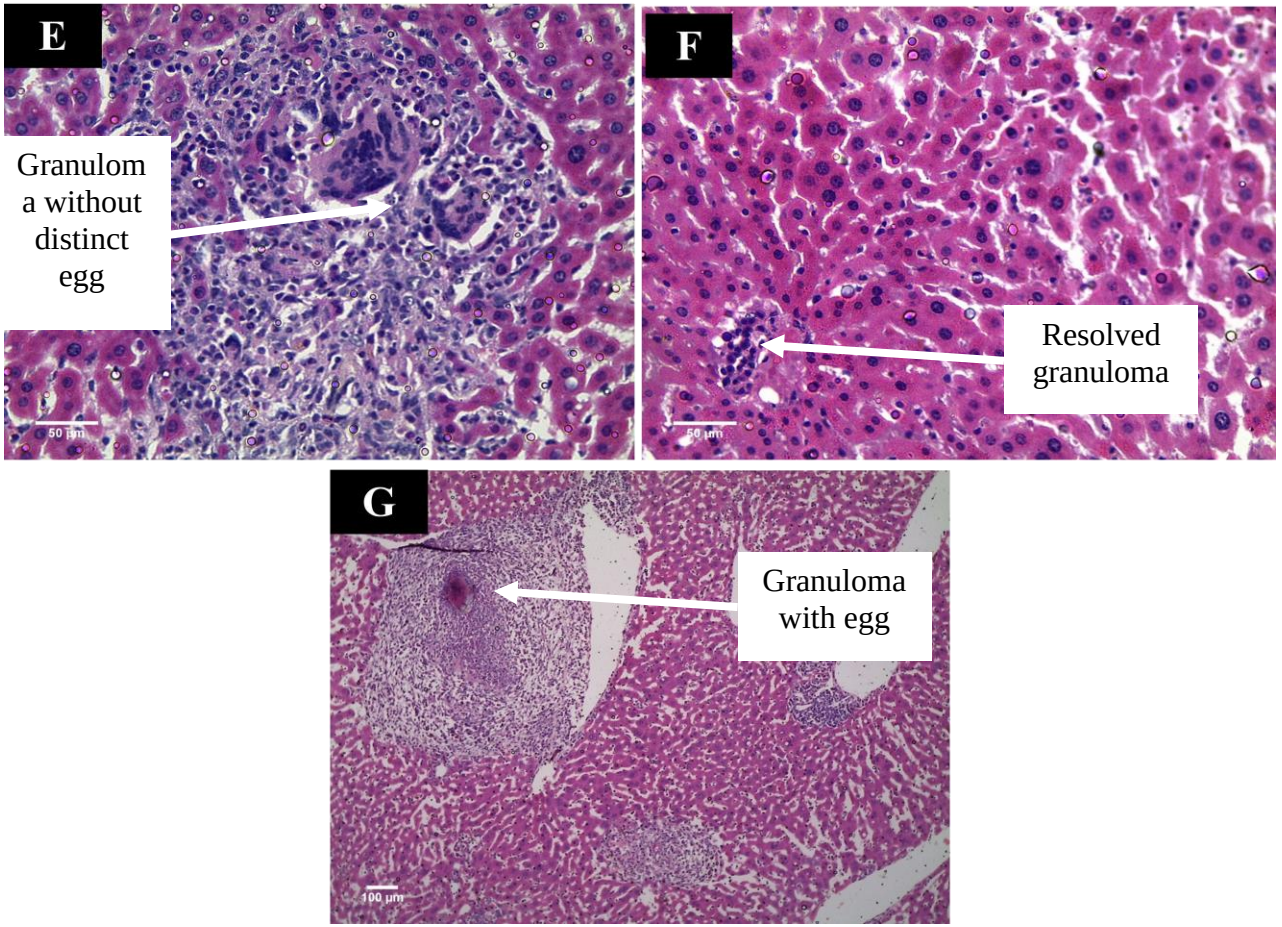


Figure 6. Histological sections of livers of BALB/c mice groups treated with plant extracts, praziquantel and infected control where treatment was withheld. Granuloma with intact egg was observed for the infected control and resolving granulomas for the treatment groups. Note that histological section for infected control was taken at a lower magnification than the others in order to visualize the entire granuloma

Key: A (Praziquantel); B (*B. micrantha* water extract); C (*C. ambrosoides* methanol extract); D (*C. ambrosoides* water extract); E (*O. americanum* hexane extract); F (*O. americanum* water extract); G (Infected control)

3.3.5 IgG profiles of the different treatment groups

There was no statistical difference for IgG profiles using SWAP antigen in adult worm treatment study (P>0.05). The mean OD readings for *C. ambrosoides* methanol extract, *B. micrantha* water extract, *C. amrosoides* water extract, praziquantel, Infected control, Naïve, *O. americanum* water extract and *O. americanum* hexane extract was 0.586, 0.537, 0.517, 0.510, 0.448, 0.438, 0.433 and 0.397 respectively.

The mean OD readings in juvenile worm treatment study were generally lower when compared to the adult study except for 0.619 *B. micrantha* water extract. Mean OD readings for *C. ambrosoides* methanol extract, *C. ambrosoides* water extract, *O. americanum* water extract, praziquantel and *O. americanum* hexane extract were 0.515, 0.494, 0.431, 0.390 and 0.331 respectively.

IgG profile against the schistosomula 0-3 hour release protein had mean OD readings that were lower than both SWAP and SEA. There was no statistical difference in adult or juvenile worm treatment groups (P>0.05). The OD readings in the adult worm treatment groups for *B. micrantha* water extract, *C. ambrosoides* methanol extract, praziquantel, *O. americanum* hexane extract, Infected control, *C. ambrosoides* water extract, *O. americanum* water extract and Naïve was 0.266, 0.226, 0.226, 0.214, 0.210, 0.202, 0.194 and 0.180 respectively.

In juvenile worm treatment study, the mean OD readings for *B. micrantha* water extract, *C. ambrosoides* methanol extract, *C. ambrosoides* water extract, *O. americanum* hexane extract, *O. americanum* water extract and praziquantel were 0.257, 0.202, 0.185, 0.185, 0.172 and 0.170 respectively.

Anti-SEA IgG profile mean OD readings were also not significantly different for adult or juvenile worm treatment groups. Naïve OD reading was however statistically lower when compared with the adult treatment groups. OD readings for *C. ambrosoides* methanol extract, *B. micrantha* water extract, *C. ambrosoides* water extract, praziquantel, *O. americanum* water extract, infected control and *O. americanum* hexane extract treatment groups were 0.519, 0.461, 0.427, 0.428, 0.353, 0.330, 0.301 and 0.284 respectively

For juvenile worm treatment study, OD readings were *B. micrantha* water extract, *C. ambrosoides* water extract, *C. ambrosoides* methanol extract, *O. americanum* water extract, praziquantel, *O. americanum* hexane extract were 0.473, 0.437, 0.411, 0.381, 0.367 and 0.323 respectively

The graphical representation of IgG profiles of the treatment groups for the three antigens is also represented in Figures 7 and 8 below.

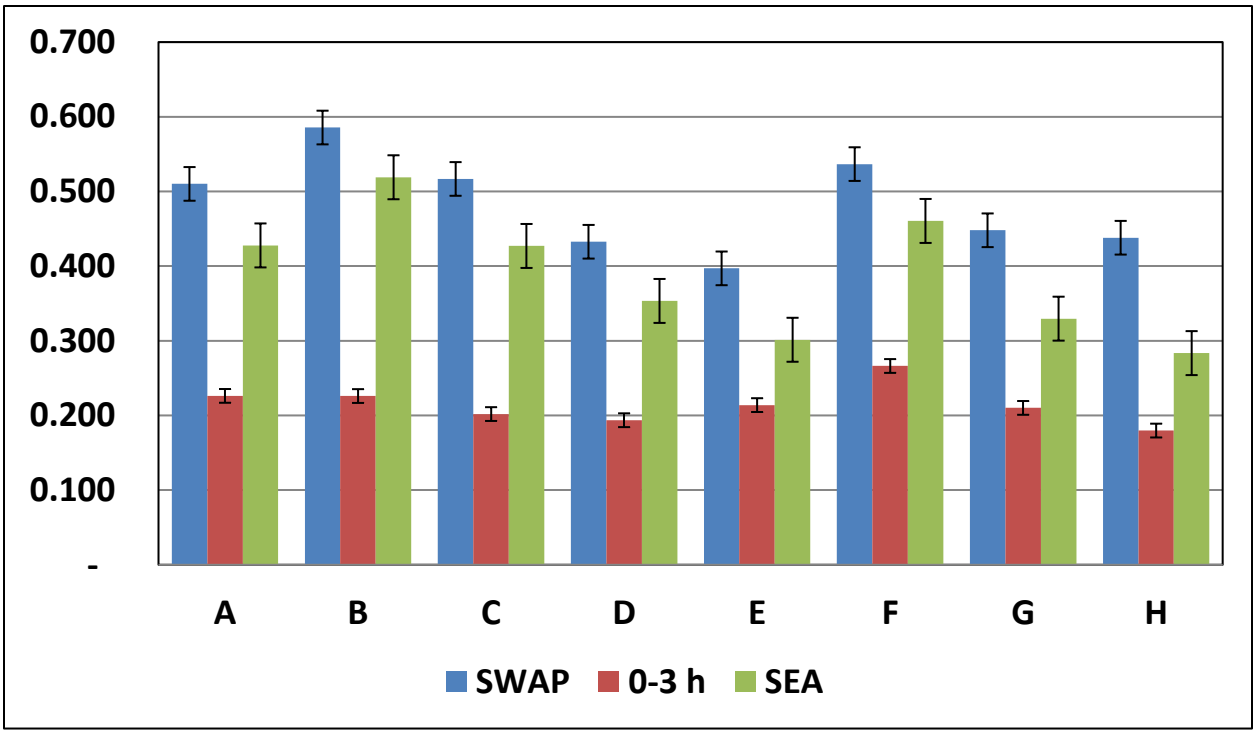


Figure 7. IgG mean OD readings of mice infected with S. mansoni and treated on week 4 (adult) post infection (Error bars represent Standard Error)

Key: A - Praziquantel; B - *C. ambrosoides* methanol extract; C - *C. ambrosoides* water extract; D - *O. americanum* water extract; E - *O. americanum* hexane extract; F - *B. micrantha* water extract; G - infected control; H - non infected control

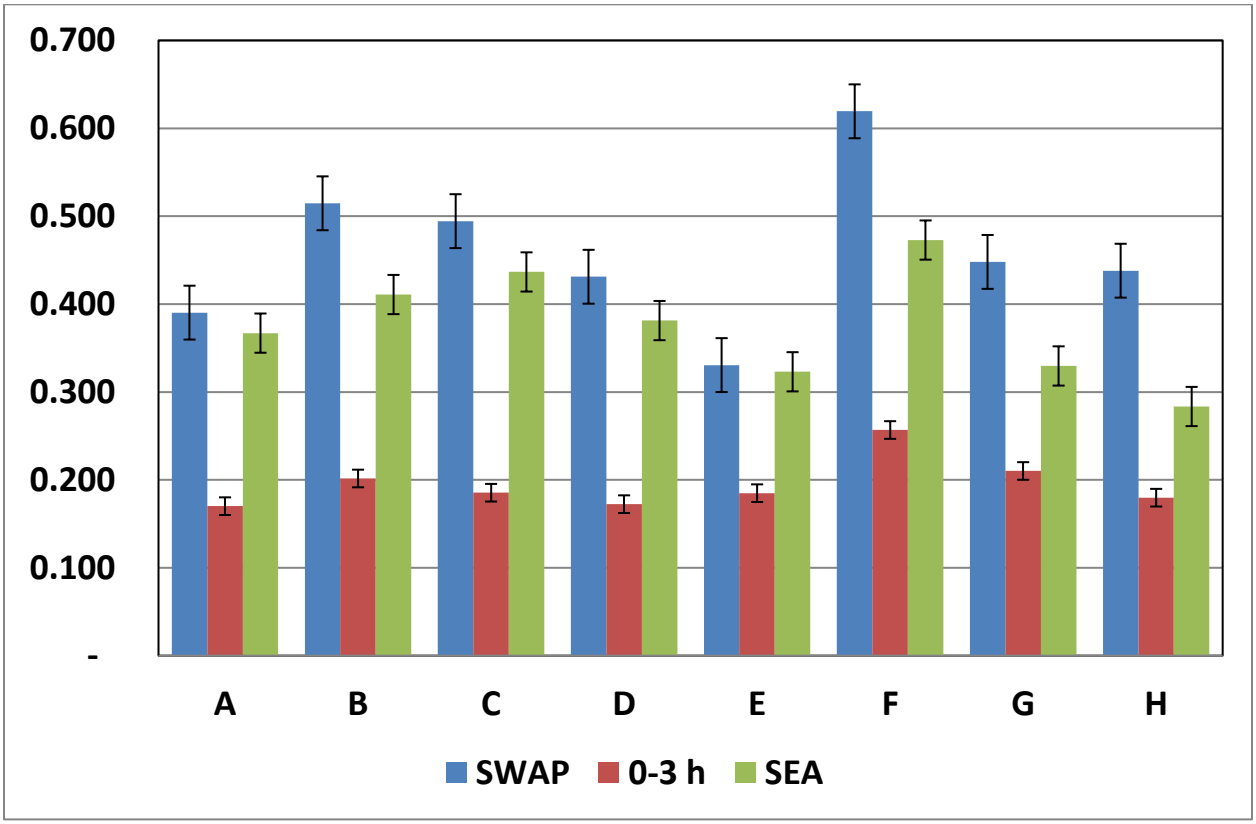


Figure 8. IgG mean OD readings of mice infected with S. mansoni and treated on week 3 (juvenile) post infection. (Error bars represent Standard Error)

