EVALUATING PHYTOCHEMICAL PROFILES, MOLLUSCICIDAL AND SCHISTOSOMICIDAL ACTIVITY OF AQUEOUS AND ETHANOL EXTRACTS OF *Vernonia amygdalina* AND *Harrisonia abyssinica*

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (APPLIED PARASITOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

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DECLARATION

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

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We confirm that the work reported in this thesis was carried out by the candidate under our supervision and has been submitted for examination with our approval.

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DEDICATION

To my late dad Pancras Otwani, you always wanted me to do this and much more.

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To my family, Oscar, Ezra and Terry: Deepest gratitude for all the days you had to do without a mother as I was studying.

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ABBREVIATIONS AND ACRONYMS

µg/l	Micrograms per liter
µm	Micrometers
ANOVA	Analysis of variance.
CDC	Center for Disease Control
Df	Degrees of freedom
GAHI	Global Atlas of Helminth infections
IPR	Institute of Primate Research
LD	Lethal dose
LT	Lethal time
Mg/l	Milligrams per liter
Ml	Milliliters
Mm	Millimeters
NMK	National Museums of Kenya
Ppm	Parts per million
PZQ	Praziquantel
SRG	Schistosomiasis Research Group
WHO	World Health Organization
FAO	Food and Agriculture Organization

ABSTRACT

Schistosomiasis is considered one of the neglected tropical diseases caused by blood flukes. The disease kills 200,000 people annually in Sub Saharan Africa and stunts cognitive and physical growth. In Kenya, the total population requiring preventive chemotherapy in the year 2015 was estimated at 2.5 million out of which 1.8 million were school aged children. Fresh water snails of genus *Biomphalaria* are the intermediate hosts of *S. mansoni*. Chemical molluscicides used to control snails also kill non target species and have long term detrimental effects to the environment. Chemotherapy is thus the most widely applied control method. Praziquantel is the only drug recommended for mass administration hence there is a high risk of developing resistance. The search for alternative molluscicidal and schistosomicidal herbs is inevitable. This study investigated the molluscicidal and schistosomicidal potential of *Vernonia amygdalina* and *Harrisonia abyssinica* known for their broad spectrum medicinal values. The plants were sourced from Bungoma County where they are traditionally used to treat worm infections. The root and stem bark were ripped off using a knife and air dried at room temperature then crushed and sieved to standardized particles, and extracted in ethanol and distilled water. The extracts were qualitatively screened for phytochemicals by reacting the plant extracts with standard reagents and observing color change. *V. amygdalina* was rich in saponins, glycosides and phenols while *H. abyssinica* had abundant phenols, and alkaloids. Batches of ten snails were exposed to each of the plant extracts at 50, 150 and 300 mg/l in 500 ml plastic containers. One positive and one negative control were set using niclosamide and distilled water respectively. The numbers of dead snails were counted and recorded after 24 hours. Ten miracidia and ten cercariae were exposed separately in each well of a 24 well microtitre plate to lower concentrations of 5, 15 and 30 µg/l and monitored for 60 minutes. This was followed by exposure to higher concentrations of plant extracts at 50, 150 and 300 mg/l and monitoring for another 60 minutes. The number of dead miracidia and cercaria were enumerated and recorded at 5, 10, 15, 20, 30, 45 and 60 minutes. Data on snail deaths were analyzed using ANOVA at $p \leq 0.05$ to compare the three dosages of plant extracts followed by the Dunnet test to compare with the positive control. *Harrisonia abyssinica* root water extracts had the highest molluscicidal activity, similar to the positive control, Niclosamide (50 mg/l $p = 1.00$, 150 mg/l $p = 0.095$, 300 mg/l $p = 1.00$). Finney probit analysis was used to calculate the LD_{50} for snails and LT_{50} for miracidia and cercariae. The root water extract of *H. abyssinica* was the most effective against snails with the lowest LD_{50} value of 2.437 mg/l while the stem ethanol extract of *V. amygdalina* was the most effective cercaricidal agent (LT_{50} of 6.72 minutes). The best miracicidal agent was 300 mg/l of *V. amygdalina* stem water extract (LT_{50} 57.73 minutes).*H. abyssinica* root extracts should be considered for development of molluscicides since they had the best LD_{50} value. The stem ethanol extracts of *V. amygdalina* can be considered for development of cercaricidal agents in combination with other plants proved to have cercaricidal properties since the extract was lethal at high dosages. Miracidia were relatively tolerant to extracts from the two plants hence the plants may not be good candidates for miracicidal activity as stand-alone extracts. This study provides baseline information which can be used by pharmaceutical companies, researchers and the ministry of health in their quest to develop new molluscicides and schistosomicides.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Schistosoma mansoni are blood trematodes. Trematodes are commonly referred to as flukes. Schistosomes belong to the family Schistosomatidae which includes species that are among the most dreaded parasites of humans (Schmidt and Roberts, 2013).Schistosomes cause schistosomiasis, also known as bilharzia or snail fever (WHO, 2010; CDC, 2012).

Five clinically important species cause majority of human infections (Cao *et al.*, 2010). *Schistosoma mansoni, Schistosoma japonicum, Schistosoma mekongi* and *S.intercalatum species* mature in the hepatic sinusoids and migrate to the portal vein and its tributaries, notably the interior mesenteric vein, causing intestinal schistosomiasis. *Schistosoma haematobium* infection typically involves the bladder, lower ureters, seminal vesicles and less frequently the vas deferens, prostate and female genital system. Adult worms reside in the urinary bladder plexus causing urinary schistosomiasis. The two main species of concern in Kenya are *S. mansoni* and *S. haematobium* (GAHI, 2010).

Schistosomiasis is considered a neglected tropical disease and affects more than 250 million people in tropical and sub tropical regions of the world. Sub-Saharan Africa accounts for approximately 90% of worldwide cases (WHO, 2017). Disease assessments indicate that schistosomiasis accounts for up to seventy million disability adjusted life years lost annually, considering the amount of end organ pathologies in the liver for *S. mansoni and S. japonicum* and the bladder and kidney for *S.haematobium* coupled with chronic morbidities associated with impaired child growth and development, chronic inflammation, anaemia and other nutritional deficiencies (King and Dangerfield, 2008).

There is a risk of infection in fresh water of southern and sub Saharan Africa including great lakes, rivers as well as small water bodies. Transmission also occurs in South America and some Caribbean countries (CDC, 2000). In Kenya, it is estimated that 16 million people are at risk of schistosomiasis (MOPHS, 2011).

Snails do not transmit the parasite from one host to another but are an indispensable intermediate host for the development of the parasite (Molgaard *et al.,* 1999). Each schistosome species uses a different snail species as an intermediate host hence availability of a suitable snail intermediate host determines the endemicity of a particular species of Schistosoma. Snails of the genus *Biomphalaria* including *B. pfeifferi* act as the intermediate hosts of *S. mansoni* in Africa. *B. pfeifferi* is the most important and most widely distributed (De Clerq *et al*., 2000). Reservoir hosts for *S. mansoni* such as monkeys and rodents are an important epidemiological factor of the disease in Africa and tropical America (Schmidt and Roberts, 2013).

The main drug used for treatment of all types of schistosomiasis is Praziquantel which is relatively expensive. The drug faces challenges such as not being able to kill juvenile schistosomes and risk of resistance, being the only drug suitable for mass treatment in Africa control programs (Danso-Appiah *et al.*, 2008). In the year 2015, approximately 218 million people required preventive treatment globally, Kenya accounted for 2.5 million people. However, only 66.5 million were reported to have been treated where Kenya accounted for 527,256 giving a national coverage of 20.7% (WHO, 2017).

Control of schistosomiasis has involved the use of chemical molluscicides to eliminate the snail intermediate hosts. The major challenge to use of chemicals has been their high cost

and toxicity to non target organisms (Schmidt and Roberts, 2013). The search for cheaper and environmentally friendly molluscicides and schistomicides from natural sources has increased over the years and plants are a major source of biologically active compounds which can give lead structures to develop new drugs (Megalhaes *et al*., 2010). This study investigated *H. abyssinica* and *V. amygdalina* as promising alternative sources of molluscicides and schistosomicides against *Biomphalaria* snails and *Schistosoma mansoni*.

1.2 Problem statement

Schistosomiasis is a significant helminth infection which has proved difficult to control for centuries (Mahmoud, 2001; Hotez, 2009).The main approach used for control is chemotherapy, using Praziquantel (WHO, 2016). Praziquantel does not prevent re infection, is inactive on juvenile schistosomes and has limited effect on liver forms (Utzinger *et al*., 2003) hence development of resistance is a feared possibility (Danso-Appiah *et al*., 2008).The search for new drugs to combat the intermediate host and parasites is inevitable. *Vernonia amygdalina* and *Harrisonia abyssinica* are both broad spectrum herbs locally available in forests in many parts of Kenya. *H. abyssinica* root decoction treats bacterial infections as well as exhibiting molluscicidal activities (Kokwaro, 2009; Mubo and Osiyemi, 2012) while *V. amygdalina* has been used as a remedy for worms, malaria, schistosomiasis and as a molluscicide (Ojewole and John, 2004; Asemota *et al*., 2015; Tugume *et al*., 2016; Asante *et al*., 2016). The efficacy of these plants against miracidia and cercariae has not been evaluated hence there is need to do so owing to the eco friendly nature of plants and the numerous medicinal uses they exhibit.

1.3 Study justification

Many plants are known to treat bilharzia including, *Acalypha ciliata, Asparagus buchananii, Cadaba kirkii, and Delonix elata* (Kokwaro, 2009; Ojewole and John,

2004).*Vernonia amygdalina* and *Harrisonia abyssinica* contain various phytochemicals which are responsible for their lethal or curative properties. The type and quantity of phytochemicals is influenced by other factors such as the geographical location of the plant. No data is available on the phytochemical properties of *Vernonia amygdalina* and *Harrisonia abyssinica* which grow abundantly in Bungoma County and are used by local communities to treat several ailments. This study was designed to determine the phytochemical profiles of the root and stem bark extracts of the two plants and evaluate their potential as molluscicides and schistosomicides. The study has provided baseline information on the plant extracts' potential as alternative molluscicides and schistosomicides targeting *B. pfeifferi* snails and *S. mansoni* miracidia and cercariae.

1.4 Research questions

i) What are the phytochemical profiles of aqueous and ethanol extracts of *Vernonia amygdalina* and *Harrisonia abyssinica*?

ii) What is the molluscicidal effect of aqueous and ethanol extracts of *Vernonia amygdalina* and *Harrisonia abyssinica* on *Biomphalaria pfeifferi* adult snails?

iii) What is the schistosomicidal effect of aqueous and ethanol extracts of *Vernonia amygdalina* and *Harrisonia abyssinica* on *Schistosoma mansoni* miracidia and cercariae?

1.5 Null hypotheses

i) Aqueous and ethanol extracts of *Vernonia amygdalina* and *Harrisonia abyssinica* have no molluscicidal effect on *Biomphalaria pfeifferi*.

ii) Aqueous and ethanol extracts of *Vernonia amygdalina* and *Harrisonia abyssinica* have no schistosomicidal effect on *Schistosoma mansoni* miracidia and cercariae.

1.6 Objectives of the study

1.6.1 General objective

To determine phytochemical profiles, molluscicidal and schistosomicidal effects of *Vernonia amygdalina* and *Harrisonia abyssinica* extracts.

1.6.2 Specific objectives

i) To determine the phytochemical profiles of aqueous and ethanol extracts of *V. amygdalina* and *H. abyssinica*.

ii) To determine the molluscicidal effects of aqueous and ethanol extracts of *V. amygdalina* and *H. abyssinica* as potential molluscicides against *B. pfeifferi* adult snails.

iii) To determine the schistosomicidal effects of aqueous and ethanol extracts of *V. amygdalina* and *H. abyssinica* as potential schistosomicides against *S. mansoni* miracidia and cercariae.

1.7 Significance of the study

This study evaluated the molluscicidal effects of ethanol and water extracts of *V. amygdalina* and *H. abyssinica* on *B. pfeifferi,* and the schistosomicidal effects of the two plant extracts against miracidia and cercariae of *S. mansoni.* The findings provide baseline information to pharmaceutical companies, health care providers and other stake holders on the potential for inclusion of the two plant extracts in the manufacture of molluscicides and schistosomicides. The information is useful to researchers who might want to test the compatibility of phytochemical products derived from the two plants in polyherbal therapy which involves combining various agents from different plant sources for therapeutic purposes thus producing maximum therapeutic efficacy with minimum side effects (Ebong *et al.,* 2008).

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of Schistosomiasis

Schistosomiasis is the second most prevalent disease in Africa after malaria (Sanaa, 2011). Globally, 200,000 deaths are attributed to schistosomiasis annually and about 700 million people are at risk of the disease in 76 endemic countries as their agricultural work, domestic chores and recreational activities expose them to infested water (WHO, 2016). The situation is exacerbated by low socio-economic and hygiene standards, including inadequacy of clean water supply, lack of proper medical attention, poor sanitation, ignorance and poverty, collectively known to have direct impact on disease distribution (Steitmann *et al.*, 2006).

