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Evaluation of schistosomacidal activity of lannea schimperi and searsia longipes stem bark extracts

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EVALUATION OF SCHISTOSOMACIDAL ACTIVITY OF *Lannea schimperi* AND *Searsia longipes* STEM BARK EXTRACTS

Nicolaus O. Mbugi

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Award of the Degree of Master's in Life Sciences of Nelson Mandela African Institution of Sciences and Technology

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ABSTRACT

Schistosomiasis is one of the chronic threatening but neglected parasitic disease caused by trematode worms under genus *schistosoma*. Praziquantel is the only drug recommended by WHO for the treatment of all forms of human schistosomiasis. Praziquantel is currently facing challenges, among which selective efficacy and resistance, making it of high imperative searching for new anti-schistosomal drugs. This study aimed to evaluate schistosomacidal activity of *Lannea schimperi* and *Searsia longipes* extracts against cercariae, schistosomula and adult stage of *Schistosoma mansoni*. Bioassays were conducted *in vitro* in 24 well plates for cercariae and schistosomula and 6 well plates for adult worms.

Lannea schimperi and *Searsia longipes* expressed highest activity against schistosomula at 1 to 2 mg/ml and 2 mg/ml respectively with the mortality rate of 100% after one hour. *Searsia longipes* and *Lannea schimperi* exhibited 100% activity against adult worms at the dose range of 0.5 to 2 mg/ml and 1 to 2 mg/ml respectively after 48 hours of exposure. One hundred percent mortality of the cercariae was observed at the concentration range of 1 to 2 mg/ml and 2 mg/ml for *Searsia longipes* and *Lannea schimperi* respectively. Both extracts exhibited good margin of safety on Swiss albino mice with LD_{50} above 2000 mg/kg bwt. *Lannea schimperi* and *Searsia longipes* expressed significant cytotoxicity on brine shrimp larvae with IC_{50} of 150.0478 and 280.7875 µg/ml respectively. Extracts have revealed to contain flavonoids, saponnins, tannins and glycosides. To the best of my knowledge, schistosomacidal activities of *Lannea schimperi* and *Searsia longipes* are reported for the first time.

DECLARATION

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

Signed…….. Date……

Nicolaus O. Mbugi

This thesis has been submitted for examination with our approval as University supervisors:

Signed……Date……

Dr. Musa Chacha

NM-AIST, Tanzania

Signed……Date……..

Dr. Christopher Anjili

KEMRI, Kenya

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by The Nelson Mandela African Institution of Science and Technology a dissertation entitled: "Evaluation of schistosomacidal activity of *Lannea schimperi* and *Searsia longipes* stem bark extracts".

Dr. Musa Chacha _____ _____

Signature and Name of Supervisor **Date**

Dr. Christopher Anjili _____ _____

Signature and Name of Supervisor **Date**

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DEDICATION

Dedication goes to my wife Paulina and my son Declan. To my mother and my sisters for their tolerance, support and endurance throughout the period on which this study was undertaken

CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

&	And
<	Less than
=	Equal
>	Greater than
±	Plus or minus
≥	Greater or equal
^{0}C	Degree Celsius
ANOVA	Analysis of Variance
Bwt	Body weight
cm	Centimeter
DNA	Deoxyribonucleic acid
E	East
EDTA	Ethylenediaminetetraacetic Acid
F	Female
GHS	Global Harmonized System
HIV	Human Immunodeficiency virus
Hrs	Hours
I.U	International unit
IC_{50}	Inhibitory concentration 50
Kg	Kilogram
LD_{50}	Lethal dose 50
LSM	*Lannea schimperi*

M	Male
m	Meter
mg	Milligram
ml	Milliliter
NHT	National Herbarium of Tanzania
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NIMR	National Institute for Medical Research
NM	Nicolaus Mbugi
OECD	Organization for Economic Co-operation and Development
RPMI	Roswell Park Memorial Institute
S	South
SLM	*Searsia longipes*
Spp	Specie
TLC	Thin Layer Chromatography
TPRI	Tanzania Pesticide Research Institute
WHO	World Health Organization

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CHAPTER ONE

INTRODUCTION

1.1 Background

Schistosomiasis (Bilhazia) is one of the chronic threatening but neglected parasitic disease caused by trematode worms under genus *schistosoma*. It is prevalent in tropical and subtropical regions and affects more than 74 countries globally (Ross *et al.,* 2002). Schistosomiasis infects more than 210 million peoples worldwide, whereby 91.4% occurs in African countries contributing to about 200 000 deaths annually (WHO, 2002; Rollinson *et al.,* 2013; Simões *et al.,* 2015). Furthermore, it's estimated that more than 800 million people are at risk of getting this disease globally (Simões *et al.,* 2015). In Tanzania schistosomiasis is endemic all over the country; however the extent of endemicity is more pronounced in islands and regions bordering Lake Victoria. Rollinson and coworkers (2012) reported the prevalence of schistosomiasis to be around 51.5%, which is relatively high compared to the previous reports. Hence, due to the high prevalence general Tanzanian population is therefore considered to be at risk of acquiring schistosomiasis (Mazigo *et al.,* 2012). Despite the seriousness of this disease in health and financial perspective, there are limited numbers of drugs for treatment of the aforementioned disease (WHO, 2013). This is because most of people affected with schistosomiasis are poor, hence little attention and efforts are dedicated to treatment, prevention and control of this disease.