Key: A - Praziquantel; B - *C. ambrosoides* methanol extract; C - *C. ambrosoides* water extract; D - *O. americanum* water extract; E - *O. americanum* hexane extract; F - *B. micrantha* water extract; G - infected control

3.3.6 Cytokine profiles of the different treatment groups using Flow cytometry

All cytokine concentrations in the various groups are represented in Tables 9 and 10.

Statistical difference for adult efficacy study was observed for all cytokine concentration groups except for IL-4 which was only detectable in mice treated with *B. micrantha* water extract. Infected control and naïve group cytokine concentrations were lower when compared with all treatment groups except for IL-2 where *C. ambrosoides* water extract and *B. micrantha* water extract treatment groups had lower concentrations.

Statistical difference for juvenile efficacy study was observed for TNF and IL-5. IL4 was only detectable in mice treated with *C. ambrosoides* methanol extract. Infected control had IL-2 concentration of 4.91 pg/ml, which was higher than for four treatment groups including praziquantel. Naïve group IL-2 concentration was lower than for all treatment groups except *B. micrantha* water extract. These differences were however not statistically significant. There was also no statistical difference for IFN-γ but concentration for infected control was relatively low and undetectable for naïve group.

Table 9. Mean cytokine concentrations of mice treated against adult worms

	Cytokine concentration in pg/ml				
	Th1			Th2	
Treatment groups	**TNF**	**IFN-γ**	**IL-2**	**IL-5**	**IL-4**
Praziquantel	12.68±2.93	2.88±1.74	6.13±0.23	5.63±3.3	-
C. ambrosoides (water extract)	6.67±5.53	3.73±0.17	1.96±9.12	12.28±7.09	-
C. ambrosoides (methanol extract)	105.03±78.75	37.12±34.27	7.78±3.09	8.31±1.31	-
B. micrantha (water extract)	12.51±2.45	2.49±3.54	2.46±0.07	29.8±3.37	11.56±5.95
O. americanum (water extract)	5.66±4.72	4.07±1.21	-	13.74±0.36	-
O. americanum (hexane extract)	9.27±1.16	-	14.6±0.12	11.74±3.12	-
Infected control	3.53±2.39	0.26±0.21	4.91±1.64	-	-
Naïve	3.81±2.21	-	2.72±9.43	2.46±3.76	

Table 10. Mean cytokine concentrations of mice treated against juvenile worms

	Cytokine concentration in pg/ml				
	Th1			Th2	
Treatment groups	**TNF**	**IFN-γ**	**IL-2**	**IL-5**	**IL-4**
Praziquantel	8.54±3.15	1.2±0.21	3.66±0.82	4.76±1.35	-
C. ambrosoides (water extract)	58.68±45.39	1.32±0.08	5.78±4.71	8.48±1.77	-
C. ambrosoides (methanol extract)	6.3±2.17	0.86±0.54	6.92±3.51	9.14±4.93	6.16±2.41
B. micrantha (water extract)	11.54±2.46	1.7±0.95	2.54±2.29	17.86±4.6	-
O. americanum (water extract)	9.47±4.07	2.22±0.2	4.66±3.51	13.45±3.17	-
O. americanum (hexane extract)	9.59±0.51	0.99±2.12	4.59±1.7	9.45±1.72	-
Infected control	3.53±2.39	0.26±0.02	4.91±8.51	-	-
Naïve	3.81±2.21	-	2.72±9.43	2.46±3.76	-

3.4 Discussion

While the pathology for the plant extracts was not as remarkable as those for the praziquantel treated groups, the observation of resolving granulomas could be an indicator that the extracts have comparable properties. It is likely that at higher doses than the administered 150 mg/kg, the

effect on pathology would be more pronounced. Alternatively, pathology of mice done serially over a few weeks would better show the disease progression. The IgG responses were observed to be similar across the treatment groups, infected control and naïve despite variation in worm numbers suggesting insignificant IgG effect in early stage infection. Naïve OD readings were potentially due to non specific binding

Interestingly, praziquantel profile was not highest for any of the cytokines while infected control was low in all groups except for IL-2. While it is known that innate immunity can develop following prolonged exposure owing to responses against dead adult worms, praziquantel has been proven to elicit protective immune responses (Mutapi *et al.,* 2005; Mitchell *et al.,* 2012, Bourke *et al.,* 2012).

The balance of Th1 and Th2 responses is important in the pathology of schistosomiasis. Th1 responses are associated with the acute stage of the infection being a reaction towards the worms while Th2 responses occur later due to eggs laid by mature worms (Cintron-Rivera, 1956; Warren, 1973; Nash *et al.,* 1982; McManus and Loukas, 2008). Th2 associated cytokines IL-4, IL-5 and IL-13 all play important roles in pathogenesis of schistosomiasis with IL-4 and IL-13 directing granuloma formation (Chiaramonte *et al.,* 1999a). IL-13 on the other hand is primarily the stimulus for tissue fibrosis (Chiaramonte *et al.,* 1999b; Fallon *et al.,* 2000). Prominence of Th1 cytokines especially IFNγ has an effect of down regulating Th2 cytokines and consequently a reduction of granuloma size and fibrosis (Cheever *et al.,* 1992; Cheever *et al.,* 1998). IL-5 serves to recruit eosinophils but has no obvious effect on granuloma size. IL-4 leads to formation of IgE with varied effect on granuloma while IL-2 increases granuloma size and fibrosis

(Cheever et al., 1992). Although TNF has not been widely studied, it is thought to have a role in periportal fibrosis (Cheever *et al.*, 1992; Ramadan *et al.*, 2013). This suggests the inflammation causing cytokines may be important in acting against the miracidia within eggs and thereafter, other cytokines resolve the granuloma implying the importance of the cytokine balance. This suggests the plant extracts elicited cytokine responses that compared well with praziquantel and may potentially be protective.

The suggestion that the plant extracts used in this study could potentially promote immunological resistance is interesting. This would mean antischistosomal drug candidates based on *B. micrantha*, *C. ambrosoides* and *O. americanum* extracts will have comparable seroprotection qualities to that experienced after treatment with praziquantel.

CHAPTER 4

4 Evaluation of toxicity of plant extracts with antischistosomal activity

4.1 Introduction

Natural products have proven to be reliable sources of therapies and new chemical entities (NCEs) in medical history (De Pasquale, 1984; Paterson and Anderson, 2005; Chin *et al.,* 2006; Newmann and Cragg, 2007). It is therefore not curious that drugs used to treat 87% of categorised human diseases have a natural products origin and a snapshot of the period between 2000 to 2005 had 23 new drugs with other NCEs still undergoing trials (Newman *et al*., 2003; Chin *et al.,* 2006). Given the potential challenges of having praziquantel as the only commercially available anti-schistosomal drug, it is crucial that studies aimed at identifying NCEs which will complement praziquantel be undertaken (Fenwick *et al.,* 2003; El-Subbagh and Al-Badr, 1998; Ismail *et al.,* 1999; Danso-Appiah, 2002; Utzinger and Keiser, 2004).

B. micrantha, *C. ambrosoides* and *O. americanum* have been used as remedy for a variety of ailments including as wormicidal applications (Hiltunen and Holm, 2005; Yadav *et al.,* 2007; Orwa *et al.,* 2009). Earlier chapters of this work and as reported separately, studied the effects of these plants against *Schistosoma* infection (Moilo *et al*., 2014; Waiganjo *et al*., 2014). However, limited toxicological studies have been undertaken to determine if bioactive molecules are safe particularly if intended for clinical use (Aneela *et al.,* 2011).

Organisation for Economic Co-operation and Development (OECD) guidelines further elaborate that it is important to undertake these studies so that a decision to adopt products for clinical use or not can be determined (OECD 1987, 2001a,b).

In this chapter brine shrimp (*Artemia salina*) lethality test was undertaken to determine LC_{50} followed by acute toxicity experiments which included eye irritation, dermal irritation and skin sensitization. Finally subchronic (28 day) study was performed to determine if the extracts had an adverse effect on albino rats' (*Rattus albus*) livers.

4.2 Materials and methods

All the studies in this chapter were performed at University of Nairobi's Department of Public Health, Pharmacology and Toxicology.

4.2.1 Brine shrimp bioassay

This test was performed as described by Sorgeloos *et al.,* (1978). Briefly, 1g of *A. salina* eggs were hatched by incubation at room temperature in 3.3% artificial brine (Sera marine salt, Sera Company, Heinsberg, Germany) for 72 h. The extract solution was constituted by dissolving 100 g of extract in 10ml ddH_2O and topped up to 15ml using artificial brine. This formed a stock solution of 1000 mg/ml for subsequent dilutions.

Serial dilutions were constituted in 15 ml test tubes by adding 500µl, 50µl and 5µl stock solution to 4.5ml, 5ml and 5ml artificial brine respectively. Ten *Artemia salina* larval 1 stage larvae were added in each test tube and kept in light for 24 h followed by counting of surviving larvae. This

test was performed in five replicates and Finney's probit analysis used for the determination of LC_{50} using Finney computer program (Finney, 1976).

4.2.2 Acute dermal irritation study

This was performed as per OECD guidelines (2002a). Three months old albino New Zealand White rabbits (*Oryctolagus cuniculus*) with average weight of 3.3 kgs were used for this study, where after 7 days of acclimatization, they were shaved on two sides on the dorsal side with one abraded and the other left intact. The rabbits were maintained in cages at 20ºC ± 3ºC and relative humidity at above 30% -but less than 70%-, with natural light. The animals were fed on pellets (Unga group Ltd, Nairobi, Kenya) with water *ad libitum*.

Water moistened paste of 0.5g of *C. ambrosoides* and *O. americanum* water extracts was applied on the two sites of each animal and covered with gauze held in place by a non-irritating tape. Care was taken to ensure the animal could not access the patch or inhale the applied extract. The animals were returned to the cages for the duration of the exposure (4 h) after which the gauze was removed and the area cleaned gently using water.

The animals were observed for signs of erythema and oedema at 24 h and 72 h post exposure period. As recommended in the OECD sequential testing strategy, the test was initially performed on one animal for either treatment followed by two replicates after confirming absence of severe effects and responses. The grading of erythema and oedema was based on a subjective score on a scale of 0 to 4 as described in OECD guidelines (2002a).

4.2.3 Acute eye irritation study

Three months old albino New Zealand White rabbits (*Oryctolagus cuniculus*) with average weight of 3.3 kgs were also used for this study as described in OECD (2002b). The animals were allowed to acclimatize for a minimum of 7 days. Three animals were used for each of the two extracts and maintained in cages as described in the section above. The conjuctival sac was brought into contact with the extracts -0.5 g dissolved in water to form 0.1 ml by sonication- by inserting 0.1 ml solution inside the lower eye lid then holding both eye lids together for a brief moment to allow for even distribution. The other eye which was not exposed to the extracts was used as a negative control. As described in above section, OECD sequential testing strategy was applied.