Population movements such as migration to urban areas are introducing the disease to non endemic areas (WHO, 2017). United States is a non endemic area (CDC, 2012) but 400,000 cases are introduced each year by immigrants and travelers returning from endemic regions (Neal, 2004). Other countries like China reported outbreaks in 1984 and 1995 with over 800,000 cases, and in1998 after a flood (Xu *et al*., 2008). Brazil had an acute outbreak in the year 2003 in the metropolitan area of Belo Horizonte where 11 cases were reported (Enk *et al*., 2009). Figure2.1 shows the geographical distribution of different schistosome species.

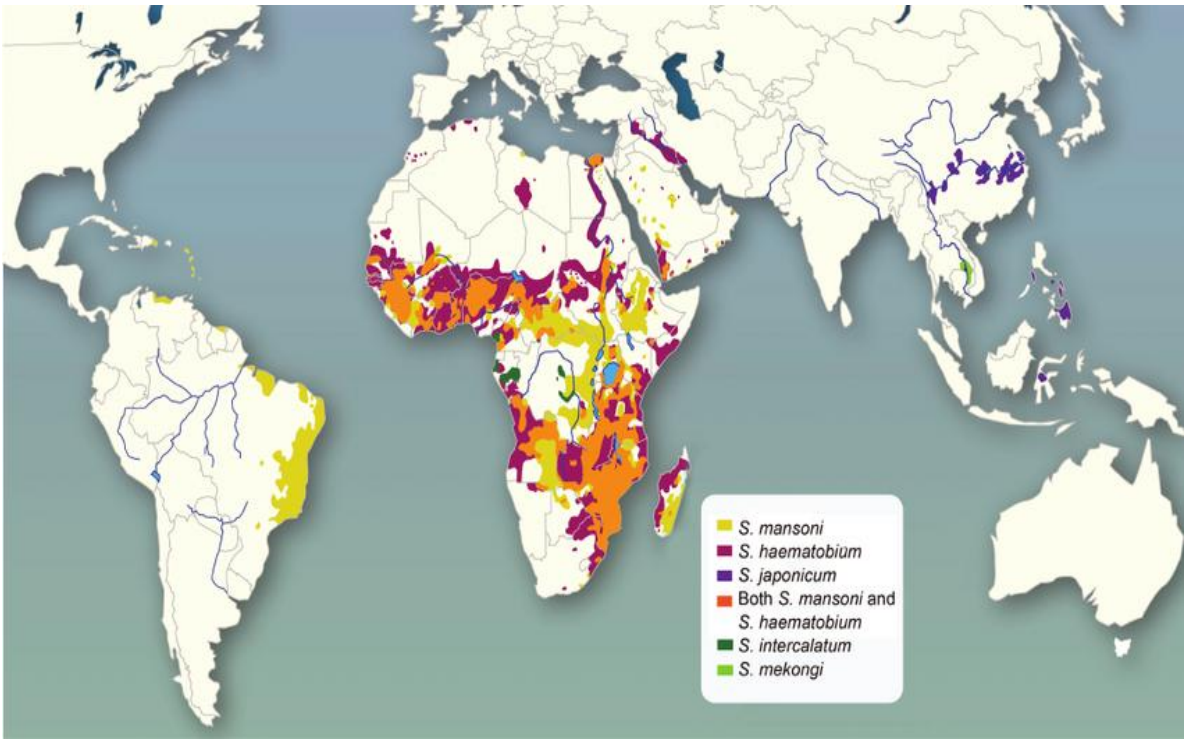


Figure 2.1 Geographical distributions of human schistosomes (GAHI, 2016)

In Kenya, schistosomiasis occurs mostly in western, Coast and selected foci in Central part of the country. Two species have been reported (GAHI, 2010). *Schistosoma mansoni* is found predominantly in Western and Central Kenyan while in the Coastal region infections are exclusively by *Schistosoma haematobium* (Brooker *et al.*, 2011). Schistosomiasis prevalence ranges from 5% to 65% indifferent communities and contributes to significant morbidity (Karanja *et al*., 1998).A study done in schools within Mbita and adjacent islands of Lake Victoria showed a mean *S. mansoni* prevalence of 60.5% (Odiere *et al.,* 2012*).*

Transmission necessitates a set of cultural, social and health habits that facilitate spread of infection (Cohen and Powderly, 2004).Human urine or faeces in water containing intermediate hosts is the most important epidemiological factor. Farmers who wade in irrigation water, fishermen in lakes and streams, children who play in dirty water and

people who bathe and wash clothes in streams are more at risk to acquire infection (WHO, 2017).

Population increase and corresponding needs for power and water result to development of schemes and environmental modification facilitating transmission (WHO, 2017). Studies done in Coast and Western Kenya indicated that together with other prevailing factors, terrestrial aquatic environments such as ponds, streams, rivers, swamps and to a less extent dams are the main in-land *S. haematobium* transmission sites (Odiere *et al*., 2011). In endemic areas, children encounter infection as early as four years and the prevalence increases with age, peaking at 15-20 years (Guyatt *et al*., 1994).

Availability of a suitable snail host determines endemicity of a particular species of *Schistosoma*. For *S. mansoni*, different species of the genus *Biomphalaria* including *B. pfeifferi*, *B. alexandrina, B. sudanica* and *B. glabrata* are the intermediate hosts and are endemic in Africa, Middle East and Asia while *Tropicorbis centrimetralis* is the intermediate host endemic in Brazil. *S. haematobium* is transmitted by species of *Bulinus, Physopsis* and *Planorbarius* while *S. japonicum* is transmitted by *Oncomelania* species only found in Asia (Schmidt and Roberts, 2013). Plate 2.1 below shows some shells of snail species that cause for schistosomiasis.

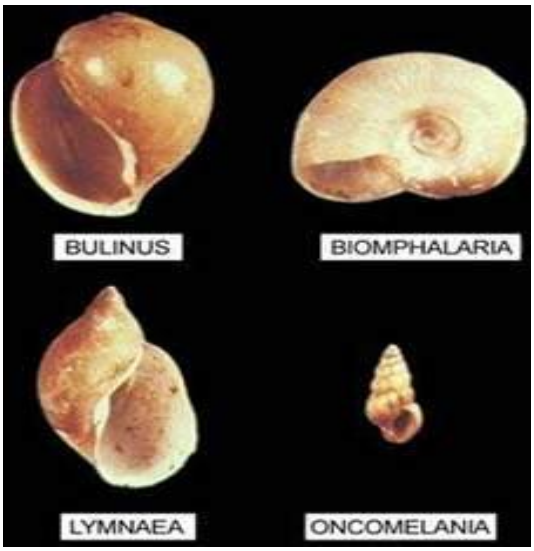


Plate 2.1: Diagram showing shells of snail genera that are intermediate hosts of schistosomiasis (Danish bilharziasis Lab, 1996)

Reservoir hosts are an important epidemiological factor in schistosomiasis. *S.haematobium* is host specific with no natural reservoirs but *S. mansoni*, has been successfully experimentally infected in more than seven orders of mammals, although monkeys and rodents are important natural reservoirs in Africa. *S. japonicum* is the least host specific and can develop in dogs, cats, horses, swine, cattle and rodents (Schmidt and Roberts, 2013).

2.2The life cycle of human Schistosomes

The life cycle of schistosomes includes an intermediate (snail) and a definitive mammalian host. The life cycle begins when the eggs shed through urine or feces from an infected human reach fresh water. These eggs hatch under optimal environmental conditions to release free-living ciliated larvae, known as a miracidia, that proceed to locate and invade a specific snail intermediate host (Walker, 2011). Developmental stages in the snail include two generations of sporocysts and production of a free living stage called cercariae (CDC, 2012). The cercariae burst out of the snail into water, where they find the parasite's

definitive host and burrow into the skin. Once in their definitive host, these parasites enter the circulation and begin to develop as either male or female parasites. The male and female worms pair up within the host's circulation, another, and then begin laying eggs (Walker, 2011; Miteva, 2012). When these eggs traverse the intestine or bladder and are released from the host, the life cycle is completed (Figure 2.2)

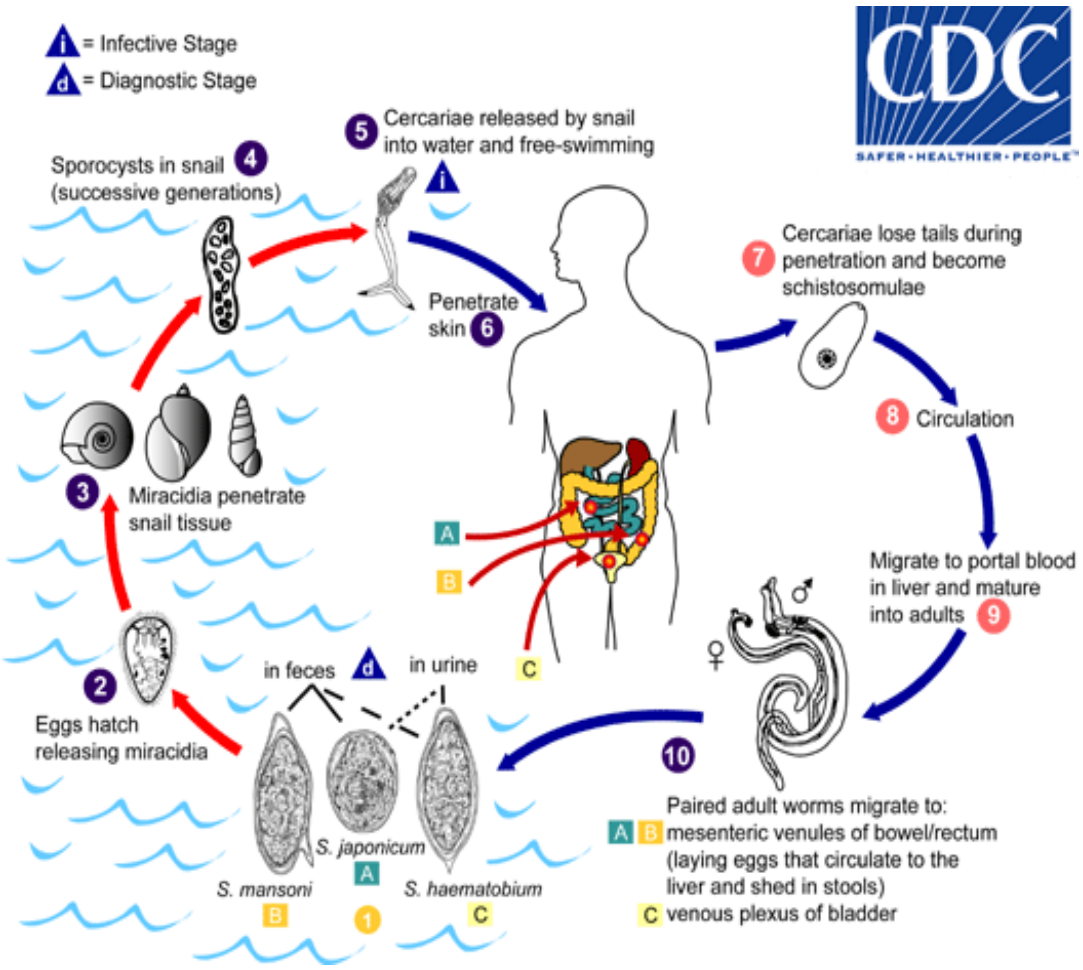


Figure 2.2: The life cycle of human schistosomes (CDC, 2012)

2.2.1 The egg of schistosomes

Infected human wastes contain the parasites ova which are non operculate, yellow or yellow- brown in colour. The eggs are fully embryonated when passed from the host. The

eggs of the five human infecting schistosomes have morphological characteristics that can be used to differentiate them (CDC, 2012). *Schistosoma mansoni* egg is about 114-180µm by 45-73µm. It is elongate with a prominent lateral spine near the posterior end (Plate 2.2). The anterior end is tapered and slightly curved. It is found in faeces and rarely in urine. Eggs are discharged at irregular intervals and may not be in every stool. The eggs are rare in chronic stages of infection.

Schistosoma japonicum egg is 68-100µm by 45-80µm. It is oval with a small lateral spine often seen as a small hook or knob located in a depression on the shell (Plate 2.2). Eggs are found in faeces and are usually covered with debris. *Schistosoma mekongi egg* is spherical with a small lateral spine that is not always visible or may appear as a small knob in a depression. It is 51-73µm by 39-66µm in size. It is found in faeces and closely resembles *S. japonicum* except its smaller and may be coated with debris (CDC, 2012)

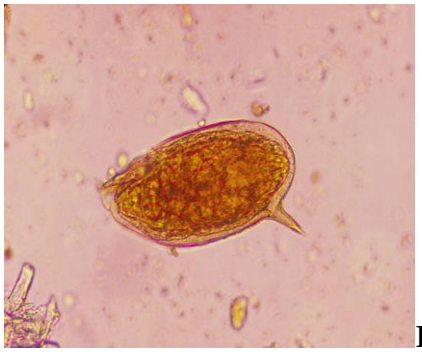
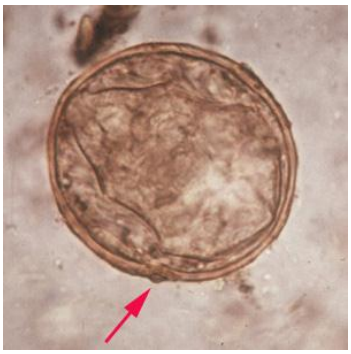
A  **B** 

Plate 2.2: Scanning electron micrograph showing A : *S. mansoni* , B: *S.japonicum* eggs (Red arrow shows a knob with the lateral spine)(CDC, 2012)

The egg of *S. haematobium* is elongated with a rounded anterior end and a sharp terminal spine at the posterior end (Plate 2.3). It measures about 110m-170µm by 50-70µm. The egg is usually found in urine and occasionally in faeces. *S. intercalatum* egg measures 140-

240µm by 50-85µm. It is elongated with tapered anterior end, Sometimes spindle shaped. The terminal spine is long, slender with a bent tip. It resembles *S. haematobium* except it is longer and thinner. It is found in faeces and may have debris adhering to the shell.