The eyes were observed at 24 h, 48 h, 72 h, 4 days and 7 days intervals post exposure and grading of ocular lesions; ulceration, opacity, necrosis and opaqueness on a scale of 0 to 4 done as indicated in the OECD guideline (2002b).

4.2.4 Skin sensitization study

This study was performed in 2 months old guinea pigs (*Cavia porcellus*) with average weight of 743g, using a modified technique adapted from OECD guidelines (1992). The animals were maintained in cages at $20^{o}C \pm 3^{o}C$, with natural lighting, being fed on rodent pellets (Unga Ltd, Nairobi, Kenya) with water *ad libitum*. Three animals were used for each extract as well as control group.

Briefly, following a period of acclimatisation of not less than 7 days, the animals were shaved on their back on the shoulder region taking care not to abrade the skin. They were then injected intradermally with 0.1 ml solution of each extract on the shaved area 3 times a week on alternate days for 3 weeks totalling 10 treatments. The solution was prepared by measuring 0.5 g extract, topped up to 0.1 ml with water, dissolved by sonication and then filtered through 0.45 µm nylon membrane (Acrodisc® Premium 25 mm syringe filter, Pall corporation, USA). Control animals were injected with normal saline which served as sham treatment. Two weeks post exposure a challenge injection was administered and observation for clinical signs such as erythema, oedema, corrosion or general inflammation was made at 24 h, 7 days, 14 days, 28 days and 35 days.

The observations were graded according to Magnusson and Kligman for the evaluation of challenge patch test reactions with grading from 0-3 (OECD, 1992).

4.2.5 Twenty eight days subchronic toxicity study

This study was performed using 6 weeks old albino rats (*Rattus albus*) with average weight of 134g for a duration of 28 days with daily dosing as described in OECD guidelines (1995). Eight rats were used for each extract comprising of 4 individuals of either sex maintained in cages at 22^{o}C ± 3^{o}C and relative humidity at above 30% -but less than 70%-, with natural lighting. They were fed with standard rodent pellets (Unga feeds, Nairobi, Kenya) with water *ad libitum*.

Before the first dose was administered on day one, blood was collected from the orbital sinus (Janet hoff, 2000) and stored for later analysis as a baseline. Oral doses were administered for 28 days at a concentration of 500 mg/kg and on the final day, blood was again collected from the

orbital sinus and stored for final reading analysis then the animals were euthanized following ethical procedures. This was used as a preliminary study where if there was a noted effect, other haematological and pathological studies would have been undertaken. Biochemical analysis was performed on the collected blood using aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as indicators of liver cells damage (Boone *et al.,* 2005; Diallo *et al.,* 2010; Sur *et al.,* 2015).

AST and ALT concentrations post exposure were categorised relative to means of initial concentration which was assumed to be the upper limit of the normal (ULN). These were categorised as proposed by Thapa and Walia (2007) as follows; severe (> 20 times ULN), moderate (3-20 times ULN) and mild (1-3 times ULN).

4.2.6 Disposal of test animals.

All animals used in these studies were euthenized using pentobarbitone then disposed by incineration at the University of Nairobi's department of veterinary pathology and parasitology.

4.3 Results

4.3.1 Brine shrimp bioassay

The average mortality for each extract with increasing concentration is shown in Table 11 below. Toxicity was classified as suggested by Rand (2003).

Table 11. Brine shrimp mortality at various concentrations of extracts of *B. micrantha*, *C. ambrosoides* and *O. americanum*

Concentration (µg/ml)	*B. micrantha* (water extract)	*C. ambrosoides* (methanol extract)	*C. ambrosoides* (water extract)	*O. americanum* (hexane extract)	*O. americanum* (water extract)
0	0	0	0	0	0
10	1.6	1.2	0.4	0.4	0
100	5.2	4.4	1.2	1.4	0
1000	9.2	9.6	6	4.2	3.4
LC_{50} (µg/ml)	77.24	104.63	696.44	887.59	2254.60
Toxicity categorization	Toxic	Moderately toxic	Moderately toxic	Moderately toxic	Slightly toxic

4.3.2 Acute toxicity studies

4.3.2.1 Acute dermal irritation test

C. ambrosoides water extract was largely not irritating to rabbit skin. There was however very slight erythema at 24 h post exposure on the abraded skin. Rabbits exposed to *O. americanum* water extracts on the other hand did not exhibit any effect. The results are summarized in Table 12 below.

Table 12. Effects of dermal irritation of intact and abraded rabbit skins by aqueous extracts of *C. ambrosoides* and *O. americanum*

		24 Hours				72 Hours			
		Intact		Abraded		Intact		Abraded	
Replicates	**Reaction**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
	Erythema	-	-	+	-	-	-	-	-
1	Edema	-	-	-	-	-	-	-	-
	Erythema	-	-	+	-	-	-	-	-
2	Edema	-	-	-	-	-	-	-	-
	Erythema	-	-	+	-	-	-	-	-
3	Edema	-	-	-	-	-	-	-	-

Key:

A : *C. ambrosoides* water extract

B : *O. americanum* water extract

+ Reaction observed

- No reaction

4.3.2.2 Acute eye irritation study

Mild changes were observed to the iris and some reddening where rabbits eyes were exposed to *C. ambrosoides* water extract as shown in Table 13. This however dissipated by 48 h post exposure and appeared normal thereafter. There were no other observable effects on the cornea and conjunctiva.

Table 13. Acute eye irritation results of rabbit eyes exposed to *C. ambrosoides* water extract

	CORNEA					**IRIS**					**CONJUNCTIVAE**				
	Opacity					**Changes**					**Chemosis**				
R	**24 h**	**48 h**	**72 h**	**4 Days**	**7 Days**	**24 h**	**48 h**	**72 h**	**4 Days**	**7 Days**	**24 h**	**48 h**	**72 h**	**4 Days**	**7 Days**
1	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	Area involved					**Redness**					**Discharge**				
	24 h	**48 h**	**72 h**	**4 Days**	**7 Days**	**24 h**	**48 h**	**72 h**	**4 Days**	**7 Days**	**24 h**	**48 h**	**72 h**	**4 Days**	**7 Days**
1	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-

Key:

R - Replicates

+ Reaction observed

- No reaction observed

Table 14. Acute eye irritation results of rabbit eyes exposed to *O. americanum* water extract

	CORNEA					**IRIS**					**CONJUNCTIVAE**				
	Opacity					**Changes**					**Chemosis**				
R	**24 h**	**48 h**	**72 h**	**4 Days**	**7 Days**	**24 h**	**48 h**	**72 h**	**4 Days**	**7 Days**	**24 h**	**48 h**	**72 h**	**4 Days**	**7 Days**
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Area involved					**Redness**					**Discharge**				
	24 h	**48 h**	**72 h**	**4 Days**	**7 Days**	**24 h**	**48 h**	**72 h**	**4 Days**	**7 Days**	**24 h**	**48 h**	**72 h**	**4 Days**	**7 Days**
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Key:

R - Replicates

+ Reaction observed

- No reaction observed

Rabbits' eyes exposed to *O. americanum* water extracts did not exhibit any observable effects as shown in Table 14 above.

4.3.2.3 Skin sensitization

No visible changes were observed for the skin sensitization study as shown in Tables 15 and 16 below.

Table 15. *C. ambrosoides* water extract skin sensitization results

Replicates	Reaction	24 h	7 Days	14 Days	28 Days	35 Days
1	Erythema	-	-	-	-	-
	Edema others	-	-	-	-	-
2	Erythema	-	-	-	-	-
	Edema others	-	-	-	-	-
3	Erythema	-	-	-	-	-
	Edema others	-	-	-	-	-

Key:

+ Reaction observed

- No reaction observed

Table 16. *O. americanum* water extract skin sensitization results

Replicates	Reaction	24 h	7 Days	14 Days	28 Days	35 Days
1	Erythema	-	-	-	-	-
	Edema others	-	-	-	-	-
2	Erythema	-	-	-	-	-
	Edema others	-	-	-	-	-
3	Erythema	-	-	-	-	-
	Edema others	-	-	-	-	-

Key:

+ Reaction observed

- No reaction observed

4.3.2.4 Subchronic toxicity

The results of subchronic toxicity are shown in Tables 17 and 18 below

Table 17. *C. ambrosoides* water extract subchronic toxicity study results for biochemical test

		AST (iu)		ALT (iu)				
No	**Sex**	**Initial**	**Final**	**Initial**	**Final**	**Dose**	**Initial wt (gms)**	**Final wt (gms)**
1	F	134.00	122.40	67.43	65.60	500mg/kg	215.60	202.30
2	F	103.40	220.20	50.51	48.90	500mg/kg	220.40	203.60
3	F	106.20	218.10	54.60	52.70	500mg/kg	185.60	187.10
4	F	117.50	145.60	62.80	59.40	500mg/kg	204.60	193.50
Mean		**115.28±6.95**	**176.58±25.0**	**58.84±3.84**	**56.65±3.69**			
5	M	137.00	128.70	71.79	69.80	500mg/kg	192.40	180.40
6	M	102.60	221.90	53.56	51.75	500mg/kg	181.30	178.40
7	M	110.40	218.10	56.50	53.60	500mg/kg	162.10	154.20
8	M	120.30	150.10	62.30	58.40	500mg/kg	159.30	156.80
Mean		**117.58±7.41**	**179.70±23.7**	**61.04±4.02**	**58.39±4.05**			

AST level is 1.53 times for females and 1.52 for males which corresponds to ‘mild’ category. ALT on the other hand was 0.96 times for females and 0.95 times for males suggesting there is no effect.

Table 18. *O. americanum* water extract subchronic toxicity study results biochemical test

		AST (iu)		ALT (iu)				
No	**Sex**	**Initial**	**Final**	**Initial**	**Final**	**Dose**	**Initial wt (gms)**	**Final wt (gms)**
1	F	78.20	101.10	54.25	48.90	500mg/kg	143.50	132.50
2	F	135.30	132.40	51.30	46.36	500mg/kg	140.60	120.70
3	F	86.20	101.30	46.24	42.44	500mg/kg	149.60	138.60
4	F	91.20	110.60	58.30	43.21	500mg/kg	138.90	129.00
Mean		**97.73±12.8**	**111.35±7.35**	**52.52± 2.54**	**45.23± 1.50**			
5	M	80.40	103.80	56.39	41.71	500mg/kg	122.50	110.60
6	M	138.80	154.90	56.75	43.22	500mg/kg	187.50	176.40
7	M	87.40	102.60	47.54	43.62	500mg/kg	125.20	118.60
8	M	93.40	112.80	58.10	47.50	500mg/kg	134.00	127.40
Mean		**100.00±13.2**	**118.53±12.3**	**54.70±2.41**	**44.01± 1.23**			

AST level is 1.14 times for females and 1.18 for males which corresponds to the 'mild' category. ALT on the other hand was 0.86 times for females and 0.80 times for males suggesting there was no effect.

These results therefore suggest the water extracts of *O. americanum* and *C. ambrosoides* have little to no effect on the liver and therefore relatively safe.

4.4 Discussion

Due to the use of these plants in traditional medicine, one could assume that they are completely safe. However, studies have shown that there is a risk such plants may have adverse effects despite their widespread use (Ertekin *et al.,* 2005; Koduru *et al.,* 2006). This therefore suggests the importance of determining the toxicological profile of extracts studied for their potential use in drug development.

In this study, brine shrimp toxicity was used as a screening test before other more elaborate studies could be performed. This led to the elimination of *B. micrantha* water extract which was found to be toxic at the same time allowing for the selection of least toxic extracts of *O. americanum* and *C. ambrosoides*.

C. ambrosoides water extract was observed to be a mild dermal irritant on the abraded skin at 24 h resolving by 48 h and remaining normal thereafter. There was also mild irritation observed on the iris accompanied with some redness. No sign of skin sensitization was however observed. *O. ambrosoides* water extract on the other hand was not found to be neither a dermal irritant nor an eye irritant and there were no observable changes for skin sensitization. These results suggest that both these extracts are relatively safe when exposed to eyes or skin with *O. americanum* water extract having a slight edge.