A 

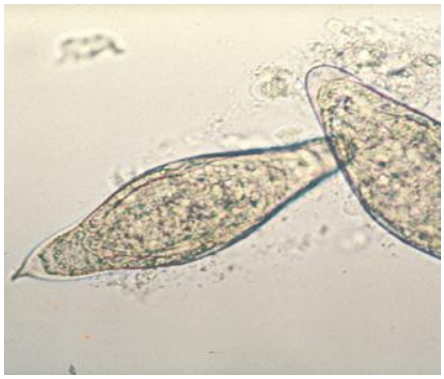
B 

Plate 2.3: Photograph showing A: *S. haematobium*, B: *S. intercalatum* eggs (Walker, 2011; CDC, 2012)

2.2.2Schistosome miracidia

The eggs hatch spontaneously to tiny ciliated organisms called miracidia on exposure to lower osmolarity of fresh water. Leucine amino peptidase enzyme released by miracidia within the egg helps digest the egg capsule from inside. The eggs cannot hatch inside the host's body because this enzyme is inhibited by sodium chloride. Miracidia are piriform with a retractable apical papilla at the anterior end bearing five pairs of duct openings (Plate 2.4). They have a prominent apical gland that secretes histolytic enzymes (Schmidt and Roberts, 2013).

The surface of miracidia is covered by flat ciliated epidermal cells used for locomotion. They are very active and can swim at a rate of 2mm per second. They must swim to find a suitable snail host rapidly since they can survive as free living organisms only for a few hours. Mucus produced by snails is a powerful attractant. Miracidia attach to the snail by their apical papillae which contract and extend in an auger like motion. It takes about thirty

minutes to penetrate and cytolysis of snail tissue can be seen as the miracidia penetrate. On penetration, miracidia undergo several changes including shedding of their epithelium and formation of a new tegument with microvilli. They then develop to a mother sporocyst (Schmidt and Roberts, 2013).

2.2.3 The sporocyst

The sporocyst has no digestive system but absorbs nutrients through the intimate contact with the host. Mother sporocysts give rise to daughter sporocysts where cercariae begin to emerge 25 to 30 days after snail infection (Schmidt and Roberts, 2013).

2.2.4 Cercaria

Cercariae are the infective stages to humans, they are sexually differentiated and non-feeding. Their energy requirements are met by stored glycogen in the tail and body. They have a mouth surrounded by an oral sucker and a bifurcated tail at the posterior end for swimming (Plate 2.4). The fully developed tegument is covered by a trilaminate plasma membrane surface coat which has a glycocalyx equipped with spines and sensory papillae (Schmidt and Roberts, 2013). The tegument is thinner at the tail than the body and helps stick cercariae together during penetration. It also protects cercariae from their own enzymatic reactions and is essential for survival in water. There is no morphological evidence that the tegument is an absorptive structure (Dawes, 1973).

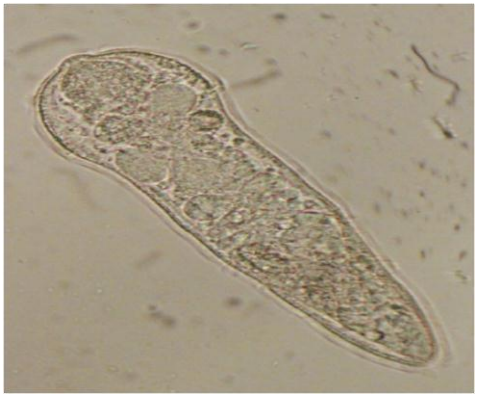
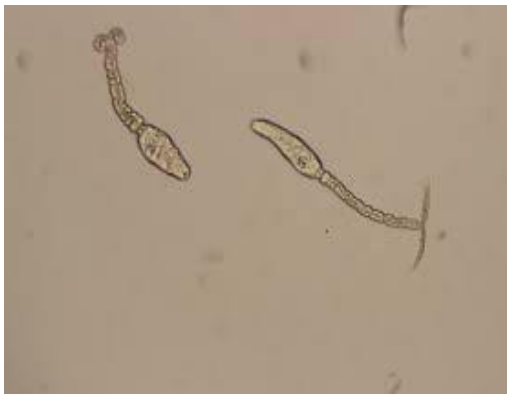
A  B 

Plate 2.4: Scanning electron micrographs showing A: Miracidium, B: Cercariae

Shedding of cercariae from snails exhibits pronounced diurnal periodicity. Usually, there is a single emission every 24 hours. For human schistosomes, increased light intensity within normal temperature (15- 30^{o}) stimulates release of cercariae with a peak emission some hours later. This causes the buildup of cercariae in water to coincide with periods of high human contact (Mahmoud, 2001).

The excretory system is well developed (Schmidt and Roberts, 2013). It comprises a pair of flame cells in the head and one pair at the base of the tail, from which tubules lead to a posterior excretory bladder where the main excretory duct runs through the stem of the tail to two branches that open at the tip of the furcae (Mahmoud, 2001).

Schistosoma mansoni cercariae which directly penetrate into warm blooded definitive hosts concentrate in a thermal gradient near a heat source of 34ºC. Cercariae die after their energy reserves of glycogen get exhausted before locating and penetrating a definitive host. Survival time is affected by temperature and frequency of external stimuli provoking swimming activity. The proportion penetrating definitive hosts and developing to adult worms begins to diminish by 8 hours (Mahmoud, 2001).

2.2.5 Schistosomules and adult schistosomes

When cercariae penetrate human skin, they transform structurally and physiologically and develop into schistosomules. The changes include loss of tail, release of pro-acetabular gland contents, loss of cercarial glycocalyx, development of double layer outer membrane and presentation of new glycoproteins (Walker, 2011). Schistosomules remain in the skin for at least 48 hours before gaining access to systemic circulation where they begin to feed on blood, and move to the hepato-portal system. They then grow rapidly and the tegument matures to an adult worm. The worms pair up and migrate to the walls of the gut to lay eggs (Schmidt and Roberts, 2013).

Adult worms are 7-20 mm long and are intimately associated. Females reside in the gynaecophoric canal of the male (Plate 2.5).Worms have two terminal suckers for attachment and a complex syncytial tegument that plays a role in host immune system evasion and excretion. They also possess a blind digestive tract and well developed excretory, neural and reproductive system.

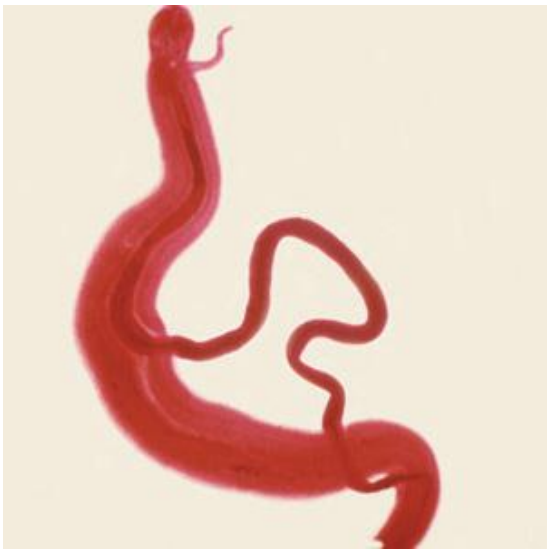


Plate 2.5: Picture showing paired adult schistosomes (CDC, 2012)

Egg production begins 4-6 weeks post infection and continues for up to 15 years. Females produce hundreds of eggs daily, a proportion of which escape from the host via the gut or bladder wall to enter excreta or urine. Eggs not voided are trapped in organs causing immunological reactions (Gryseels *et al*., 2006).

2.3 Pathogenesis and clinical features

Pathogenesis is caused by trapped eggs in body organs. Eggs traverse the gut or bladder wall surrounded by a granuloma which disperses upon reaching the lumen. Granulomas from eggs that do not reach the lumen remain intact leaking antigens over a considerable length of time (Schmidt and Roberts, 2013). The progress and outcome of the disease are as a result of complex interplay of immunopathology involving both the Th_1 and Th_2arms of the immune response (Cheever, 1993).

The disease develops in three phases (CDC, 2016). The first phase is also called the migratory phase. It occurs before production of eggs and is usually asymptomatic.

Penetration of cercariae may however cause cercarial dermatitis, characterized by a skin rash that may appear within 24 hours of exposure to water containing cercariae. This condition manifests differently depending on whether the individuals' immune system has been sensitized by cercariae before (Schmidt and Roberts, 2013). Non sensitized people may develop a few macules that disappear shortly and may not be noticed while sensitized individuals usually develop erythma, papules and pruritus that may last more than a week (Cohen and Powderly, 2004).

The acute stage also called Katayama fever ensues from 14-84 days post exposure , this is the time when schistosomes start producing eggs (CDC, 2012) thus substantially increasing the amount of antigens release. Katayama fever is a serum illness caused by large antigen-antibody complex deposition and pro-inflammatory cytokines in response to the shower of egg antigens (Dejesus *et al*., 2004). The stage is marked by fever, fatigue, rash, headache, myalgia, gasterointestinal discomfort and respiratory symptoms. Usually eosinophilia is present with hepato and splenomegaly (CDC, 2012; Schmidt and Roberts, 2013).

The chronic phase is mostly in patients from endemic areas who are usually asymptomatic but may have bloody diarrhea with mild abdominal pain and lethargy in cases of intestinal schistosomiasis. With urinary schistosomiasis, there may be pain on urination and blood in urine. In most cases the patient's immune response is modulated through a balance between collagen synthesis and degradation so that granulomatous reactions do not become so severe. In about 8% cases of *S. mansoni* and *S. japonicum* development of egg granulomas and fibrosis impedes portal blood flow. As eggs accumulate and fibrotic reactions in the liver continue, a periportal cirrhosis and portal hypertension ensue (Cohen and Powderly,

2004). There is marked splenomegaly due to eggs and partly due to chronic passive congestion in the liver. Ascites is common at this stage (Schmidt and Roberts, 2013).

A proportion of the eggs are carried to distant organs such as the lungs and nervous system forming tubercles. Eggs of *S. japonicum* reach the brain more often than other species. Sixty percent of all neurologic disease in schistosomiasis and almost all brain lesions are due to *S. japonicum.* For *S.haematobium* the onset of bloody urine is gradual and becomes marked as the disease develops and the bladder wall becomes ulcerated. Chronic heavy infection leads to genital, uretal, kidney involvement and lesions in other parts of the body. Major disease manifestations are urinary tract blockages, chronic urinary bacterial infections, bladder cancer and bladder calcification (Schmidt and Roberts, 2013).In females genital schistosomiasis can affect the cervix, fallopian tubes and vagina increasing susceptibility to other infections (CDC, 2012).The worms have a predilection for the portal rather than the systemic veins owing to the former's higher oxygen content and nutrients. The females migrate further against the blood stream towards the region of the distal colon and rectum driven by the oxygen gradient, to lay their eggs. Egg deposition in the sub mucosa leads to granuloma formation, congestion, edema, polyp formation and ulceration. These lead to abdominal cramping and bloody diarrhea (Cao *et al.*, 2010). Females move to the bladder wall directed by the higher oxygen gradient generated by its urinary content. They lay their eggs with the terminal spikes directed towards the bladder lumen to facilitate extrusion. Eggs that fail to exit to the lumen remain trapped in the bladder wall, causing pathologic lesions by releasing their antigens and provoking granuloma formation. Granulomas coalesce to form tubercles, nodules and masses that often ulcerate (Barsoum,

2005). The characteristic clinical presentation is terminal hematuria, usually associated with increased frequency of micturition and dysuria (Barsoum *et al*., 2013).

2.4 Diagnosis

The traditional method of diagnosis is detection of eggs in stool for all species except *S. Haematobium* for which eggs are shed in urine (Colley *et al*., 2014). The different species have varying egg characteristics that can be used to differentiate them under a microscope. Microscopy is sensitive in regions of high endemicity but sensitivity declines in regions with low prevalence and in persons with acute or low infection (Wami *et al*., 2014; Colley *et al*., 2014). Eggs can be first detected after a pre- patent period of 6-8 weeks. Egg shedding varies from day to day and the quantity of shed eggs depends on the parasite burden (Hussein *et al*., 2012; Van Meensel *et al*., 2014) this explains the low sensitivity of microscopy leading to under evaluation of active infections and prevalence.

Serological tests have been developed to detect host antibodies and schistosome antigens. Antigen detection for eggs, schistosomular, adult worms in blood, urine or sputum is now a proven, highly effective method of diagnosis. Circulating antigens released in vomitus of worms, can be demonstrated in blood from 3 weeks after infection, and detected in Elisa with serum or Urine (Elsherbiny *et al.*, 1999).

Schistosome antibodies can be detected using various methods. Elisa can be used to detect reactivity between antibodies in patient serum and extracted antigens from different life cycle stages. A *Schistosoma mansoni* cercarial antigen preparation (SmCTF) was successfully evaluated to detect anti-schistosome antibodies in human sera (Smith *et al*., 2012).

IHT detects reactivity between antibodies in serum of infected individuals and schistosomal antigen coated red blood cells. The method is simple, sensitive and is used in large scale community surveys in endemic areas (Zhou *et al*., 2007). Another Antibody detection method, IFA detects Immunoglobin M and Immunoglobin G antibodies in acute and chronic stages of schistosomiasis especially in low prevalence areas but requires expensive microscopes and reagents (Burlandy *et al*., 2003). Antibody tests are not specific for active disease because people living in endemic areas may have elevated anti body titers for long periods (Cohen and Powderly, 2004).

Schistosome DNA can be detected in host stool, urine or organ biopsy samples by Polymerase Chain Reaction (PCR). PCR amplifies a specific target gene segment and is a very specific method for direct detection of schistosome DNA in fecal samples (Gordon *et al*., 2012).

Colonoscopy and cytoscopy allow for biopsies of infected tissue to be taken in order to show eggs and granulomatous tissue but it may be difficult to distinguish current versus past infection (Cohen and Powderly, 2004).

2.5 Differential diagnosis

Acute schistosomiasis may be wrongly diagnosed as prolonged febrile illnesses like typhoid fever, brucellosis and malaria. Strongyloidosis and trichinosis also cause fever with eosinophilia which makes them difficult to distinguish from schistosomiasis. Genitourinary schistosomiasis may be confused with other infections causing hematuria like malignancy, nephritic syndromes and bacterial infections while intestinal schistosomiasis may be confused with other infectious causes of diarrhea, peptic ulcer disease, pancreatitis or

appendicitis. Differential diagnosis for hepatosplenic schistosomiasis includes visceral leishmaniasis, hemoglobin apathies and viral hepatitis (Davis, 2000).

2.6Control of schistosomiasis

Control of schistosomiasis aims to prevent new infections by interrupting the parasites' life cycle. This may be achieved through many possible ways including reduction of snail intermediate hosts and their habitats and also sanitation to prevent water contamination. All these methods have advantages and disadvantages as discussed below.

2.6.1 Chemotherapy

This is currently the main method of control. Praziquantel (PZQ), a heterocyclic pyrazine isoquinolone is currently the drug of choice recommended by the World Health Organization (WHO, 2016). The dosage is 40mg/kg of body weight. Effective treatment requires a mature antibody response to the parasite hence host immune response also impacts one's response to treatment (CDC, 2012). PZQ is not effective on juvenile schistosomes; it achieves 60-90% cure rates (Andrews, 1981).

In the year 2005, the Kenyan Ministries of Health and Education initiated a parasite control program with the aid of Japan International Co-operation Agency and Kenya Medical Research Institute targeting *S. mansoni* and Soil Transmitted Helminths in school aged children. A total of 43,928 children were dewormed with PZQ and albendazole. The prevalence rates decreased from 47.4% to 8.6% Central Kenya (Kihara *et al* 2007). However, two years after the program, the prevalence rates had shot to 53.7% (Masaku *et al*., 2015).

There is no clinically relevant resistance of adult schistosomes to the drug but there is a concern that resistance may occur due to growing drug pressure (Fenwick *et al.*,

2006).PZQ position as the only drug for mass treatment in Africa control programs and the fact that it never achieves 100% cure rates (Danso-Appiah *et al*., 2008) is a threat as this may lead to spread of the disease.

Inadequate treatment coverage is a serious impediment for mass drug administration implementation. In the year 2011, the 54th world health assembly officially endorsed chemotherapy as the key public health strategy to combat schistosomiasis with a goal of achieving 75% to 100% drug coverage rate in school aged children (Fenwick and Jourdan, 2016). However, the global coverage was 20.74 by the year 2014 (Olveda *et al*., 2016). WHO recommends mass drug administration delivery using a school based or community wide approach depending on prevalence of infection but in one study both approaches yielded significant impact on prevalence and intensity of the disease (Onkanga *et al*., 2016).

2.6.2Prevention of infection to definitive host

Preventing infections to humans and other susceptible animals is the ultimate goal for every control effort done directly or indirectly. Various methods have been employed to directly prevent infections to the definitive host such as mass education, environmental management, biological and chemical control of the snail intermediate host among others.

2.6.2.1Mass education

Mass education is effective but very difficult depending on the hard task of persuading masses of poor uneducated people to change their customs (Schmidt and Roberts, 2013). A study done in Kumba, Cameroon observed that infected people had more knowledge of infection than uninfected people in the same area. This could be interpreted as a case of

health communication that has worked under conditions that have not allowed people to change their health habits (Sama and Ratard, 1994).

2.6.2.2Use of vaccines

Currently, there is no effective vaccine against schistosomiasis available for human use. To be an effective control tool, the vaccine must protect vaccinated people thus interrupting transmission. The vaccine should also be cheap and effective on a single application because the countries affected are among the poorest in the world and must be stable to survive storage (CDC, 2012).

Parasite derived antigens such as Glutathione -S- transferase 26(GST26), GST28 (Esch, 2007), Sm 23 and Sm 24 (McManus and Loukas, 2008) have been tested for vaccine development without much success in humans. Other tried vaccine candidates include Paromysosin, irV-5 and TP1 (Molyneux, 2007).

The major challenge for schistosome vaccine development is the actual identification of antigens that mediate protection. The size of schistosome genome at 280MB DNA encoding up to 20,000 genes makes this task difficult (Wilson *et al*., 2004).

2.6.2.3 Environmental infrastructure

This reduces contact between the populations at risk and contaminated water. Construction of wash houses and safe water supplies have the advantage of reducing or eliminating other water borne diseases as well as reducing schistosomiasis. This method is effective if coupled with educating masses on the importance of using such facilities.

Elimination of schistosomiasis has been achieved in Japan and Venezuela through water and sanitation projects (Cohen and Powderly, 2004). Construction and use of improved latrines can in the long term reduce schistosomiasis transmission by reducing post-

excretion water contact. The ventilated pit latrines of Zimbabwe which are fly proof and odour free are an improvement to the usual pit latrine. The pit latrine can also serve as a bathing facility forming an alternative to bathing in the stream (WHO, 1985).

Provision of clean piped water in public stand pipes also had a positive impact in Zimbabwe. The pipes had to be supplemented with concrete washing slabs to prevent the public from washing clothes in the streams hence reducing stream water contact by 50%. The disadvantage of this method is the large cost of implementing such schemes (Schistosomiasis Research Group, 2010).

2.6.3Vector control

This method reduces re-infection. Snail control may be undertaken by environmental management, biological control and application of molluscicides.

2.6.3.1Environmental management

This involves altering the rate of water flow by clearing vegetation in drainage canals, stream channelization, seepage control and canal lining. Periodic removal of vegetation from irrigation canals reduces the snail population since this forms their primary shelter. Alteration of water levels in irrigation canals by partial or complete drainage not only reduces the amount of aquatic vegetation, but also strands and kills snails by desiccation (WHO, 1985).

Lining of canals with rubber or cement is often necessary to prevent seepage or silting. Water bodies around villages such as burrow pits that may be filled with water and rivers near towns can be canalized to make edges unsuitable for snails. The disadvantage of this method is that it is not practical to employ it in all snail habitats and altering the

environment may make it suitable for other disease vectors, for instance increasing rate of water flow will inhibit snails but may favor colonization by *Simulium* larvae transmitting river blindness (Schistosomiasis Research Group, 2010).

2.6.3.2 Biological control

Introduction of potential predators and competitor snails has been successful. Competitor snails like *Marisa cornuarietis* compete for food and prey on *Biomphalaria* snail eggs. *Marisa cornuarietis* has been used in Puerto Rico as a control agent (Schistosomiasis Research Group, 2010). Other competitor snails are *Melanoides tuberculata* and *Thiara granifera*. A crayfish, *Procambarus clakii* feeds on *Biomphalaria* species (Schimidt and Roberts, 2013). *Astatoreochromis alluaudi* is a cichlid fish that has been used in trials in Kenya and Cameroon with some initial success. After a long time of trial, the fish appeared to be ineffective because of the reduction in the fishes' pharyngeal apparatus used to crush snail shells. This led to a shift in the fish's prey preference to more profitable prey items like benthic and pelagic macro fauna. The fish also reproduces at a slow pace which limits its use in large scale trials (Slootweg *et al*., 1994).

Growth of some plants on the sides of water ways has been used to control snails. The berries of *Phytolacca dodecandra* have been shown to naturally kill snails when they fall into water. The presence of *P. dodecandra* on the sides of rivers in Ethiopia is associated with a reduction in local snail populations (Schistosomiasis Research Group, 2010).

2.6.3.3 Application of molluscicides

There is no perfect molluscicide. The minimum requirements for a good molluscicide are; toxicity to snails at low concentration, absence of toxicity to mammals (neither presenting

acute nor chronic problems of toxicity), lack of adverse effects when it enters the food chain and stability in storage for at least 18 months (De Souza, 1999).

Molluscicides have had a history of success and failure in control of schistosomiasis. Pentachlorophenical and compounds containing tin and lead were discarded due to toxicity to non target organisms. N-trytylmorphine was one of the most active compounds on adult snails but not on eggs. Copper has also been used with some success but it loses toxicity in the presence of organic material and high PH. Niclosamide is currently the preferred molluscicide but it is costly (De Souza, 1999).

The limitations associated with use of molluscicides include not being practical for use in large water bodies like lake shore, where it is difficult to apply the chemical in right concentrations throughout the water body. This may be possible only in small water bodies where transmission foci are well defined in geographical terms and periods of active transmission are known (Molyneux, 2007).

The other limitation of mollusciciding is that once it ceases, snails have the ability to breed and reach pre-control levels in 2 to 3 years. The high rate of multiplication of *Bulinus* and *Biomphalaria* species is related to their ability to reproduce by both cross and self fertilization (Molyneux, 2007).

2.7 Previous studies on plant mollilusciciides

The high cost of synthetic molluscicides, along with concern over the possibility of snail resistance and toxicity to non target species, has given a new impetus to the study of plant molluscicides (Sharma Massoud and Habib, 2003 *et al*., 2009). A large proportion of pharmaceutical products are designed from plants (Cowan, 1999; Raskin *et al.,* 2002).

Plants manufacture chemical substances that are used for their own defense from attacks by insects, and pathogens (Abdalla *et al.,* 2011).Medicinal plants are focused as a source of molluscicidal agents since they are less expensive and less hazardous to the environment than their synthetic counterparts. Use of plants also stimulates growth of small scale industries in the developing countries (Gehad *et al.,* 2009).

Plants such as *Phytolacca dodecandra* (Endod) have been studied for molluscicidal activity. An aqueous solution of powdered leaves of the plant contains triterpenoid oleanoic acid and glycoside which are lethal to Planorbid snails within 24 hours at a concentration of 15-30mg per liter. The component is rapidly biodegraded and lacks ovicidal activity at levels lethal to adult snails (Schistosoma Research Group, 2010). Other plants that have been tested for molluscicidal and schistosomicidal activity include *Datura stramonium* and *Sesbania sesban* (Momeana *et al*., 2011), *S. sinaicum* and *S. villosum* (Gehad *et al.,* 2009).

2.8 *Vernonia amygdalina*

Vernonia amygdalina belongs to family Asteraceae. It is a perennial, rapidly regenerating shrub with height between 1 meter and 6 meters (Farombi and Olotunde2011), a pale green bark and tormentose twigs. The leaves are ovate with entire or dentate margins and sparsely pubescent beneath. Florets are cream or white (Plate 2.6). The plant can be found growing at lake shores, rivers and along fences (Beentje, 1994).

Plate 2.6: **Picturesof*Vernonia amygdalina* plants showing the stem, roots and florets**

Vernonia amygdalina contains very high concentrations of carbohydrates, saponins, alkaloids, tannins, proteins and steroids (+++). Flavonoids and glycosides in high concentration (++), and resins in low concentration (+) (Ugwoke and Ame, 2010; Audu *et al*., 2012).Glycosides posses anti- oxidant activity and play a role in cancer prevention. They also offer some protection against diabetes and arthrosclerosis. *V. amygdalina* leaves are used as vegetable to stimulate the digestive system as well as reduce fever. They are also used as local medicine against leeches that transmit bilharzia, amoebic dysentery, gastrointestinal disorder and microbial infections. Chimpanzees eat the leaves when attacked by parasites (Audu *et al*., 2012). Extracts possess invitro anti-helminthic and anti-parasitic properties). Water extracts of the plant have been reported to show greater efficacy against many livestock helminthes than commercially available drugs such as ivermectin, levamisole and albendazole.