Having ruled out adverse reaction on topical application, it was important to study the effect on liver injury using aminotrasferases AST and ALT as indicators of hepatocellular necrosis (Thapa and Walia, 2007). Both extracts had mild effect on the liver evidenced by the mild elevation of AST for both sexes and no elevation for ALT. This suggests the extracts have minimal to no effect on internal organs and the effect is not sex dependent. In conclusion, *C. ambrosoides* and *O. americanum* water extracts were found to have a desirable toxicological profile suggesting they can be used for development of new antischistosomal drug candidates that will be relatively safe to use.

CHAPTER 5

5 Phytochemical analysis of the plant extracts

5.1 Introduction

Natural products have been in use for therapeutic purposes for a long time (De Pasquale, 1984). However, the interest of using them as sources of NCEs by pharmaceutical companies was low in favour of combination chemistry (Newmann and Cragg, 2007). This is however now changing due to combination chemistry not being as productive as initially thought (Paterson and Anderson, 2005, Chin *et al.,* 2006) and advancement of separation as well as structural elucidation technology (Bilia *et al.,* 2002). It is therefore reported that natural products have become important sources of NCEs (Newman *et al*., 2003; Chin *et al.,* 2006; Newmann and Cragg, 2007). More specifically, plants sources contributed 25% of drugs prescribed worldwide and 11% of drugs considered by World Health Organisation (WHO) as essential (Rates, 2001).

Studies aimed at phytochemical analysis of *Ocimum* sp and *C. ambrosoides* have been undertaken confirming the plants are rich in diverse phytochemicals (Vieira and Simon, 2000; Javanmardi *et al.,* 2002; Javanmardi *et al.,* 2003; Jardim *et al.,* 2008; Monzote *et al.,* 2011; Barros *et al.,* 2013). It is also known that plant phytochemicals vary depending on soil conditions and geographical location among other variables suggesting the importance of analysing the Kenyan plants (Arya *et al.,* 2010; Martínez-Díaz *et al.,* 2015).

Since plants extracts are a mixture of several phytochemicals with different polarities. It is important to first separate these phytochemicals through chromatography techniques then analyse them for identification of pure compounds (Sasidharan *et al.,* 2011).

Routinely, there are four techniques used by organic chemists for structural elucidation. These are ultraviolet spectroscopy (UV) which has been used since 1930s, infrared spectroscopy (1940s), followed by nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS) in the following two decades (Harwood and Claridge, 1997).

In this chapter, phytochemical analysis was carried out to identify the compounds in the crude plant extracts.

5.2 Materials and methods

5.2.1 Qualitative phytochemical analysis

The extracts prepared in section 2.2.2 were exposed to preliminary phytochemical screening carried out using standard procedures as described below.

5.2.1.1 Test for Tannins

To 0.5g of extract, 10ml of ddH_2O was added, stirred and then filtered. A few drops of 1% ferric chloride were added to 2ml of filtrate. Occurrence of blue-black, green or blue-green precipitate was considered as evidence of the presence of tannins (Evans, 2002).

5.2.1.2 Borntrager's test for Anthraquinone

To 0.2g of extract, 10ml of benzene was added, shaken and filtered. 5ml of 10% ammonia solution was added to filtrate and shaken. Pink, red or violet colour in lower ammoniacal phase indicated the presence of free hydroxyl anthraquinones (Evans, 2002).

5.2.1.3 Liebermann-Burchard test for Steroids and Terpenoids

To 0.2g of extract, 2ml of acetic acid was added, the solution cooled in ice followed by addition of concentrated sulphuric acid. Violet to blue or bluish-green colour was considered as evidence of the presence of steroids or terpenoids (Nath *et al*., 1946).

5.2.1.4 Test for Saponins

To 1g of extract, 5ml of ddH_2O was added, the mixture warmed in water bath followed by vigorous shaking. Persistence of frothing was considered as a preliminary confirmation of the presence of saponin (Wall *et al*., 1952).

5.2.1.5 Test for Flavonoids

To 0.5g of extract, 3 pieces of magnesium strips were added followed by a few drops of concentrated hydrochloric acid. Reddish coloration indicated positive test for flavonoids (Sofowara, 1993).

5.2.1.6 Test for Alkaloids

A portion of extract was stirred with 5ml of 1% hydrochloric acid and filtered. To 1ml of filtrate, a few drops of Dragendorff's reagent were added. Buff coloured precipitate was considered as confirmation of alkaloids presence (Harborne, 1973).

5.2.2 Structure elucidation using spectroscopy and spectrometry techniques

Due to cost prohibition, only two best extracts that were relatively non-toxic while having antischistosomal properties were selected for the subsequent study.

5.2.2.1 Mass spectrometry (MS) analysis of the crude extracts

5.2.2.1.1 Headspace gas chromatography/mass spectrometry (GC/MS) analysis

Agilent 7697A headspace sampler coupled with Agilent 7000 GC/MS Triple Quad (Agilent technologies, USA) equipment was used in this analysis. The headspace vials were approximately half filled with the solid extracts taking care to ensure the vial caps were properly sealed with a crimper. The vials were then loaded on the tray on the headspace whose parameters were set as shown in Table 19. The gas aliquots from the headspace were directly injected in the GC/MS whose parameters were set as shown in Tables 20 and 21. Agilent MassHunter deconvolution software and NIST11.L spectral library were used to screen the chromatograms for the determination of compounds.

Table 19. Description of Agilent 7697A headspace sampler parameters

PARAMETER	DESCRIPTION
Temperature	
Oven	200 ^{o}C
Loop/Valve	110 ^{o}C
Transfer line	115 ^{o}C
Time	
GC Cycle time	7 min
Vial equilibrium time	7 min
Injection time	0.5 min
Vial	
Fill pressure	0 kPa
Loop fill mode	default
Vial size	20 ml
Shaking	18 shakes/min

Table 20. Gas chromatography parameters

PARAMETER	DESCRIPTION
AUXILIARY HEATERS	
Thermal auxiliary 1 (G3520 Transfer line)	115ºC
Thermal auxiliary 2 (MSD Transfer line)	280ºC
COLUMNS	
Flow	1.2 ml/min
Pressure	63.068 kPa
Average velocity	39.723 cm/sec
Holdup time	1.2587 min
Post run	1.2 ml/min
INLETS	
Heater	250ºC
Pressure	63.068 kPa
Total flow	244.2 ml/min
Septum purge flow	3 ml/min
Split ratio	200
Split flow	240 ml/min

Table 21. Gas chromatography column program

	Rate ºC/min	Value ºC	Hold time (min)	Run time (min)
Initial		40	1	1
Ramp 1	10	200	1	18
Ramp 2	5	280	9	43

5.3 Results

5.3.1 Phytochemical screening of plants extracts

The results of crude plant extracts analysis for the presence of six bioactive chemical groups are summarized in Table 22. Briefly, *B.micrantha* water extract was observed to have high quantities of steroids and/or other terpenoids as well as Saponins. Flavonoid, Alkaloids and Tannins were found in moderate quantities and Anthroquinone was not detected.

C. ambrosoides methanol extract had high quantities of Tannins, Anthroquinones, Flavonoids and Saponins. Steroids and/or other terpenoids were found in low quantities while it was negative for Alkaloids. The water extract of the same plant was found to have abundant Saponins, moderate quantities of Alkaloids and Steroids and/or other terpenoids. Tannins were present in low quantities while it was negative for Anthraquinone and Flavonoids.

O. americanum water extract was observed to have high quantities of Tannins, moderate Alkaloids and low quantities of Saponins. Tests for presence of Anthroquinone and Flavonoids were negative although one anthraquinone molecule was identified in subsequent MS analysis. The hexane extract of the same plant had high quantities of Steroids and/or terpenoids while it was negative for the other five chemical groups.

Table 22. Phytochemicals screening of five plant extracts from three plants

Extract	Tannin	Anthroquinone	Flavonoids	Steroids/ Terpenoids	Saponin	Alkaloids
B. micrantha(water)	++	-	++	+++	+++	++
C. ambrosoides(methanol)	+++	+++	+++	+	+++	-
C. ambrosoides(water)	+	-	-	++	+++	++
O. americanum(water)	+++	-	-	-	+	++
O. americanum(hexane)	-	-	-	+++	-	-

Key:
+++ High concentration
++ Medium concentration
+ Low concentration
- Negative

5.3.1.1 Mass spectrometry results of *C. ambrosoides* water extract

The total ion concentration (TIC) chromatogram shown in Figure 9 below was screened with Agilent's masshunter deconvolution software combined with NIST11.L library leading to identification of 43 unique molecules. These are represented in Table 23.

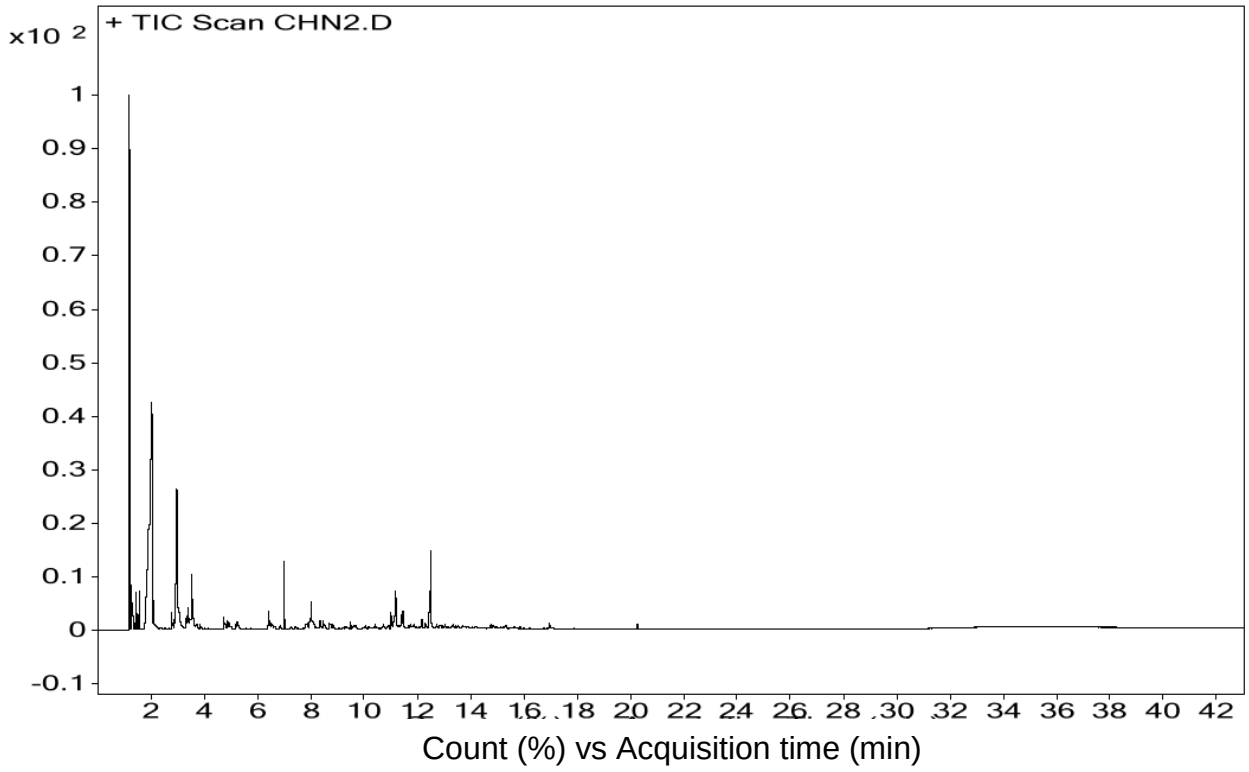
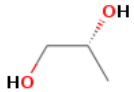
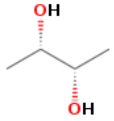
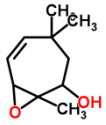
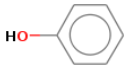


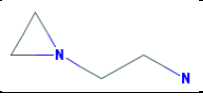
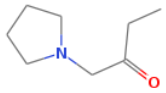
Figure 9. Total ion current (TIC) chromatogram of *C. ambrosoides* water extract