2.10 *Harrisonia abyssinica*

Harrisonia abyssinica belongs to family Rutaceae and is commonly known as hook thorn. The plant is widely spread in tropical regions of Africa. The bark is pale gray to brown. The leaves are alternate, with two to seven pairs of leaflets that are hairy. The petiole has two curved spines at the base, margins entire or variably toothed. The plant is variable in size, shape, or hairiness of the leaves. The flowers are cream yellow while the fruit is red, globose or lobbed. It is an evergreen much branched shrub or small tree that is sometimes climbing (Plate 2.7).

Plate 2.7: Pictures of *Harrisonia abyssinica* aerial parts with raw (Green) and ripe fruits (Red)

The plant grows in dry evergreen forest, wooded grassland, riverine forests or coastal areas (Kokwaro, 2009). The stem bark contains a high number of steroids and ketosteroids. The root bark contains limnoids which exhibit a wide range of biological activities such as insecticidal, antibacterial, anti-malarial and anti-cancer properties. Throughout Africa, the root powder, decoction or infusion is taken to treat venereal diseases, fever, malaria, diarrhoea, urinary problems and intestinal worms. Other ethnoveterinary uses include

treating East Coast Fever (Ejobi *et al.*, 2007), heart water, mange, pleuropneumonia (Grade *et al*., 2009) and dermatophilosis (Nalule *et al*., 2009).

H. abyssinica combined with *V. amygdalina* and salt treats diarrhea in cattle (Tabuti *et al*., 2003). Other medicinal uses of the two plants are outlined in table 2.1 below.

Table 2.1 Table showing some medicinal uses of *Vernonia amygdalina* and *Harrisonia abyssinica*

Plant	Medicinal uses	Reference
Vernonia amygdalina	Treating worms	Innocent and Deogracious, 2006; Ademola and Elof, 2011
	Anti-malaria	Njan *et al*.,2008; Asanta *et al*., 2016
	Stomachache	Tugume *et al*., 2016
	Schistosomiasis	Ojewole and John, 2004
	Liver diseases	Makazayire *et al*., 2011
	Diabetes, constipation, high blood pressure	Asanta *et al*., 2016
	Candidiasis	Mustapha *et al*., 2013
	Menstration pain, STDs	Fasuyi, 2006
	Wound healing	Yineger and Yewhalaw, 2007
	Molluscicide	Ojewole and John, 2004
Harrisonia abyssinica	Anti fungal, anti bacteria, anti viral activity	Mubo and Osiyemi, 2012
	STDs, fever, Urinary problems	Burkill, 2000
	Malaria	,,
	Worms	Ejobi *et al.*, 2007
	Molluscicide	Sonibare and Osiyemi, 2012

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample analysis facilities

The research was done at the Institute of Primate research (IPR), Nairobi. Plant extractions were carried out at University of Nairobi School of Biological Sciences while phytochemical analysis was done at the National Museums of Kenya (NMK), in the phytochemistry laboratory.

3.2 Study design

The study adopted the Latin square true experimental design. Three concentrations of each plant part were used with a replicate thus each concentration formed a block of 8 samples including one negative and one positive control which were set for molluscicidal assays and similar controls for miracicidal and cercaricidal assays.

3.3 Plant collection and drying

The plants were identified by a herbalist from their natural habitat in Lusanjela village, Western Kenya as herbs that are used to treat worm infections and other stomach ailments. Small branches from each plant were then cut and labeled with a specimen voucher number then taken to the National Museums of Kenya herbarium for identification. The stem and root of each plant were then harvested by uprooting the plants using a hoe. A sharp knife was then used to separate the roots from the stems. One plant was used for *Vernonia amygdalina* while four plants were used for *Harrisonia abyssinica*. The stems and roots were washed separately in clean running water. They were then spread on newspapers under shade at room temperature (24-26^{0}C) and left to dry (Poojory *et al*., 2015; Dhanani *et al*., 2017). This took about two months.

The dry plant parts were crushed using a Mekon micromealer single phase and passed through a 0.5mm mesh to standardize the particles (Micheal *et al*., 2013). The powder was stored in screw topped glass bottles and kept at room temperature.

3.4Ethanol extraction

Two kilograms of powder from the roots and stem of *V. amygdalina* and *H. abyssinica* were placed in separate clean large bottles and two liters of 70% ethanol added until the samples were submerged then left to soak for 72 hours. The soaked powder was filtered and the process of soaking and filtering repeated three times for each plant part (Micheal *et al*., 2013; Ganguly *et al*., 2017). Two liters of ethanol were used for each of the second and third soaking. The three filtrates of each plant part were pooled separately and clarified by filtration through whatman filter paper then concentrated in a vacuum in a rotary evaporator to form a dry extract.

3.5 Aqueous extraction

This was done by a method previously used by Adeoga *et al*. (2015) and Dhanani *et al.* (2017) with modifications. Two kilograms of the powder from the root sand stem of each plant were placed in two separate clean large bottles and three liters of distilled water added to each bottle until the samples were completely submerged, then left to soak for 72 hours. The soaked plant parts were filtered and this process of soaking and filtering repeated three times for each plant part. Two liters of distilled water were used for each of the second and third soaking. The three extracts from each plant part were then pooled separately and freeze dried using a Lablyo plus freeze drying machine to give a dry extract.

3.6 Phytochemical screening

The extracts were screened qualitatively for presence of alkaloids, saponins, tannins, flavonoids, triterpenes, cardiac glycosides, glycosides, steroids, anthraquinones, phenols, flavones and resins. These were identified using characteristic colour changes by methods described by Harborne (1998) and Ayoola *et al*. (2008). Each plant extract was tested individually with specific chemical reagents according to standard procedures (see detailed sections for each below).Each test was qualitatively expressed as negative (-), positive (+); the intensity of the characteristic colour was expressed as ++ or +++ (Ayuk *et al*., 2015).The data was then recorded for each extract as in table 3.1.

Table 3.1: Ranks given to plant extracts according to the intensity of colour change with standard reagents.

Rank	Observation	Interpretation
-	No observed colour change	Phytochemical not detected
+	Slight Positive colour change	Trace phytochemical
++	Strong positive colour change	Phytochemical present
+++	Very strong positive colour change	Present highly present

3.6.1 Test for tannins

This was done using the lead acetate test. Five milligrams of powdered extract was weighed and placed in a test tube. One a milliliter of 1% lead acetate were then added and

the mixture shaken for one minute by hand. Formation of a yellow or red precipitate indicated presence of tannins (Trease and Evans, 2002).

3.6.2 Test for alkaloids

Half a gram of extract was weighed and added to 2ml of 1% hydrochloric acid, and stirred. The mixture was then heated in a boiling water bath for 10 minutes and filtered when hot, then treated with 1 ml of Dragendorfs reagent. Turbidity or precipitation showed presence of alkaloids (Sofawara, 1993).

3.6.3 Test for Cardiac glycosides

One milliliter of glacial acetic acid was placed in a test tube and 0.5 milliliters of ferric chloride solution added. One hundred grams of plant extract was weighed and added to the test tube then shaken to dissolve. The mixture was then under layered with 1ml of concentrated sulphuric acid using a Pasteur pipette. Formation of a brown ring at the interphase of the two layers with the lower acidic layer turning blue green upon standing was a positive test for cardiac glycosides (Trease and Evans, 2002).

3.6.4 Test for sterols and triterpenes

Half a gram of each extract was weighed and placed in a test tube. About 3 ml of hexane was added to remove fats from the extract. The supernatant was poured and the residue extracted in 2 ml dichloroethane. The solution was then dehydrated with magnesium sulphate anhydride. The mixture was then treated with 0.5 ml acetic anhydride followed by 1ml of concentrated sulphuric acid. A gradual appearance of green blue color indicated the presence of sterols while color change from pink to purple indicated triterpenes (Trease and Evans, 2002).

3.6.5 Test for flavonoids and flavones

Two grams of plant extract were weighed and put into a test-tube. Four milliliters of 50% methanol were added to dissolve. The solution was warmed and magnesium added. A red colour was observed for flavonoids and orange colour for flavones (Trease and Evans, 2002).

3.6.6 Test for anthraquinones.

One gram of extract was dissolved in 2 ml of 70% acetone to a final concentration of 50mg/ml. Two milliliters of the test sample was then shaken with 4ml of hexane to defat. The upper lipophillic layer was then separated and treated with 4ml of 30% ammonia. Samples whose lower layer changed to violet then pink were positive for anthraquinones (Sofawara, 1993).

3.6.7 Test for glycosides

Two milligrams of plant extract were dissolved in 1 ml of water and then 1 mg of aqueous sodium hydroxide added. Formation of a yellow colour indicated presence of glycosides (Sofawara, 1993).

3.6.8 Test for saponins

Half a gram of extract was shaken in 2 ml of distilled water for five minutes. Frothing that persisted for at least half an hour indicated presence of saponins (Sofawara, 1993).

3.6.9 Test for resins

To two milligrams of extract, 5ml of acetic acid anhydride was added and dissolved by gentle heating. After cooling, 0.5 ml of sulphuric acid was added. A bright purple color produced indicated presence of resins (Sofawara, 1993).

3.7 Preparation of plant extracts for molluscicidal assays

Three concentrations were prepared for each of the stem ethanol, stem water, root ethanol and root water extracts by weighing the extracts from the two plants (That is : 4 extracts × 2 plants = 8 extracts), dissolving in distilled water, and stirring using a magnetic stirrer as shown in table 3.2.

Table3.2 Concentrations of plant extracts used for molluscicidal assays

Extracts weight(g)	Amount of water added in ml	Final concentration
0.05	1000	50 mg/l
0.15	1000	150 mg/l
0.3	1000	300 mg/l

The three concentrations made for each of the eight extracts gave a total of 24dissolved extracts. Each of the dissolved extracts was then divided into two (500ml portions) for use in two replicates, giving a total of 48 experimental containers.

3.8Collection and laboratory maintenance of *Biomphalaria pfeifferi* snails

Five hundred and fifty *Biomphalaria pfeifferi* snails were collected from Mwea irrigation scheme water canals using scoopers (Diakite *et al.*, 2017).A layer of wet cotton wool was placed in a plastic container with holes and snails placed on the cotton wool. Another layer of wet cotton wool was placed on the snails below and more snails added and covered. This was repeated until the container was full (Micheal *et al.*, 2013). The container was then transported to IPR snail laboratory in room temperature boxes for use in the experiments.

Plastic tanks of 5 liters capacity were washed using 3% hydrochloric acid and rinsed thoroughly with chlorine free water from IPR well. Sand and gravel were collected from the snails' natural habitat in Mwea and sterilized by heating at 150ºC for eleven hours, cooled then layered on tanks. Unchlorinated (snail) water from IPR wells was then added. *B. pfeifferi* snails were washed with snail water. They were screened by exposing them to light (100 watts bulb) for five consecutive weeks. The snails were then distributed to the prepared tanks and 20daphnia added to each snail tank for aeration. The snails were housed in a temperature controlled room (25-28^{0}C) with 12 hours light and 12 hours darkness periods (Micheal *et al*., 2013).The snails were fed on dried lettuce (FAO, 2013) and maintained at IPR snail laboratory.

3.8.1Determination of molluscicidal effects of plant extracts

Molluscicidal evaluation was done according to WHO recommended guidelines for molluscicidal tests (WHO, 1965). Forty eight half liter plastic containers with perforated lids were filled with distilled water. Batches of 10 snails were pooled together in each clean 500 ml plastic containers. Dried lettuce was added to each container and left to stand for 24 hours.

Distilled water was drained then the snails were challenged with50, 150 and 300 mg/l of each plant extract for 24 hours. The snails were not fed during the treatment period. Previous studies had shown that healthy snails can live for up to 5 days without feeding (Odetunji and Salawu, 2010).One negative control and one positive control were set using distilled water and niclosamide respectively.

After forty eight hours, the extracts were drained, distilled water was added and the snails were given a 24 hour recovery period. Dead snails were then identified by lack of reaction

to irritation of the foot with an applicator stick and absence of heartbeat (Gehad *et al.*, 2009) when observed under a dissecting microscope.

The number of dead or surviving snails was recorded for each of the treatments and controls. The fully recovered snails were kept in snail water for further use while those that died were discarded.

3.9 Preparation of plant extracts for miracicidal and cercaricidal assays

Six concentrations of plant extracts were prepared by weighing 50µg, 150µg and 300 µg then 0.05g, 0.15g and 0.30g of each plant extract and dissolving in water as shown in table 3.3.

Table 3.3 Concentrations of crude plant extracts for cercaricidal and miracicidal experiments.

Extracts weight	Amount of water added in ml	Concentration
50 µg	10	5 µg/ml
150 µg	10	15 µg/ml
300 µg	10	30 µg/ml
0.05 g	1000	50 mg/l
0.15 g	1000	150 mg/l
0.30 g	1000	300 mg/l

3.9.1 Collection of *S. mansoni* eggs and hatching of miracidia

Miracidia for use in this experiment were obtained by hatching the eggs of *Schistosoma mansoni*. The eggs were obtained from faeces of chronically infected baboons (*Papio anubis*) which are maintained at the IPR Animal Science Department**.**

Faeces were collected from chronically infected baboons in trays left under the cages for 24 hours. About 500 g of faeces were placed in a one liter plastic container. Half a liter of normal saline was then added and thoroughly mixed. The suspension was passed through 2 sieve meshes (size 600 and 200 µm) and the filtrate collected in a tray. The filtrate was then placed in clean 100 ml urine jars and left for 30 minutes in the dark to settle after which the supernatant was poured out. The sediment was then re-suspended in 100 ml of saline and again allowed to stand for 30 minutes (Micheal *et al*., 2013).

Using a pasteur pipette, the sediment was sucked and carefully layered on a petri dish containing water to cover half of the surface then placed under artificial light (20-25ºC) for 30 minutes for miracidia to emerge based on the strong phototrophic behavior exhibited by miracidia (Jurburg *et al*., 2008).

3.9.2Assaying miracicidal effects of plant extracts

An aliquot of 10 miracidia were picked from the petridish under a dissecting microscope and dispensed in each well of a 24 well microtitre culture plate. The miracidia were then treated with two milliliters of *V. amygdalina* and *H. abyssinica* extracts at 5, 15 and 30 µg/l followed by a higher concentration of 50, 150 and 300 mg/l. A replicate for each concentration was made. Each preparation was observed under a dissecting microscope for miracidia motility at 5, 10,20,30,45 and 60 minutes. Immobile miracidia were enumerated and the data recorded at each time point for each extract concentration. At the end of 60 minutes, iodine was added to immobilize all miracidia. The total number of miracidia was then counted as a confirmation of accuracy of the counting procedure. However, if all miracidia became immobile before 60 minutes, the experiment was terminated at that point.

3.9.3 Obtaining cercariae for cercaricidal assays

Miracidia used to infect snails in order to get cercariae for cercaricidal assays were obtained by a procedure described in section 3.9.1 above. The procedures below were then followed to obtain cercariae from *Schistosoma mansoni* miracidia.

A petri dish with hatched miracidia was placed under a dissecting microscope. Five miracidia were picked from the petri dish using a drawn out glass pipette mounted with a rubber bulb. The miracidia were dispersed into each well of a 24 well microtiter culture plate (Knight *et al*., 2015). One snail was transferred to each well using a forceps and the plate covered to prevent the snails from crawling out. The plates were then left for 30 minutes for miracidia to penetrate after which snails were maintained in a 24 hours light and 24 hours darkness cycle for 3 weeks. At the fourth week, they were placed in the dark to avoid trickle shedding of cercariae (Micheal *et al*., 2013).

3.9.2 Shedding of cercariae from infected snails and bioassays

After 5 weeks (prepatent period), snails were removed from the dark and placed in 5 beakers containing 10 ml of snail water. The beakers were placed under light (100 watts lamp) shaded with glass and snails left for 30 minutes to release cercariae. The cercariae suspension was pooled in 100 ml beaker giving a total of 50 ml of cercariae suspension. This was then mixed well.

3.9.4 Assaying cercaricidal effects of the plant extracts.

About 10 ml of cercariae suspension was poured into a petridish and put under a dissecting microscope. A batch of ten cercariae were picked using a drawn out pipette with a rubber bulb and placed in each well of a 24 well microtiter plate then exposed to each concentration of plant extracts prepared for cercaricidal assays at 5, 15 and 30 µg/ml

followed by 50, 150 and 300mg/l (Table 3.3). The samples were checked at minute 5, 10, 15, 20, 30, 45 and 60 for sunken immobile cercariae. The dead cercariae were enumerated and recorded.

3.10 Data analysis

Data on snail mortality were analyzed using Statistical Package for Social Science, Version 16. The mean, standard errors and standard deviations of the various mortalities observed after treating snails with the various extracts of the two plants at different concentrations were computed using the program. Data from each plant extract was then subjected to one way ANOVA to determine whether there were significant differences between the three dosages used. Once significant differences were identified, data was subjected to the Dunnet test to determine whether snail mortality any of the concentrations of a given plant extract was similar to the positive control (Niclosamide). The significance level used in the analysis was $P \leq 0.05$.

Molluscicidal, miracicidal and cercaricidal data was subjected to Finney probit analysis using Biostat 2009 to determine Lethal Dosage 50 (LD_{50} – Concentration of a plant extract that can kill 50 % of snails and Lethal Time 50 (LT_{50} – time taken by a specific concentration of a plant extract to kill 50 % of miracidia or cercariae).The percentage mortality and concentrations were fed into the program where mortalities were converted to probits and concentrations or time to logarithms. Logarithms were then plotted against probits to get a straight line. From the regression lines a probit value equivalent to 50 % mortality gives a logarithm which corresponds to LD_{50} or LT_{50}. The lower the LD_{50} or LT_{50}, the more effective the plant extract is in killing snails or miracidia and cercariae respectively.

CHAPTER FOUR

RESULTS

4.1 Phytochemicals present in extracts of *Vernonia amygdalina*

All extracts of Vernonia amygdalina were highly rich in saponins. Glycosides and phenols were also highly abundant apart from the water extract of the stem and root that had trace amounts of glycosides and phenols respectively. Cardiac glycosides were present in all the four extracts in trace quantities while tannins and steroids were absent in root water and stem water extracts. Triterpenes were only found in water extracts while alkaloids were not detected in all the extracts. Phytochemicals present in the root were also present in the stem (Table 4.1).

Table 4.1 Phytochemicals present in *Vernonia amygdalina* stem and root extracts

Extract	**Tannins**	**Cardiac glycosides**	**Steroids**	**Triterpenes**	**Phenols**	**Saponins**	**Glycosides**	**Alkaloids**
Root ethanol	++	+	+++	_	+++	+++	+++	_
Root water	_	+	_	+++	+	+++	+++	_
Stem ethanol	+++	+	++	_	+++	+++	+++	_
Stem water	_	+	_	+++	+++	++	+	_

Key

_Phytochemical absent + = Trace ++ = Present +++ = highly present

4.1.2 Phytochemicals present in *Harrisonia abyssinica*

Phenols were the most abundant phytochemicals in *H. abyssinica* (+++) in all extracts except the root ethanol extract where they were not detected. Cardiac glycosides were found in all the extracts but in trace amounts (+). On average, saponins were the least detected phytochemical only trace amounts found in the root ethanol extract.

There was no uniformity in phytochemicals present in the root and stem; root extracts had high amounts of alkaloids while the stem lacked alkaloids. The stem had tannins and glycosides while these two phytochemicals were not detected in the root extracts. Triterpenes were lacking in all *H. abyssinica* extracts (Table 4.2).

Table 4.2 Phytochemicals present in *Harrisonia abyssinica* stem and root extracts

Extract	**Tannins**	**Cardiac glycosides**	**Steroids**	**Triterpenes**	**Phenols**	**Saponins**	**Glycosides**	**Alkaloids**
Root ethanol	_	+	+	_	_	+	_	+++
Root water	_	+	_	_	+++	_	_	+++
Stem ethanol	++	+	+	_	+++	_	++	_
Stem water	+++	+	_	_	+++	_	+	_

Key

_ Phytochemical absent + = Trace ++ = Present +++ = highly present

Four phytochemicals (flavonoids, anthraquinones, flavones and resins) out of the twelve under investigation were not detected in both plants.

4.2 Molluscicidal effects of *Harrisonia abyssinica* plant extracts

For *H. abyssinica,* the water extract of root was the most effective, killing all the 10 snails (100%)exposed to it at the lowest and highest concentrations (50mg/l and 300mg/l). 150mg/l of this extract killed 9 out of 10 snails (90%). Other extracts that caused 100% mortality are the root ethanol extract (300 mg/l) and stem water extract (300 mg/l) snail mortality for these two extracts increased with an increase in concentration. The maximum number of snails killed by the stem ethanol extract was 70% (Table 4.3).

The calculated Lethal Dosage 50 (LD_{50}) for the plant extracts gave similar results. LD_{50} is the minimum dosage required to kill 50% of the snails by a given plant extract. The water extract of the root had the lowest calculated LD_{50} of 2.437mg/l followed by the stem ethanol extract (70.53 mg/l). The water extract of the stem recorded the highest LD_{50} of 127.71mg/l (Table 4.3).

Table 4.3 Average number of dead *B. pfeifferi* after 24 hour exposure to *Harrisonia abyssinica* extracts

Plant extract	**Number of *B. pfeifferi* dead after 24 hours (n = 10) (Mean±SE)**			
Conc. mg/l	**Root water**	**Root ethanol**	**Stem water**	**Stem ethanol**
50	**10*** ±0	2 ±0.88	1±0.5	**4***±1.15
150	**9*** ±0.5	8 ±0.88	**7**±1.45	**7***±1.73
300	**10*** ±0	**10*** ±0	**10***±0	**6***±2.6
LD_{50}(mg/l)	2.437	104.65	127.71	70.53

The root water extract was the most effective (LD_{50} = 2.437).

Values with * were not significantly different from Niclosamide, the positive control

The graphical presentation of these results is in appendix IV.

Analysis of Variance at p <0.05significance level was used to compare snail mortality of the three concentrations of each plant extract. This revealed that there was no statistically significant difference in terms of snail mortality for the three concentrations of the root water extract(ANOVA df= 3; P = 0.95) and stem ethanol extract (ANOVA df = 3; P = 0.152).On the contrary, there were significant differences in terms of snail mortality among the three concentrations of *H. abyssinica* root ethanol and stem water extracts used (ANOVA df = 3; P = 0.000).This means that the highest concentration (300mg/l) had higher snail mortality compared to 150mg/l and 50mg/l.

Significant differences called for Post hoc ANOVA to compare the results with the positive control (Niclosamide).This was done using Dunnet test which showed that the three concentrations of *H. abyssinica* root water extracts (50 mg/l, P = 1.00; 150mg/l, P = 0.095; 300 mg/l, P = 1.00)and three concentrations of *H. abyssinica* stem ethanol extracts (50 mg/l, P = 0.082; 150 mg/l, P = 0.480; 300 mg/l, P = 0.230) had molluscicidal activity which were similar to that of niclosamide, the commercial molluscicide used as positive control.

Other concentrations whose molluscicidal activity was not significantly different from that of Niclosamide were 300 mg/l *H. abyssinica* root ethanol extracts (P = 1.00) and 300 mg/l *H. abyssinica* stem water extracts (P = 1.00).

4.2.2 Molluscicidal effects of *Vernonia amygdalina*

The only extract that killed 100% of snails was 300 mg/l of the root water extract followed by 300mg/l of stem ethanol extract (80%). For the root extracts, 50mg/l and 150 mg/l killed the same number of snails. The number of snails killed by the stem extracts gradually

increased with an increase in concentration of extracts. Calculation of the LD_{50}using probit analysis showed that the water extract of the stem had the lowest LD_{50}value of 150.92mg/l followed by the stem ethanol extract with an LD_{50} of 184.67 (Table 4.4).The results indicate that 300 mg/l of root water extract was the best dosage killing 100% of snails exposed to it equivalent to niclosamide (Table 4.4).

Table 4.4 Average number of dead *B. pfeifferi* after 24 hour exposure to *Vernonia amygdalina* extracts

Plant extract	**Number of *B pfeifferi* dead after 24 hours exposure (n = 10) (Mean ± SE)**			
Conc. mg/l	**Root water**	**Root ethanol**	**Stem water**	**Stem ethanol**
50	1 ±0	2 ±0.88	1 ±0	2±1.15
150	1 ±0	2 ±0.33	5 ±1.15	4±0.88
300	**10*** ±0.33	**6*** ±1.15	**7*** ±0.57	**8***±1.15
LD_{50}(mg/l)	**209.30**	**265.33**	**150.92**	**184.67**

Stem water extract was the most effective (LD_{50}150.92 mg/l),

Values with * were not significantly different from Niclosamide.

A graphical presentation of these results is in Appendix IV

Using ANOVA, all the comparisons among the different concentrations for the four extracts gave statistically significant differences. The root water, root ethanol and stem water extracts had a P value of 0.000 while the stem ethanol extract had a P value of 0.001.This implies that 300mg/l of all *Vernonia amygdalina* extracts had better molluscicidal activity compared to 150mg/l and 50mg/l respectively.POST hoc ANOVA using Dunnet test revealed that molluscicidal activity for 300mg/l of root water extract

(P = 0.054) and 300mg/l of the stem Ethanol extract (P = 0.352)were not different from that of niclosamide. All the rest were significantly different from niclosamide meaning their molluscicidal effect was low.

4.3 Miracicidal effects of the plant extracts.

There were no deaths of miracidia after 1 hour of exposure to all extracts of the two plants at concentrations of 50, 150, and 300µg/l. On increasing the concentrations by 10^6 to 50, 150, and 300mg/l as used in molluscicidal assays, no miracidia died before 45 minutes except in the water extract of *V. amygdalina* stem where 1 miracidium died at 45minutes.

Two concentrations of *Vernonia amygdalina* stem water extract (150 mg/l and 300 mg/l) recorded the highest death rates each killing a maximum of 6 miracidia at the end of 60 minutes. The lowest concentration of this extract (50 mg/l) killed 4 miracidia. Four miracidia were also killed by 300mg/l of root ethanol extracts (Table 4.5). The rest of the extracts from this plant did not kill miracidia by the end of 60 minutes (see Appendix V).

The 300mg/l of stem water had the lowest calculated LT_{50} of57.73 minutes. LT values for 50 mg/l and 150 mg/l of the root ethanol extract could not be computed because the two concentrations did not kill miracidia by the end of the experiment (Table 4.5).