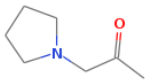
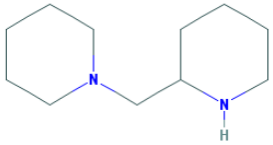
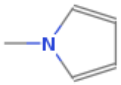
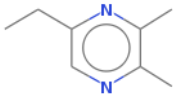
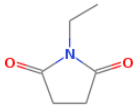
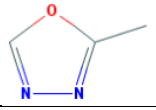
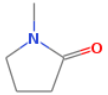
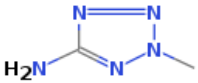
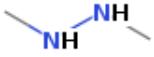
Table 23. Head space GCMS results for *C. ambrosoides* water extract

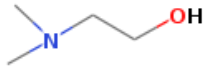
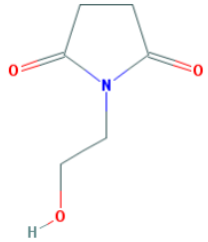
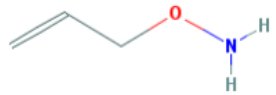
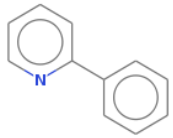
Alcohols

Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
(S)-(+)-1,2-Propanediol		C3H8O2	76.10	76.60	45.10	2.80
2,3-Butanediol, [S-(R*,R*)]-		C4H10O2	90.10	87.49	45.10	3.40
8-Oxabicyclo[5.1.0]oct-5-en-2-ol, 1,4,4-trimethyl-		C10H16O2	168.10	72.51	43.10	12.50
Phenol		C6H6O	94.00	91.64	94.10	6.40

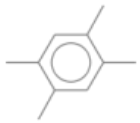
Alkaloids

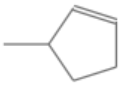
Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
(2-Aziridinylethyl)amine		C4H10N2	86.10	68.92	44.10	1.20
1-(1'-pyrrolidinyl)-2-butanone		C8H15NO	141.10	89.30	84.10	6.50

1-(1'-pyrrolidinyl)-2-propanone		C7H13NO	127.10	87.27	84.10	4.90
1-(Piperidin-2-ylmethyl)piperidine		C11H22N2	182.20	53.08	59.10	3.50
1H-Pyrrole, 1-methyl-		C5H7N	81.10	90.34	81.10	2.80
2,3-Dimethyl-5-ethylpyrazine		C8H12N2	136.10	86.58	135.10	7.80
2,5-Pyrrolidinedione, 1-ethyl-		C6H9NO2	127.10	88.53	56.10	8.70
2-Methyl[1,3,4]oxadiazole		C3H4N2O	84.00	60.36	84.10	4.40
2-Pyrrolidinone, 1-methyl-		C5H9NO	99.10	69.96	98.10	3.30
5-Amino-2-methyl-2H-tetrazole		C2H5N5	99.10	76.74	99.10	8.00
Hydrazine, 1,2-dimethyl-		C2H8N2	60.10	63.51	45.10	2.00

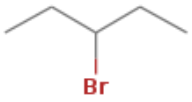
Methylamine, N,N-dimethyl-		C3H9N	59.10	84.28	58.10	1.30
N,N-Dimethylaminoethanol		C4H11NO	89.08		58.10	2.90
N-[2-Hydroxyethyl]succinimide		C6H9NO3	143.10	84.68	100.00	12.20
o-Allylhydroxylamine		C3H7NO	73.10	58.21	41.20	1.50
Pyridine, 2-phenyl-		C11H9N	155.10	64.99	155.00	13.30

Alkenes

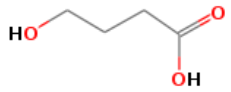
Chemical Name	**Chemical structure**	**Molecular formula**	**Molecular weight**	**Score %**	**M/Z**	**Retention time**
Benzene, 1,2,4,5-tetramethyl-		C10H14	134.10	58.24	117.10	8.80

Cyclopentene, 3-methyl-		C6H10	82.10	84.46	67.10	1.90

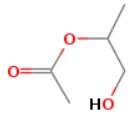
Alkyl halides

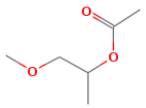
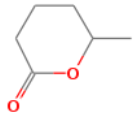
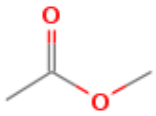
Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
Pentane, 3-bromo-		C5H11Br	150.00	58.44	71.10	7.20

Carboxylic acid

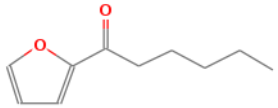
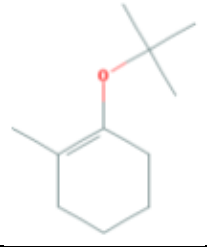
Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
Butanoic acid, 4-hydroxy-		C4H8O3	104.00	89.75	42.10	5.20

Esters

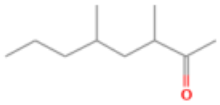
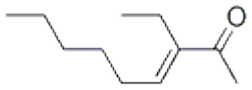
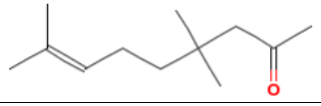
Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
1,2-Propanediol, 2-acetate		C5H10O3	118.10	72.33	43.10	4.90

1-Methoxy-2-propyl acetate		C6H12O3	132.10	64.68	43.10	5.60
2H-Pyran-2-one, tetrahydro-6-methyl-		C6H10O2	114.10	90.63	42.10	8.10
Acetic acid, methyl ester		C3H6O2	74.00	88.03	43.10	1.50

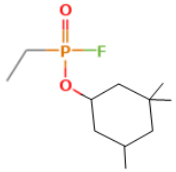
Ethers

Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
2-Hexanoylfuran		C10H14O2	166.10	70.54	95.10	11.20
Allyl ethyl ether		C5H10O	86.10	75.83	57.10	2.10
Cyclohexene, 1-(1,1-dimethylethoxy)-2-methyl-		C11H20O	168.20	67.88	112.10	11.20

Ketones

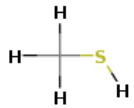
Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
3,5-Dimethyl-2-octanone		C10H20O	156.20	69.23	72.10	8.30
3-Nonen-2-one, 3-ethyl-		C11H20O	168.20	65.36	43.10	12.30
4,4,8-Trimethyl-non-7-en-2-one		C12H22O	182.20	68.68	124.10	17.00

Organophosphorous

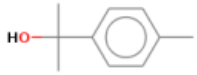
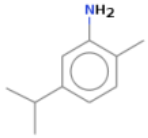
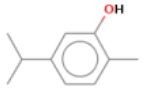
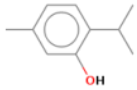
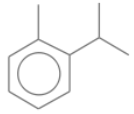
Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
3,5,5-Trimethylcyclohexyl ethylphosphonofluoridate		C11H22FO2P	236.10	64.31	113.10	8.00

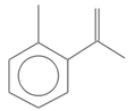
Organosulphide and sulphur

Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time

Methanethiol		CH4S	48.00	75.36	47.10	1.30
Cyclic octaatomic sulfur		S8	255.80	81.49	64.00	20.30

Terpenes and Terpenoids

Chemical Name	**Chemical structure**	**Molecular formula**	**Molecular weight**	**Score %**	**M/Z**	**Retention time**
Benzenemethanol, .alpha.,.alpha.,4-trimethyl-		C10H14O	150.10	81.63	43.10	9.50
o-Toluidine, 5-isopropyl-		C10H15N	149.10	78.47	134.10	11.50
Phenol, 2-methyl-5-(1-methylethyl)-		C10H14O	150.10	87.20	135.10	11.10
Thymol		C10H14O	150.10	88.30	135.10	11.00
o-Cymene		C10H14	134.10	91.32	119.10	7.00

o-Isopropenyltoluene		C10H12	132.10	90.95	132.10	8.00

5.3.1.2 Mass spectrometry results of *O. americanum* water extract

The TIC chromatogram shown in Figure 10 below was analyzed as described in section 5.3.2.3 above leading to the identification of 49 molecules that are listed in Table 24.

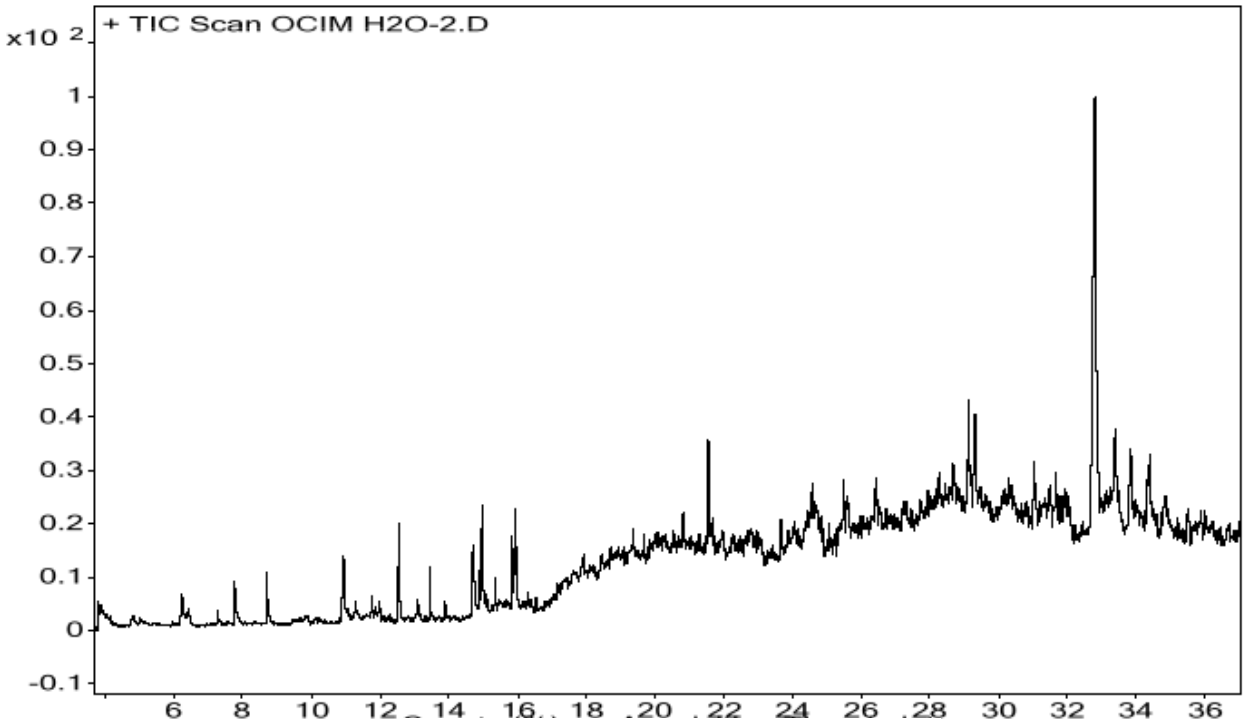
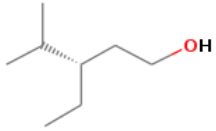
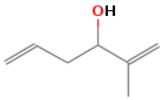
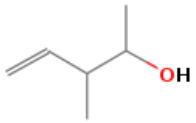


Figure 10. Total ion current (TIC) chromatogram of *O. americanum* water extract

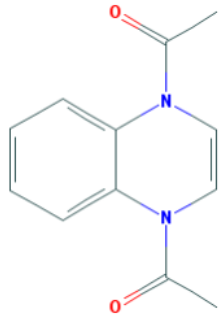
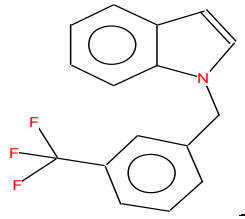
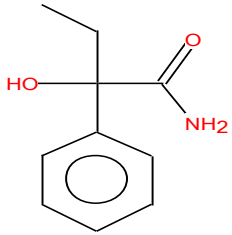
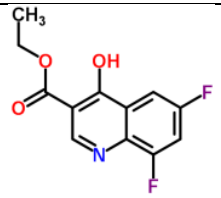
Table 24. Head space GCMS results for *O. americanum* water extract

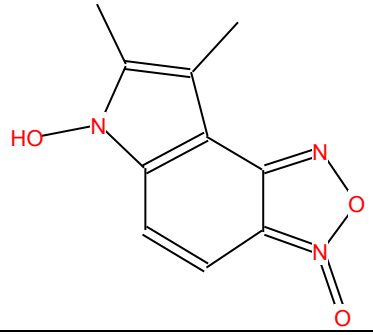
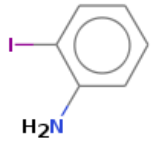
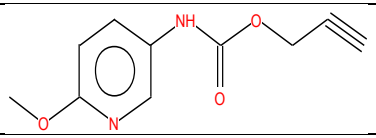
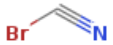
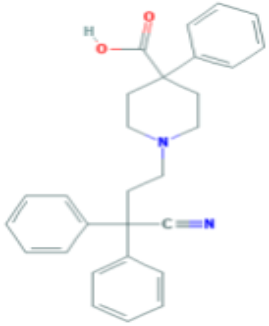
Alcohols

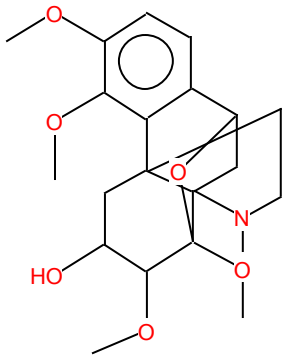
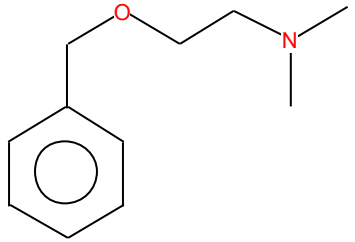
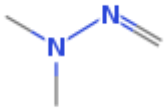
Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
(S)-3-Ethyl-4-methylpentanol		C8H18O	130.10	82.51	55.10	6.20
2-Methyl-1,5-hexadiene-3-ol		C7H12O	112.09	3.30	97.10	32.70
4-Penten-2-ol, 3-methyl-		C6H12O	100.09	14.80	57.20	31.80

Alkaloids

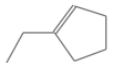
Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time

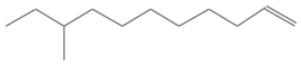
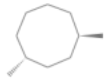
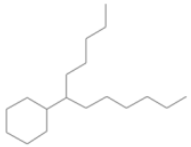
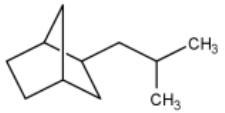
1,4-Diacetyl-1,4-dihydroquinoxaline		C12H12N2O2	216.09	42.80	207.00	34.90
1H-Indole, 1-[[3-(trifluoromethyl)phenyl]methyl]-		C16H12F3N	275.09	52.00	95.10	29.30
2-Hydroxy-2-phenylbutyramide		C10H13NO2	179.09	20.60	135.10	33.30
3-Quinolinecarboxylic acid, 6,8-difluoro-4-hydroxy-, ethyl ester		C12H9F2NO3	253.06	61.70	207.00	31.90

6-Hydroxy-7,8-dimethyl[1,2,5]oxadiazole[3,4-e]indol-3-oxide		C10H9N3O3	219.06	17.60	219.20	24.00
Benzenamine, 2-iodo-		C6H6IN	218.95	12.30	219.20	24.00
Carbamic acid, N-(6-methoxy-3-pyridyl)-, 2-propynyl ester		C10H10N2O3	206.07	1.00	207.00	35.70
Cyanogen bromide		CBrN	104.92	47.50	253.00	33.40
Difenoxin		C28H28N2O2	424.22	34.30	218.20	33.40

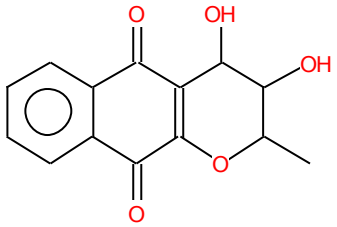
Dihydroepistephamiersine		C21H29NO6	391.20	23.70	381.30	32.80
Ethanamine, N,N-dimethyl-2-(phenylmethoxy)-		C11H17NO	179.13	74.30	73.00	34.80
Formaldehyde, dimethylhydrazone		C3H8N2	72.07	7.20	73.00	20.00

Alkanes and alkenes

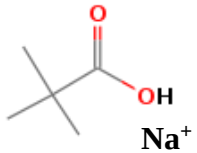
Chemical Name	**Chemical structure**	**Molecular formula**	**Molecular weight**	**Score %**	**M/Z**	**Retention time**
1-Ethylcyclopentene		C7H12	96.10	57.31	67.10	14.90

1-Undecene, 9-methyl-		C12H24	168.20	78.19	55.10	8.70
Cyclooctane, 1,4-dimethyl-, trans-		C10H20	140.20	75.24	83.00	14.70
Decane, 1-iodo-		C10H21I	268.10	56.69	57.20	20.80
Dodecane, 6-cyclohexyl-		C18H36	252.30	59.85	83.00	29.10
Norbornane, 2-isobutyl-		C11H20	152.20	68.18	95.10	15.90

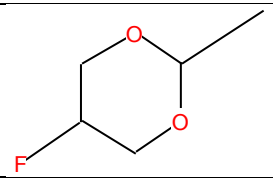
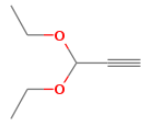
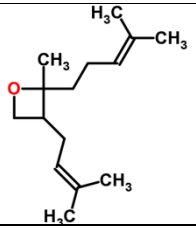
Anthraquinone

Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
2H-Naphtho[2,3-b]furan-5,10-dione, 3,4-dihydro-3,4-dihydroxy-2-methyl-, [2S-(2,3,4)]-		C14H12O5	260.07	57.40	203.10	33.40

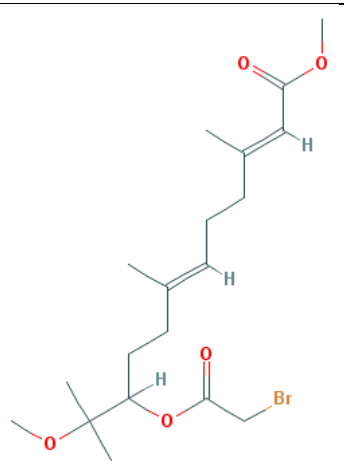
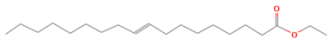
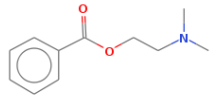
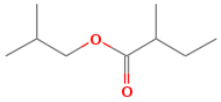
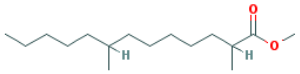
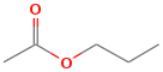
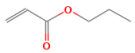
Carboxylic acid

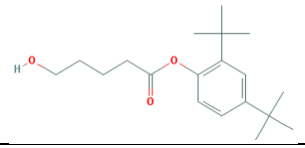
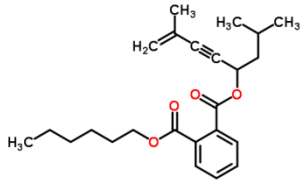
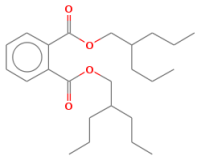
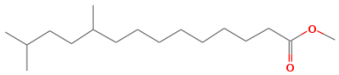
Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
Propanoic acid, 2,2-dimethyl-, sodium salt		C5H9NaO2	124.10	52.19	57.20	31.80

Ethers and epoxides

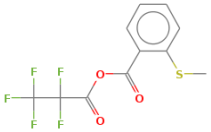
Chemical Name	Chemical structure	Molecular formula	Molecular weight	Score %	M/Z	Retention time
1,3-Dioxane, 5-fluoro-2-methyl-, cis-		C5H9FO2	120.06	2.03	119.10	34.40
3,3-Diethoxy-1-propyne		C7H12O2	128.10	67.39	55.10	3.80
2-Methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane		C15H26O	222.20	43.00	69.10	25.50

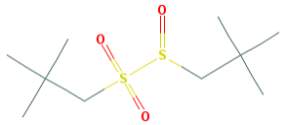
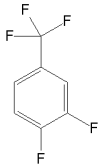
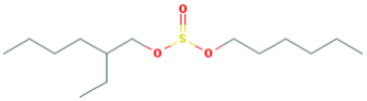
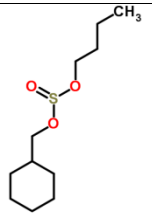
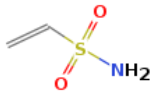
Esters

2,6-Dodecadienoic acid, 10-(bromoacetoxy)-11-methoxy-3,7,11-trimethyl-, methyl ester		C19H31BrO5	418.14	66.90	73.00	32.00
9-Octadecenoic acid, ethyl ester		C20H38O2	310.29	20.20	88.10	15.90
Benzoic acid, 2-(dimethylamino)ethyl ester		C11H15NO2	193.11	1.10	149.00	13.10
Butanoic acid, 2-methyl-, 1,2-dimethylpropyl ester		C10H20O2	172.10	51.46	57.20	23.70
Methyl 2,8-dimethyltridecanoate		C16H32O2	256.20	51.53	88.10	13.50
n-Propyl acetate		C5H10O2	102.07	76.00	74.10	15.30
n-Propyl acrylate		C6H10O2	114.10	59.27	55.10	19.30

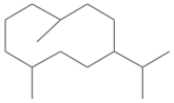
Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters		C19H30O3	306.20	75.04	191.00	7.80
Phthalic acid, 2,7-dimethyloct-7-en-5-yn-4-yl isohexyl ester		C24H32O4	384.23	45.00	149.00	25.40
Phthalic acid, cyclobutyl tridecyl ester		C25H38O4	402.30	59.72	149.00	11.90
Phthalic acid, di(2-propylpentyl) ester		C24H38O4	390.30	84.54	149.00	21.60
Tetradecanoic acid, 10,13-dimethyl-, methyl ester		C17H34O2	270.30	61.40	74.10	12.60

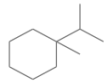
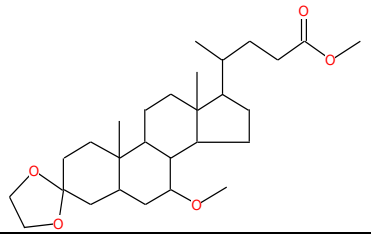
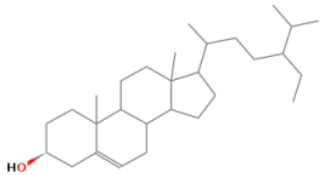
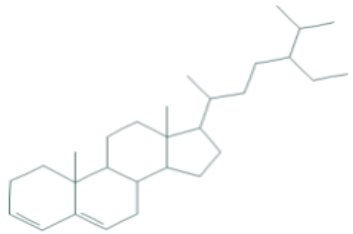
Organohalides/Organosulphides

Chemical Name	**Chemical structure**	**Molecular formula**	**Molecular weight**	**Score %**	**M/Z**	**Retention time**
2-(Methylthio)benzoic pentafluoropropionic anhydride		C11H7F5O3S	314.00	1.00	316.20	24.70

2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone		C10H22O3S2	254.10	52.60	57.20	29.10
Benzene, 1,2-difluoro-4-(trifluoromethyl)		C7H3F5	182.02	1.28	183.20	30.30
Sulfurous acid, 2-ethylhexyl hexyl ester		C14H30O3S	278.20	61.71	57.20	19.40
Sulfurous acid, butyl cyclohexylmethyl ester		C11H22O3S	234.10	56.06	55.10	15.00
Vinylsulfonamide		C2H5NO2S	107.00	10.20	91.10	33.90

Steroids and terpenoids

Chemical Name	**Chemical structure**	**Molecular formula**	**Molecular weight**	**Score %**	**M/Z**	**Retention time**
1,7-Dimethyl-4-(1-methylethyl)cyclodecane		C15H30	210.20	70.26	71.20	10.90

Cyclohexane, 1-isopropyl-1-methyl-		C10H20	140.20	59.85	55.10	15.90
Methyl 7-methoxy-3,3-ethylenedioxy-5-cholanoate		C28H46O5	462.33	57.30	79.00	32.80
Sitosterol		C29H50O	414.39	22.50	43.20	32.80
Stigmastan-3,5-diene		C29H48	396.38	7.83	107.00	32.80

5.4 Discussion

Phytochemicals that were identified for *C. ambrosoides* were alcohols, alkaloids, alkenes, alkyl halide, carboxylic acid, esters, ethers, ketones, organophosphorous, terpenoids, cyclic octaatomic sulfur and organosulphides all numbering 43 identified compounds. *O. americanum* compounds on the other hand were alcohols, alkaloids, alkenes, anthraquinone, carboxylic acid, esters, ethers, organohalides, organosulphides, steroids and terpenoids numbering 49 compounds. While this is an impressive number of phytochemical compounds, tannins, flavanoids and saponins were not identified in mass spectrometry analysis. These molecules have a high molecular weight volatile thereby making HPLC a better technique for identification of compounds (Hagerman *et al*., 1998; Oleszek and Bialy, 2006; Magiera and Zareba, 2015)

5.4.1 Tannins

These are polyphenolic compounds with molecular weight ranging from 500 to 3000 (Bate-Smith and Swain, 1962). Although they are antinutrient, they have medicinal importance such as controlling irritating bowel disorders, irritation in the small intestine, as an agent to stop bleeding, protecting exposed tissue from infection, antitumour, antiviral, antibacterial and antiparasitic (Ashok and Upadhyaya, 2012). Condensed tannins have been shown to have wormicidal activity and therefore inference can be made for this study since qualitative results showed some of these plants contained tannins (Williams et al., 2014).