Table 4.5 Number of dead miracidia after one hour exposure to *Vernonia amygdalina* extracts

Extract	Concentration (mg/l)	Number of dead miracidia at various time intervals (min)				
		30	**45**	**60**	**LT_{50}(min)**	**SD**
Root	50	0	0	0	Not computable	-
ethanol	150	0	0	0	Not computable	-
	300*	0	0	4	65.01	±6.84
Stem	50	0	0	4	65.01	±6.84
water	150	0	0	6	58.15	±5.28
	300*	0	1	6	57.73	±3.87

Stem water extract at 300mg/l was the best miracicidal agent.

* Indicates the most effective concentration for each plant.

For *H. abyssinica* extracts, all miracidia exposed were still alive at the lapse of 1 hour except for those exposed to the three concentrations of stem ethanol extracts and 300 mg/l of root water extract (killed 1 miracidium). The maximum number of miracidia killed by the stem ethanol extract was 3 at a concentration of 150 mg/l. 300 mg/l and 50 mg/ l of the stem ethanol extract killed 1 miracidium each. The lowest LT_{50} for *H. abyssinica* was 70.77 minutes recorded for 150 mg/l of the stem ethanol extract (Table 4.6). LT values for 50 mg/l and 150 mg /l of root water extracts could not be computed because they did not kill miracidia. The results indicated that miracidia exhibited a high level of tolerance to all extracts from this plant.

Table 4.6 Number of dead miracidia after one hour exposure to *Harrisonia abyssinica* extracts

Extract	Concentration (mg/l)	Number of dead miracidia at the different time intervals (min)				
		30	**45**	**60**	**LT_{50}(min)**	**SD**
Root	50	0	0	0	Not computable	-
water	150	0	0	0	Not computable	-
	300*	0	0	1	113.08	±17.81
Stem	50	0	0	1	113.08	±17.81
ethanol	150*	0	0	3	70.77	±8.16
	300	0	0	1	113.08	±17.81

Stem ethanol extract was the most lethal extract,

* Shows the best concentration for each plant extract.

4.4 Cercaricidal effects of the plant extracts

At low concentrations of 50,150 and 300µg/l no deaths were recorded for all plant extracts. When the concentrations were increased to 50, 150, and 300mg/l cercariae exhibited intolerance which was dose and time dependent.

4.4.1 Cercaricidal effects of *Harrisonia abyssinica*

The ethanol extract of the root was the most lethal to cercariae. At a dosage of 300mg/l and 150mg/l all the 10 (100%) cercariae exposed to this extract were dead at 15 minutes and 45 minutes respectively. Other extracts that killed 100 % of cercariae were 150 mg/l and 300mg/l of the stem water extract. The highest concentration of root water extract killed a maximum of 5 cercariae (Table 4.7).The results were subjected to Finney Probit analysis using Biostat 2009, to determine Lethal Time 50 (LT_{50}) for the plant extracts.

Table 4.7 Number of cercariae dead within60 minutes exposure to *Harrisonia abyssinica* extracts

Extract	Concentration (mg/l)	Number of dead cercariae at various time intervals (minutes) n = 10					
		15	**20**	**30**	**45**	**60**	**LT±SD**
Root water	50	0	0	0	0	1	113.08±**17.8**
	150	0	0	0	0	3	70.77±**8.16**
	300	0	0	0	0	5	61.07±**5.94**
Root ethanol	50	0	0	0	0	0	Uncomputed
	150	0	0	0	10	10	39.57±**3.37**
	300*	10	10	10	10	10	6.72±**5.88**
Stem water	50	0	0	0	0	9	51.34±**3.72**
	150	0	0	0	0	10	39.57±**3.37**
	300*	0	0	10	10	10	6.72±**5.89**

*Shows the most lethal concentrations (LT_{50} 6.72 minutes).

H. abyssinica extracts did not kill cercariae before 15 minutes. The stem ethanol extract did not kill cercariae at any given dosage or time (see Appendix V). The ethanol extract of the root and the water extract of the stem exhibited the highest cercaricidal activity. 300mg/l of each of these extracts had an LT_{50} of 6.72 minutes. LT_{50} for 50mg/l of the root ethanol extract could not be computed because this concentration did not kill any cercariae at any given time.

4.4.2 Cercaricidal effects of *Vernonia amygdalina*

For *Vernonia amygdalina*, the ethanol extract of the stem was the most effective killing all cercariae by the tenth minute at a dosage of 150mg/l and taking only 5 minutes to clear all cercariae at the highest dosage of 300mg/l. Other extracts that killed 100% of cercariae

were 150 mg/l and 300 mg/l of root ethanol extract at 20 minutes and 300mg/l of stem water extract at 10 minutes (Table 4.8).

Table 4.8 Number of dead cercariae on exposure to *V. amygdalina* extracts at given concentrations and time

Extract	Conc. (mg/l)	Number of dead cercariae at various time intervals (min) n = 10							
		5	10	15	20	30	45	60	LT50±SD
Root Ethanol	50	0	0	0	0	0	5	5	55±7.76
	150	0	0	0	10	10	10	10	39.57±3.37
	300	0	0	0	10	10	10	10	39.57±3.37
Stem water	50	0	0	0	0	0	0	0	Not computed
	150	0	0	0	0	2	6	9	44.79±3.23
	300*	0	10	10	10	10	10	10	6.72±5.89
Stem Ethanol	50	0	0	0	0	0	0	0	Not computed
	150*	5	10	10	10	10	10	10	6.72±5.89
	300*	10	10	10	10	10	10	10	6.72±5.89

* shows the most lethal concentrations (LT50 6.72 minutes)

Lt 50 for 50mg/l of stem water and stem ethanol extracts could not be computed because they did not kill cercariae. At a dosage of 50mg/l all the extracts of this plant did not kill any cercariae apart from the root ethanol extract that killed 5 cercariae.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1. DISCUSSION

5.1.1 Phytochemicals present in stem and root extracts of *Vernonia amygdalina* and *Harrisonia abyssinica*

This study demonstrated that *Vernonia amygdalina* was very rich in saponins, glycosides and phenols but lacked Flavonoids, anthraquinones, resins and alkaloids. The results differ from two other studies done which indicated that water and methanolic extracts of *V. amygdalina* extracts contain abundant flavonoids and alkaloids (Anyasor *et al*., 2010; Audu *et al.,* 2012). Phytochemicals are secondary metabolites and the differences in phytochemical composition of same plant species growing in different places can be attributed to differences in environmental factors such as geographical location which has an influence on soil type, precipitation, light intensity and temperature (Lumpkin, 2005; Kumar *et al*., 2017).

Steroids were only be detected in ethanol extracts while triterpenes were only extracted by water indicating that the type of solvent used dictates the phytochemicals that are extracted. Steroids are a class of lipids with a ring system of three or more cyclohexanes and several functional groups attached. The large number of carbon- hydrogens makes steroids non polar (Ophardt, 2003) hence dissolve better in non polar solvents contrary to triterpenes which have more hydroxyl groups in their structure. In another study Hammani *et al.* (2011) demonstrated that steroids from *Solunum nigrum* were best extracted by dichloromethane which is a non polar solvent.

Generally, phytochemicals present in the root of *V. amygdalina* were also present in the stem almost in equal quantities. This aspect can be used for environmental conservation due to the fact that the plant quickly sprouts when the stem is cut compared to planting a seed. Herbalists who traditionally uproot the plant to get roots for medicinal purposes can be advised to consider using the stem since it contains the same phytochemicals as the root.

Phenols were the most abundant phytochemical in *Harrisonia abyssinica* followed by alkaloids. Flavonoids, anthraquinones, resins and triterpenes were not detected contrary to a study done using methanol as a solvent where flavonoids were detected (Nthiga *et al*., 2016).Steroids were also conspicuously missing from plant extracts when water was used whereas ethanol extracts contained high concentrations of steroids. Flavonoids just like steroids are non polar with a basic structure of diphenyl propane 2 benzene rings linked by a 3 carbon chain (De la Rosa *et al*., 2010) thus this renders them less soluble in polar solvents like water.

5.1.2. Molluscicidal effects of *Harrisonia abyssinica* and *Vernonia amygdalina* extracts

The root water extract of *H. abyssinica* was the most effective in killing snails (LD_{50}2.437 mg/l). An extract with LD_{50} ranging between 0-500 mg/l indicates that the extract is highly toxic (Nguta *et al*., 2011).This implies that this extract is highly toxic to snails. The root ethanol, stem water and stem ethanol extracts from this plant had LD_{50} of less than 500 meaning that although they were less potent compared to the root water extract, they have good potential for use as molluscicidal agents. The high toxicity of *H. abyssinica* root water extract can be attributed to abundance of alkaloids and phenols. Alkaloid components were found to be toxic to *Oncomelina hupensis* by inhibiting protein synthesis and

respiratory chain oxidative phosphorylation (Wenshan *et al*., 2017). Alkaloids have been shown to kill snails in other studies (Okunji and Iwu, 1998). Srivastara (1991) also found that a quinolizine alkaloid called Virgilin from the leaves of *Calpurina aurea* killed 100 % of *Biomphalaria glabratta* at 130ppm within 48 hours.

For *Vernonia amygdalina*, the stem water extract (LD_{50}, 150.92mg/l) was the best molluscicidal agent. The root water, stem ethanol and root ethanol though toxic, they required higher dosages. The molluscicidal ability of the plants can be attributed to presence of bio active phytochemicals such as saponins, phenols and tannins. The ability of saponins to form complexes with steroids, proteins and membrane phospholipids is responsible for a large number of biological properties especially action on cell membranes causing their destruction (Schenkel *et al*., 2004).

Guidelines from WHO recommend that for any plant to be considered as a potential molluscicide, it should be able to kill 100% of snails at 100ppm or less within 24 hours (WHO , 1985). In this study, the root water extract of *H. abyssinica* killed 100% of snails at the lowest concentration of 50mg/l which is equivalent to 50ppm within the 24 hours hence it can be developed into an effective molluscicide against *Biomphalaria pfeifferi* snails.

5.1.3 Miracicidal effects of plant extracts

For *H. abyssinica* the highest death rates were seen in 150mg/l of stem ethanol extract (LT_{50} 70.77 minutes) while for *Vernonia amygdalina* 300 mg/l of stem water extract had the least LT50 of 57.73 minutes. Miracidia exhibited tolerance to the plant extracts compared to another study done using *Entada leptostachya*, where LT_{50} for miracidia was 7.69 minutes at 80 mg/l (Micheal *et al*., 2013).Studies done using *Nigella sativa* seeds

produced 100% miracidia mortality at 5mg/l after one minute of exposure (Mohamed *et al*., 2015). This indicates that extracts from the two plants in the current study may not be effective miracicidal agents when used as standalone extracts. However, the extracts can be used in combination with other plant extracts known for miracicidal properties to achieve higher potency levels.

5.1.4 Cercaricidal effects of the plant extracts

The most lethal extracts of *H. abyssinica* were 300mg/l of stem water and 300mg/l of root ethanol both recorded LT_{50} of 6.72 minutes. The same results were recorded for the stem ethanol extract of *V. amygdalina* 300mg/l (LT_{50} of 6.72 minutes). Cercaricidal effects recorded in this study are relatively higher than those obtained for *Euphobia milli* which produced 80% mortality rates after four hours exposure to 100mg/l of extracts and 73% mortality at 50mg/l (Nguta *et al.*, 2011).

The commercial molluscicide (niclosamide) has an LT_{50} of 11.9 minutes at 1mg/l on cercaria hence the individual plant extracts were not effective as cercaricidal agents due to high dosage requirements.

5.2 Conclusions

i) Phytochemicals present in both *Harrisonia abyssinica* and *Vernonia amygdalina* were saponins, tannins, steroids, glycosides, cardiac glycosides and phenols. For *H. abyssinica*, phenols were the most abundant followed by alkaloids which were present only in the root extracts. Extracts of *V. amygdalina* had abundant saponins, phenols and glycosides. Water extracts of this plant also contained high

concentrations of triterpenes which were not detected in *H. abyssinica* extracts. Flavonoids, anthraquinones, flavones and resins were lacking in both plants.

ii) The root water extract of *Harrisonia abyssinica* had very strong molluscicidal properties (LD_{50} of 2.437mg/l.).*V. amygdalina* stem water extracts killed snails at a high dosage (LD_{50} of 150.92mg/l).

iii) Miracidia were highly tolerant to extracts from the two plants. The stem water extract of *V. amygdalina* at 300 mg/l was the most effective against miracidia(LT_{50} of 57.73 minutes).The most effective extract for *H. abyssinica* was stem ethanol extract at 150mg/l (LT_{50} of 70.77 minutes).Extracts from the two plants killed cercariae but required at least 150mg/l to achieve 100% death rates. This concentration is relatively high hence they may not be considered candidates for cercaricidal work as stand alone extracts.