5.4.2 Flavonoids

Flavonoids were detected for *O. americanum* but negative for *C. ambrosoides* water extracts. These are the major plant polyphenols whose basic structure is 2-phenylbenzopyrane (Asif and Khodadadi, 2013). They have a wide variety of medical activity such as anti-HIV, antimicrobial, cardioprotection and potentially as neuroprotective agents owing to their properties as antioxidant, anti-inflamatory and metal chelating molecules (Asif and Khodadadi, 2013). Flavonoids have also been shown to have wormicidal activity suggesting *O. americanum* molecules could have such activity (Akter *et al*., 2014).

5.4.3 Steroids, Terpenes and Terpenoids

Five molecules were identified for *O. americanum* water extract in this section with three being steroids. No steroids were however detected for *C. ambrosoides* extract. Thymol which is one of the identified compounds is used as antiseptic, antihelminthic and ectoparasiticide (https://www.drugs.com/international/thymol.html). The reactive site is the hydroxyl group which is hydrophilic and can bind on microbial cell membrane thereby changing its properties (Ultee, Bennik and Moezelaar, 2002; Kordali *et al.*, 2008; Ahmad *et al.*, 2011). Inference can be made for the other identified molecules containing hydroxyl groups, carbonyl or amide which are potential reactive sites with those molecules without such as o-cymene being only weakly active (Kordali *et al.*, 2008).

5.4.4 Alkaloids

Seventeen alkaloids were identified for *C. ambrosoides* and 12 for *O. americanum* extracts. The binding of alkaloids with DNA has been studied for their importance in cancer chemotherapy (Beljanski and Beljanski, 1986; Zhou and Giannakakou, 2005; Nafisi *et al.,* 2010). This binding quality has also been shown to work as antiplasmodial, antiprotozoal and anti-parasitic nematodes (Kohler and Bachmann, 1981; Chataing *et al.,* 1998; Fennel *et al.,* 2003).

5.4.5 Saponins

While saponins were not successfully identified in this study, this class of phytochemicals has some known medical importance. For example studies have shown saponins to having adjuvant activity suggesting their importance in vaccine development (Wu *et al.,* 1992; Oda *et al.,* 2000; Song and Hu, 2009; Sun *et al*., 2009). Other medical relevance of saponins include as anticancer, antiviral, antimicrobial, antihelminthic and antimolluscidal activity against the *Schistosoma* vector *Biomphalaria glabrata*. Saponin detection in the initial qualitative study suggests there is a likelihood some have antischistosomal activity (Borel *et al*., 1987; Apers *et al.,* 2001; Avato *et al.,* 2006; Oyekunle *et al*., 2006; Wang and Yuan, 2008; Wang *et al.,* 2010; Man *et al.*, 2010; Ali *et al.*, 2011).

5.4.6 Other identified compounds

Other volatile phytochemicals identified from *O. americanum* water extract were alcohols, alkanes, anthraquinone, carboxylic acids, esters, ethers, organosulphides and organohalides. *C.*

ambrosoides water extract had alcohols, alkenes, organosulphides, cyclic octaatomic sulfur, organohalides, carboxylic acid, esters, ethers, ketones and organophosphorous.

Anthraquinone has antibiotic activity and is used in medicine as a laxative, (Nelemans, 1976; Konishi et al., 1989; Agarwal et al., 2000). These phytochemicals are important as natural antioxidants which typically has medical importance as antimicrobial, anticancer and antihelminthic among others (Candan *et al.,* 2003; Dorman and Deans, 2000; Gupta and Sharma, 2006; Korkina, 2007; Macedo *et al.,* 2010; Brewer, 2011; Özkan and Erdoğan, 2011)

Given the diversity of the phytochemicals in these two plants, more studies will be required to identify which among the identified molecules and the others that were not successfully identified have antischistosomal activity.

CHAPTER 6

6.1 General discussion

6.1.1 Comparison of this study relative to the investigational course in drug development

According to Katiyar (2012), the investigational course should take the following steps;

- Biological activity screening
- Bioassay guided fractionation of the relevant plant extracts
- Separation and structures determination
- Evaluation of druggability, chemical doability and whether patenting is possible
- Go/no go decisions based on toxicology and activity

Bridelia micrantha is an important plant in traditional medicine and has also been shown to have activity as antimicrobial and anticonvulsant (Abo and Ashidi, 1999; Bum *et al*., 2012). It is also reportedly used as one of the antidiebetic plants in traditional medicine (Abo *et al*., 2008; Gbolade, 2009). Its extracts have been successfully analysed identifying some of the compounds (Pegel and Rogers, 1968; Green *et al*., 2011). *Ocimum americanum* is also a medicinal plant known for its antimicrobial activity and studies have also shown it to also have larvicidal activity against *Aedes aegypti* (Cavalcanti *et al*., 2004; Thaweboon and Thaweboon, 2009). Significant work has been undertaken to analyse *Ocimum* species extracts confirming presence of phytochemicals with potential medical significance (Martins *et al*., 1999; Veira and Simon, 2000, 2006). The third plant in this study was *Chenopodium ambrosoides* which also has medical

use as well as being rich in phytochemical compounds (Kiuchi *et al*., 2002; Jardim *et al*., 2008; Kumar *et al*., 2007).

The broad spectrum activity of these three plants as well as their antihelminthic application from enthnobotanical sources were some of the appealing qualities considered for study of their activity against *Schistosoma mansoni* (Kliks, 1985; Mali and Mehta, 2008; Jardim *et al*., 2008). Studies by Moilo *et al* (2014) and Waiganjo *et al* (2014) reported their antischistosomal activity as well as the effective extracts. This study validated that indeed the relevant crude extracts have antischistosomal activity although similarly high efficacy rates were not reproduced

A number of bioassays were performed. Using ELISA, it was observed that there were similar IgG responses both for the extracts and praziquantel despite differences in worm recovery numbers. This as well as the observation of the cytokine profiles analysed using flow cytometry confirmed for the first time that the extracts have immunomodulatory activity which is also corroborated by the observation of resolving granulomas. Immunomodulatory quality has also been reported for some other plant extracts (Luo, 1993; Amirghofra *et al*., 2000; Ganju *et al*., 2003). This suggests that seroprotection comparable to praziquantel could be induced if phytochemical compounds in the plant extracts of this study are explored as potential antischistosomal drug candidates.

Toxicity analysis of the extracts was performed for the first time in this study. Brine shrimp bioassay led to the determination that *Bridellia micrantha* water extract was relatively toxic leading to its elimination in the subsequent tests. Acute dermal irritation, acute eye irritation, skin sensitization and subchronic toxicity studies all returned favourable results for *O.*

americanum and *C. ambrosoides* water extracts with the former being least toxic. This therefore suggests that care should be exercised when using *B. micrantha* as traditional medicine. However since it showed antischistosomal activity, there may be some merit in studying the plant extracts further for the isolation of the molecules followed by analysis to determine the ones with antischistosomal activity.

While the heavy molecules such as saponins, tannins and flavonoids were not successfully identified, this study presents one of the more exhaustive lists of identified phytochemical molecules for the two plants extracts. Further work will need to be undertaken to identify the molecules which will have antischistosomal activity as well as their druggability. It will also be important to analyse the extracts using LCMS for the identification of the heavier molecules.

This study has made significant contribution in the first three points in the investigational course as proposed by Katiyar (2012).

The question of patentability continues to raise ethical and moral issues. In many cases, the countries which have relevant research and development capability combined with the necessary funding tend not to have the plant resources. The opposite is also true where countries in Africa for example have a rich biodiversity but tend not to have the capacity to develop such resources to marketable products (Gibson, 2008). This raises human rights concerns since the local community has a right to access locally available plants for medicine, food and so on. The *Hoodia* cactus for instance is in the midst of a biopiracy row. It is the source of P57 which is an appetite suppressing drug whose rights are owned by Pfizer, a U.S. pharmaceutical giant. On the other hand, the Kung people of Southern Africa, whose knowledge was used as a lead, have had

no compensation (Willcox *et al*., 2004). This therefore suggests that should this study ultimately lead to new pharmaceutical products, all the stakeholders should be fairly compensated.

Ultimately, a decision will need to be made on whether the plant extracts should progress to the subsequent stages in drug development. This study provides evidence of the antischistosomal activity and the relative safety of the extracts which contributes towards the decision.

The extracts can also in parallel be used in traditional medicine as crude extracts. Herbal medicine is increasingly becoming popular as many people are electing to pursue natural medicine as alternatives to conventional remedies (Dubey *et al*., 2004). Safety however remains a concern with cases of adulteration and contamination with heavy metals as well as adverse reaction having been reported (Drew and Myers, 1997; Bent, 2008).

6.1.2 Drug development considerations

The cost of drug development is indeed immense, ranging from USD 92-883 million cash (USD 161-1.8 billion capitalized to the point of marketing approval) with from original idea to launch taking a duration of between 12 to 15 years (DiMasi *et al*., 2003; Huches *et al*., 2011; Morgan *et al*., 2011). According to USA's Food and Drug administration (FDA), the drug development process has 5 steps which are;

1. Discovery and development
2. Preclinical research
3. Clinical research
4. FDA Review

5. FDA post market safety monitoring

The first three points point to the drug development process while the last two refer to regulatory review. According to Steinmetz and Spack (2009), preclinical studies include manufacture of the active pharmaceutical ingredient, dosage determination, toxicology, determination of good manufacture practice and documentation for use in clinical trials. This study therefore leans more heavily on the earlier stage of discovery and development since the initial step of determining the active ingredient has not been undertaken. While toxicology was done providing significant contribution from a traditional medicine perspective, preclinical studies will require more elaborate tests including genetic toxicology and potentially safety pharmacology (Steinmetz and Spack, 2009). However it is appreciated that discovery and preclinical stages is continuous with only clinical trials having a clear boundary evidenced by filing of an Investigational New Drug (IND or its acronyms) application (Steinmetz and Spack, 2009).

Katiyar *et al* (2012) discusses that plants as sources of NCEs have led to phytochemicals of the following categories;

1. Phytochemical compounds which are used directly e.g digoxin (medication for heart condition first isolated from the foxglove plant Digitalis purpurea)
2. Phytochemicals which themselves may act as leads for other more potent compounds e.g the cancer medication paclitaxel which was originally obtained from Taxus brevifolia
3. A novel chemophore which can be converted to compounds that are druggable
4. Pure phytochemicals as marker compounds for the standardization of plant crude extracts

5. Phytochemicals for use as pharmacological tools
6. Botanical or herbal drugs e.g green tea extract

Use of botanical sources as a starting point in a drug development has certain advantages. These include the long use by humans as traditional medicine suggests efficacy as well as safety of the molecules and natural sources can lead to generation of novel molecules from the original to overcome inherent limitations (Katiyar *et al*., 2012). The major disadvantage is the environmental pressure that can be exerted by over exploitation of a natural resource following commercialization (Katiyar *et al*., 2012).

6.1.3 Druggability

In drug development, it is important to distinguish between biologically active small molecules and drugs (Keller *et al*., 2006). Drugs administered orally need to be both relatively water soluble and permeable to the gut (Lipinski, 2002). Lipinski developed general guidelines called the "Rule of 5" which is a quick way of determining if a molecule is druggable of not. The rules state as follows;

1. Should have no more than 5 hydrogen donors
2. Molecular weight should not be more than 500
3. Octanol/water partition coefficient log P of not greater than 5
4. Sum of Nitrogen and Oxygen atoms should not be more than 10
5. Transporters substrates and natural products molecules are exceptions

These rules are useful in determining potential bioavailability problems if more than one property is violated (Katiyar *et al*., 2012). The identified molecules will therefore need to be analyzed in terms of their chemical activity and druggability to progress from lead to hit.

6.2 Conclusion

This study was successful in addressing all the four objectives and presents two candidate plant extracts of low toxicity that can be studied further in development of new antischistosomal pharmaceutical products. Although considerations for not causing unnecessary discomfort to rodents led to exclusion of *B. micrantha* water extract in subsequent toxicological studies, it is worth noting that the extract had antischistosomal activity and interacted with the immune system suggesting it could still be studied for the identification of antischistosomal compounds. The results however suggest that although it is used in traditional medicine, care should be exercised due to its toxic properties.

The heavy molecules such as tannins, flavonoids and saponins were not successfully identified owing to unavailability of LCMS equipment that had a library and the prohibitive cost of incorporating standards in the available equipment. Cost estimates for sending the samples to a foreign service provider for analysis was also prohibitive being in the region of USD 8000 per sample.

6.3 Recommendations and future work

Cost permitting, it will be good to ship samples to relevant service providers outside the country to identify the heavy molecules. It will also be useful to undertake druggability studies for all

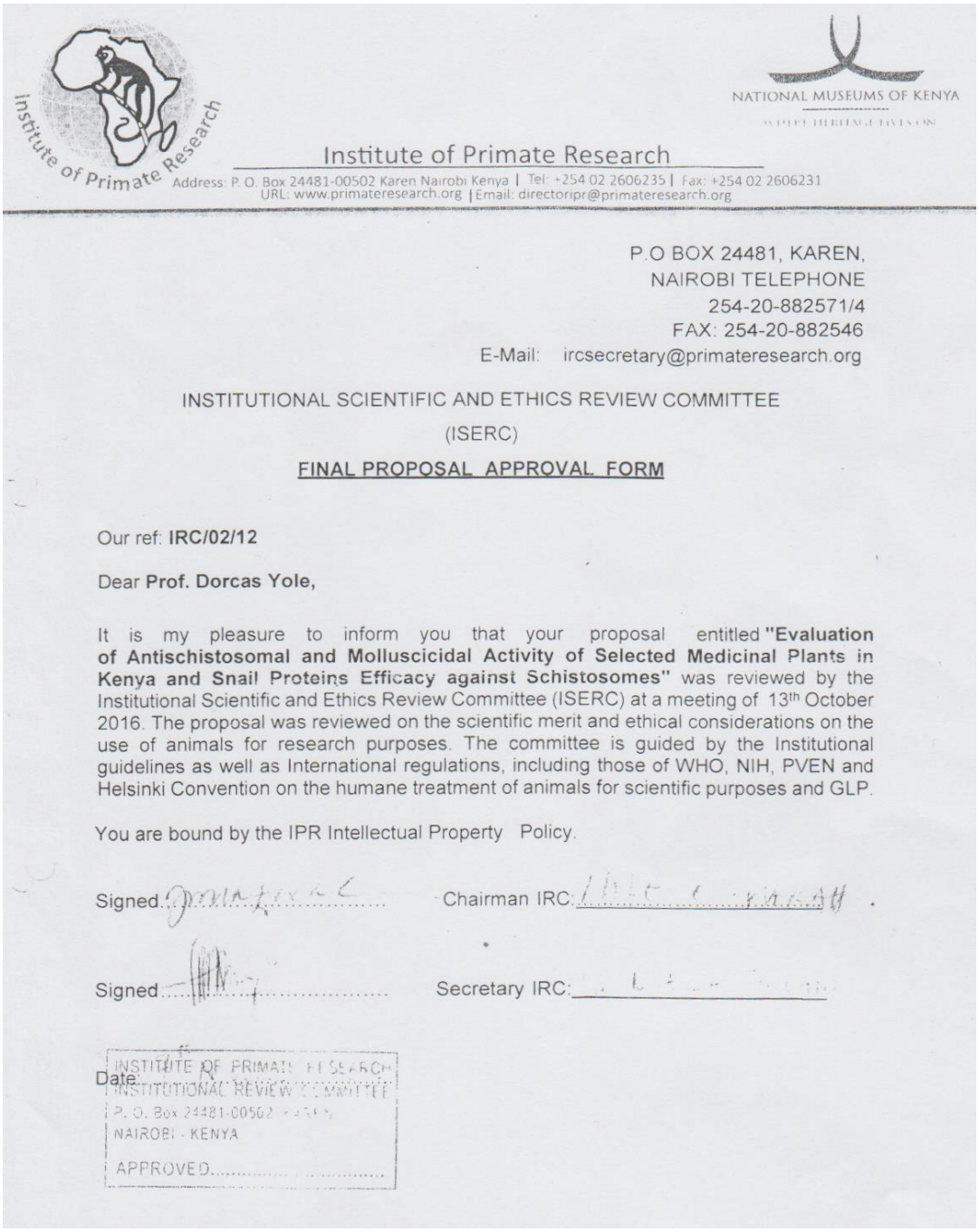
identified molecules so that the profile of the molecules interaction with druggable genes can be determined. Validation of effective dose of the crude extracts may be important. The 150 mg/kg dose suggested by Waiganjo *et al* (2014) and Moilo *et al* (2014) did not reproduce similar worm reduction results in this study. *O. americanum* and *C. ambrosoides* plant extracts can be progressed to the subsequent steps in drug development as well as be used as crude extracts as a natural remedy. Studies to identify the toxic compounds in *B. micrantha* water extracts could be undertaken since it also exhibited antischistosomal activity.

6.4 Challenges and limitations

Challenges and limitations of this study included inavailability of key facilities within the university to perform some procedures including perfusion of mice, flow cytometry and mass spectrometry. This led to collaboration with other institutions which had the necessary equipment in order to meet the research objectives. The lack of a functioning LCMS coupled with a library in the country made it impossible to identify heavy molecules in the plants extracts. With quotes to analyse the extracts out of the country being prohibitively high, a next best option of head space GCMS was opted for. Inconsistent release of funds by the donor, which at a point delayed by one full year impacted the pace and planning of research activities. To minimize this impact, activities that were not cost intensive were prioritised to try as much as possible to meet overall project completion dates.

APPENDICES

Ethical committee approval



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INSTITUTIONAL SCIENTIFIC AND ETHICS REVIEW COMMITTEE

(ISERC)

FINAL PROPOSAL APPROVAL FORM

Our ref: **IRC/02/12**

Dear **Prof. Dorcas Yole,**

It is my pleasure to inform you that your proposal entitled **"Evaluation of Antischistosomal and Molluscicidal Activity of Selected Medicinal Plants in Kenya and Snail Proteins Efficacy against Schistosomes"** was reviewed by the Institutional Scientific and Ethics Review Committee (ISERC) at a meeting of 13th October 2016. The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

You are bound by the IPR Intellectual Property Policy.

Signed..... Chairman IRC.....

Signed..... Secretary IRC.....

Date.....

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
P. O. Box 24481-00502
NAIROBI - KENYA
APPROVED.....

Publications

Print ISSN: 2319-2003 | Online ISSN: 2279-0780

IJBCP International Journal of Basic & Clinical Pharmacology

DOI: http://dx.doi.org/10.18203/2319-2003.ijbcp20164753

Original Research Article

Bioactivity and toxicity of Bridelia micrantha, Chenopodium ambrosoides and Ocimum americanum plant extracts

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Received: 08 November 2016
Accepted: 09 December 2016

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ABSTRACT

Background: *Bridelia micrantha, Chenopodium ambrosoides* and *Ocimum americanum* plant species are commonly used in traditional medicine for a number of ailments. The extracts of these plants have been shown to have anti-schistosomal activity suggesting that they could be used for the development of new chemical entities (NCEs) for the treatment of schistosomiasis. However there is limited knowledge on their toxicological profile and their use in traditional medicine may not be a satisfactory safety indication.
Methods: In this study the extracts were first screened for bioactivity using brine shrimp lethality test for the determination of LC50 followed by rodent acute toxicity and 28 day subchronic studies.
Results: *B. micrantha* water extract with a LC50 of 77µg/ml was deemed toxic while *C. ambrosoides* methanol and water extracts were moderately toxic with LC50 of 104.63µg/ml and 696.44µg/ml respectively. *O. americanum* hexane and water extracts toxicity varied from moderate to slightly toxic with LC50 of 887.59µg/ml and 2254.60µg/ml respectively. *C. ambrosoides* and *O. americanum* water extracts which were preferentially selected for subsequent studies were found to have mild to no irritation to rodent eyes and skin. Moreover, the aminotransferases AST and ALT which were used to detect liver injury suggested negligible effect.
Conclusions: This therefore confirms that *C. ambrosoides* and *O. americanum* water extracts are safe for clinical use with *O. americanum* water extract having a slight edge.

Keywords: Antihelminthic, Schistosomiasis, Toxicity

INTRODUCTION

Schistosomiasis is among the most common debilitating human disease caused by several species of the digenean blood trematode of the genus *Schistosoma*.[1] Infection is estimated at 200 million people with 120 million being asymptomatic, 20 million presenting with severe disease while 600 million are at risk of infection globally.[2] Annual mortality in Africa alone is estimated at 280,000 people and global burden is estimated at 4.5 million DALYs.[3,4]

Natural products especially of plant origin have proven to be reliable sources of therapies and new chemical entities

Journal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI: 10.21474/IJAR01/6326
DOI URL: http://dx.doi.org/10.21474/IJAR01/6326
ISSN NO. 2320-5407

RESEARCH ARTICLE

EVALUATION OF THE IN VIVO ANTI-HELMINTHIC ACTIVITY, OF BRIDELIA MICRANTHA, CHENOPODIUM AMBROSOIDES AND OCIMUM AMERICANUM EXTRACTS AGAINST SCHISTOSOMA MANSONI INFECTION IN MICE.

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Manuscript Info

Manuscript History

Received: 17 November 2017
Final Accepted: 19 December 2017
Published: January 2018

Key words:-
Schistosomiasis, Praziquantel, Histopathology, ELISA, Cytokine.

Abstract

Schistosomiasis is a chronic debilitating global disease affecting approximately 600 million people in 74 developing countries, with 800 million, mostly children at risk. Chemotherapy is the only immediate recourse to minimize the prevalence and incidence of this disease worldwide. Presently, Praziquantel is the only drug of choice for the treatment of all forms of schistosomiasis, however it shows low efficacy against schistosomula and juvenile stages. This dependence on a single drug with the likely potential for development of resistance to Praziquantel has justified the search for new alternative chemotherapies. Medicinal plants are potential candidates as sources of new drug prototypes. This study provides findings on the schistosomicidal activity of *Bridelia micrantha*, *Chenopodium ambrosoides* and *Ocimum americanum* plant extracts against *Schistosoma mansoni* infection in mice. Seven week old BALB/c mice were infected with approximately 250 cercariae and treated on the third and fourth week post infection with five crude extracts from the 3 plants for respective efficacy studies. Praziquantel treated group and infected control group served as controls. Perfusion was performed for all groups on the sixth week after infection for worm recovery. Worm recovery analysis confirmed that the three extracts have antischistosomal properties. Furthermore, pathology showed resolving granulomas and immune profiling results confirmed the extracts had immunomodulatory activity which could potentially be important in inhibiting infection.

Introduction:-

Human schistosomiasis which is caused by the trematode parasite *Schistosoma*, is included in the list of neglected diseases by the World Health Organization (WHO) and presents a significant economic as well as social impact. Chemotherapy is the only immediate recourse to minimize the prevalence and incidence of infection. Since human trials with Praziquantel were carried out in the late 1970s, the drug has gained prominence and today, it remains as the only treatment option available to 200 million people infected and 600 million at risk (Gonnert and Andrews 1977; Katz et al. 1979; Chitsulo et al. 2000; WHO, 2002; van der Werf et al. 2003; Fenwick et al. 2003; Cioli et al.

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