5.3 Recommendations

i) There is need for herbalists to consider using the stem as opposed to uprooting the whole plant to get the root especially for *Vernonia amygdalina* when preparing drugs since the phytochemicals found in the roots were also found in the stem. The remnants of cut stems regenerate quickly for sustainability purposes compared to propagation using seeds.

ii) *Harrisonia abyssinica* should be considered in development of molluscicides by pharmaceutical companies due to of the high molluscicidal properties against adult snails, established in this study

iii) The stem water extract of *Vernonia amygdalina* can be developed for use as a cercaricidal agent in snail habitats where other important aquatic fauna are not

present like in rice irrigation canals to protect farmers from infection because it kills cercariae fast but requires a high dosage.

5.4 Suggestions for further research

a) Studies should be done to purify and quantify phytochemicals which are effective in killing snails, miracidia and cercariae in each of the plant extracts.

b) There is need for studies to be done on the molluscicidal potential of combining the two and even more plants evidenced for molluscicidal activity.

c) Studies should be done on the toxicity of the two plant extracts on other aquatic fauna such as fish.

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Appendix I: Specimen Voucher Numbers for plants

Plant	Date collected	Specimen Voucher Number	
1	18th February 2014	OTWANI A. I	001
2	18th February 2014	OTWANI A. I	002

Appendix II: PLANT IDENTIFICATION

NATIONAL MUSEUMS OF KENYA

WHERE HERITAGE LIVES ON

The East African Herbarium. P.O Box 45166 - 00100 Nairobi, Kenya
Tel: 3743513, 3742131/4, Ext 2274. Fax: 3741424. Email: botany@museum.or.ke. Date: 06th March 2014

REF: NMK/BOT/CTX/1/2

Agnes Otwani
P.O Box 377 Bungoma
Tel. 0712 636 319

Dear Ms. Otwani

PLANT IDENTIFICATION

The plant specimens you brought to us on 4th March 2014 for identification have been determined as follows:

1. *Harrisonia abyssinica* Oliv. **(Family: Simaroubaceae)**
2. *Vernonia amygdalina* Del. **(Family: Asteraceae)**

The plant determinations were done by the expert from East African Herbarium (EA) based on Floras of Tropical East Africa (FTEAs) and confirmed through matching with other herbarium specimens.

Yours Sincerely

Mwadime Nyange SEP 2017

For: Head, Botany Department.

Appendix III: PHYTOCHEMICAL SCREENING OF EIGHT SAMPLES FROM TWO PLANTS.

NATIONAL MUSEUMS OF KENYA
WHERE HERITAGE LIVES ON

PHYTOCHEMICAL SCREENING OF EIGHT SAMPLES FROM TWO PLANT SPPs

SAMPLE	Alkaloids	Tannins	Cardiac glycosides	Steroids	Triterpenes	Flavanoids	Anthra quinones	Phenols	Flavones	Saponins	Glycosides	resins
V.amygdalina stem ethanol	–	+++	+	++	–	–	–	+++	–	+++	+++	–
V. amygdalina stem water extr	–	–	+	–	+++	–	–	+++	–	++	+	–
V. amygdalina root ethanol	–	++	+	+++	–	–	–	+++	–	+++	+++	–
V. amygdalina root water extr.	–	–	+	–	+++	–	–	+	–	+++	+++	–
H. abysssinica stem water	–	+++	+	–	–	–	–	+++	–	–	+	–
H. abysssinica stem ethanol	–	++	+	+	–	–	–	+++	–	–	++	–
H. abysssinica root water extr	+++	–	+	–	–	–	–	+++	–	–	–	–
H. abysssinica root ethanol	+++	–	+	+	–	–	–	–	–	+	–	–

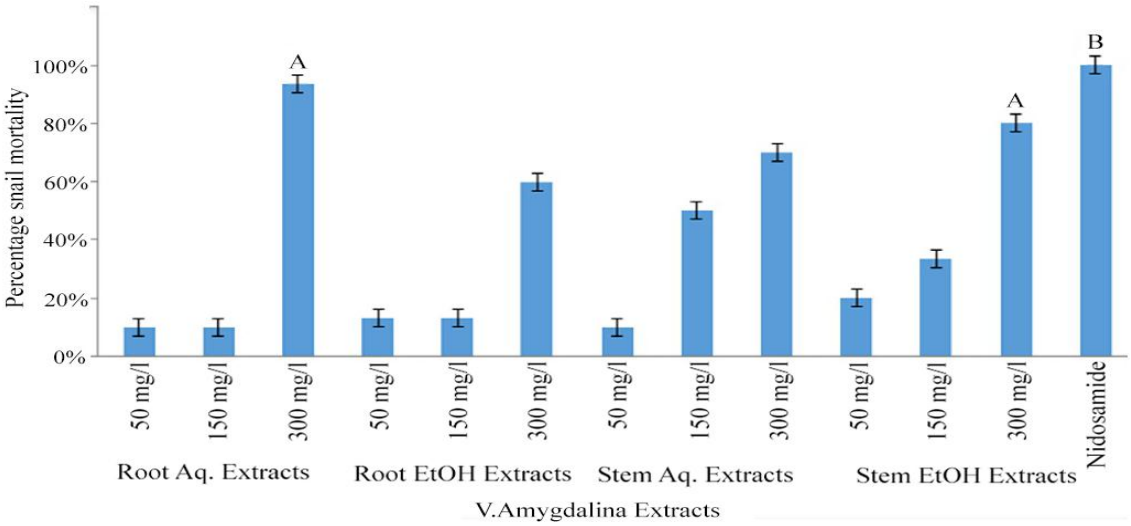
Results compiled by

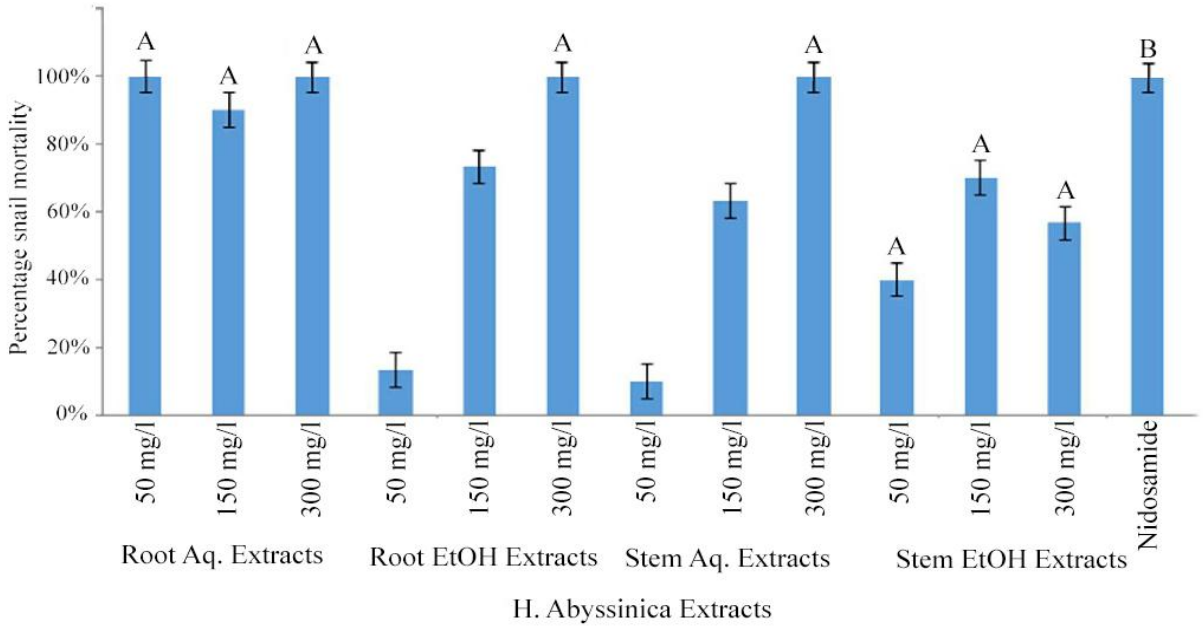
Solomon Cheboi & Daisy Nyawira
Phytochemistry section

Address P.O. Box 40658 00100 GPO Nairobi Telephone 254 20 4448930/3 . 254 20 3742161/4 . 254 20 3742131/4 Fax 254 20 3741424 Website www.museums.or.ke

Appendix IV

Percentage snail mortality on 24 hour exposure to three concentrations of *Harrisonia abyssinica*and *Vernonia amygdalina*extracts (A:Concentrations with molluscicidal activity similar to Niclosamide:B).





Appendix V: Phytochemical analysis of the two plant extracts

SAMPLE	Alkaloids	Tannins	Cardiac glycosides	Steroids	Triterpenes	Flavanoids	Anthra quinones	Phenols	Flavones	Saponnins.	Glycosides	Resins
V.amygdalina stem ethanol	–	+++	+	++	–	–	–	+++	–	+++	+++	–
V. amygdalina stem water extr	–	–	+	–	+++	–	–	+++	–	++	+	–
V. amygdalina root ethanol	–	++	+	+++	–	–	–	+++	–	+++	+++	–
V. amygdalina root water extr.	–	–	+	–	+++	–	–	+	–	+++	+++	–
H. abysssinica stem water	–	+++	+	–	–	–	–	+++	–	–	+	–
H. abysssinica stem ethanol	–	++	+	+	–	–	–	+++	–	–	++	–
H. abysssinica root water extr	+++	–	+	–	–	–	–	+++	–	–	–	–
H. abysssinica root ethanol	+++	–	+	+	–	–	–	–	–	+	–	–

KEY:

+ + + Very high concentration
+ + High concentration
+ Low concentration
- Not present

Appendix VI (a): Number of dead *B. pfeifferi* after 24 hour exposure to plant extracts.

Conc. Mg/l	H. abyssinica root water extract	H. abyssinica root ethanol extract	H. abyssinica stem water extract	H. abyssinica stem ethanol	V. amygdalina root water	V. amygdalina root ethanol	V. amygdalina stem water	V. amygdalina stem ethanol
50	10	3	2	6	1	3	1	4
150	10	9	9	4	1	2	7	5
300	10	10	10	1	9	8	6	10

Appendix VI (b): Results for molluscicidal repeat tests

Conc. Mg/l	H. abyssinica root water extract	H. abyssinica root ethanol extract	H. abyssinica stem water extract	H. abyssinica stem ethanol	V. amygdalina root water	V. amygdalina root ethanol	V. amygdalina stem water	V. amygdalina stem ethanol
50	10	0	0	2	1	0	1	0
150	8	6	4	10	1	1	3	2
300	10	10	10	10	10	4	8	6

Appendix VII: Number of dead miracidia after a given time

TIME/MIN	5	10	15	20	30	45	60	LD50	SD
1A	0	0	0	0	0	0	0	Not computed LT50>60	-
1B	0	0	0	0	0	0	0	Not computed LT50>60	-
1C	0	0	0	0	0	0	1	113.08	±17.81
2A	0	0	0	0	0	0	0	Not computed LT50>60	-
2B	0	0	0	0	0	0	0	Not computed LT50>60	-
2C	0	0	0	0	0	0	0	Not computed LT50>60	-
3A	0	0	0	0	0	0	0	Not computed LT50>60	-
3B	0	0	0	0	0	0	0	Not computed LT50>60	-
3C	0	0	0	0	0	0	0	Not computed LT50>60	-
4A	0	0	0	0	0	0	1	113.08	±17.81
4B	0	0	0	0	0	0	3	70.77	±8.16
4C	0	0	0	0	0	0	1	113.08	±17.81
5A	0	0	0	0	0	0	0	Not computed LT50>60	-
5B	0	0	0	0	0	0	0	Not computed LT50>60	-
5C	0	0	0	0	0	0	0	Not computed LT50>60	-

6A	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**Not computed LT50>60**	**-**
6B	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**Not computed LT50>60**	**-**
6C	**0**	**0**	**0**	**0**	**0**	**0**	**4**	**65.01**	**±6.84**
7A	**0**	**0**	**0**	**0**	**0**	**0**	**4**	**65.01**	**±6.84**
7B	**0**	**0**	**0**	**0**	**0**	**0**	**6**	**58.15**	**±5.28**
7C	**0**	**0**	**0**	**0**	**0**	**1**	**6**	**57.73**	**±3.87**
8A	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**Not computed LT50>60**	**-**
8B	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**Not computed LT50>60**	**-**
8C	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**Not computed LT50>60**	**-**

Appendix VIII: Number of dead cercariae after a given time of exposure to plant extracts

TIME/MIN	5	10	15	20	30	45	60
1A	0	0	0	0	0	0	5
1B	0	0	0	0	0	0	0
1C	0	0	0	0	0	0	1
2A	0	0	0	0	0	0	0
2B	0	0	0	0	0	10	-
2C	0	0	10	-	-	-	-
3A	0	0	0	0	0	0	9
3B	0	0	0	0	0	10	-
3C	0	0	0	10	-	-	0
4A	0	0	0	0	0	0	0
4B	0	0	0	0	0	0	0
4C	0	0	0	0	0	0	0
5A	0	0	0	0	0	0	0
5B	0	0	0	0	0	0	0
5C	0	0	0	0	0	0	0
6A	0	0	0	0	0	5	5
6B	0	0	0	10	-	-	-
6C	0	0	0	10	-	-	-
7A	0	0	0	0	2	6	9
7B	0	0	0	0	0	0	6
7C	0	10	-	-	-	-	-
8A	0	0	0	0	0	0	0
8B	5	10	-	-	-	-	-
8C	10	-	-	-	-	-	