According to WHO Model List of Essential Drugs (WHO, 2013), praziquantel and oxamniquine are listed as drugs for the treatment of schistosomiasis. The two drugs have a history of successful usage at the individual clinical level and in population or community-based chemotherapy (WHO, 1985). Although, praziquantel remains the drug of choice for all forms of schistosomiasis occurring in human, because of its high efficacy, low toxicity, and ease of single oral administration (Aden-Abdi *et al.,* 1987; WHO, 1993; Winstanley, 1996). Oxamniquine is listed for use when praziquantel treatment fails and it is rarely used due to its toxicity (Katz and Coelho, 2008; WHO, 2013). However, there are some reported cases pertaining to the decrease in effectiveness of the praziquantel leading to the reduced cure rates and the failure of treatment (Fallon, 1994; Botros and Bennett, 2007; Aly *et al.,* 2010). It is therefore of paramount importance to search for safe and reliable drugs to treat schistosomiasis, which will complement the available drugs (Penido *et al.,* 1999).

Medicinal plants have been utilized for centuries in Africa for treatment of many health conditions including helminthic infection such as schistosomiasis (Maroyi, 2011; Okoth, 2014). These plants are therefore regarded as a possible source of drug templates that will pave the way for the development of new drugs to combat this debilitating neglected tropical disease that affects our poor societies (Pontin *et al.*, 2008).

1.2 Problem Statement and Justification

Schistosomias is endemic in tropical and sub-tropical regions and is estimated to kill 200 000 people per year and generally infects more than 210 million people (Simões *et al.*, 2015; WHO, 2017). It was reported that around 800 million people globally are at high risk of getting Schistosomiasis (Simões *et al.*, 2015). Despite the devastating effects of this disease, praziquantel is the only drug which is currently available and recommended by WHO for the treatment of all forms of schistosomiasis in human (WHO 2015). Praziquantel is facing a challenge that it is only effective on adult schistosomes. Other stages of schistosomes like eggs, cercariae and schistosomula poses health effects such as dermatitis, enlargement of the liver, hypertension and enlargement of the spleen. It has however, reported cases of the resistant strains of schistosomes leading to failure of treatment by praziquantel (Botros and Bennett, 2007; Aly *et al.*, 2010). Hence, there is a need to discover new drugs that will complement praziquantel to treat schistosomiasis, preferably drugs that will target all stages of the parasite. In an attempt to address this challenge, *Lannea schimpheri* and *Searsia longipes* ethno medically utilized for the management of schistosomiasis investigated as possible source of anti-schistomiasis agents. The part of the plant investigated was the stem barks, and the reason for that is because this part of the plant is employed by traditional healers to prepare the decoction which is used to treat patients with schistosomiasis in traditional settings.

1.3 Significance of the Study

This study provides reliable information that will enhance the discovery of novel bioactive compounds from selected medicinal plants, which in turn, will pave the way to the development of new anti-schistosomial drug that will help in efficient treatment and management of schistosomiasis.

1.4 Objectives

1.4.1 General Objective

To evaluate anti-schistosomal activity of *Lannea schimpheri* and *Searsia longipes* stem bark extracts

1.4.2 Specific Objectives

i. To determine *in vitro* anti-schistosomal activity of *Lannea schimperi* and *Searsia longipes* stem bark extracts
ii. To determine acute toxicity and cytotoxicity level of the *Lannea schimperi* and *Searsia longipes* stem bark extracts
iii. To screening phytochemical compounds of the stem bark extracts of the *Lannea schimperi* and *Searsia longipes*

1.4.3 Research Questions

i. Which concentrations of the stem bark extracts from *Searsia longipes* and *Lannea schimperi* exhibit anti-schistosomal activity?
ii. What are the acute toxicity and cytotoxicity level of the *Lannea schimperi* and *Searsia longipes* stem bark extracts?
iii. What are the phytochemical compounds in stem bark extracts of *Searsia longipes* and *Lannea schimperi*?

CHAPTER TWO

LITERATURE REVIEW

2.1 Botanical Descriptions and Ethno-botanical Uses of *Lannea schimperi* and *Searsia longipes*

2.1.1 *Lannea schimperi*

Lannea schimperi is a plant species under genus *Lannea* which comprises about 40 plant species. This genus belongs to the family *Anacardiaceae* and most species appear as shrubs or trees and widely distributed in Africa tropical regions (Okoth, 2014). It grows up to 8 m high and possesses short trunk and twisted branches. The barks are grey or black in color, fibrous, fissured, white striped and splash red. Stems have lenticellate, and pinkish woolly haired pubescent. Leaves are alternate, imparipinnate, and can have 2 to 4 pairs of opposite leaf lets with a length up to 35 cm. Nerves on the leaves are pinnate, prominent with 8 to 15 pairs of lateral nerves. Flowers are yellowish in color and fruits are red with a length up to 1 cm (Arbonnier, 2004).

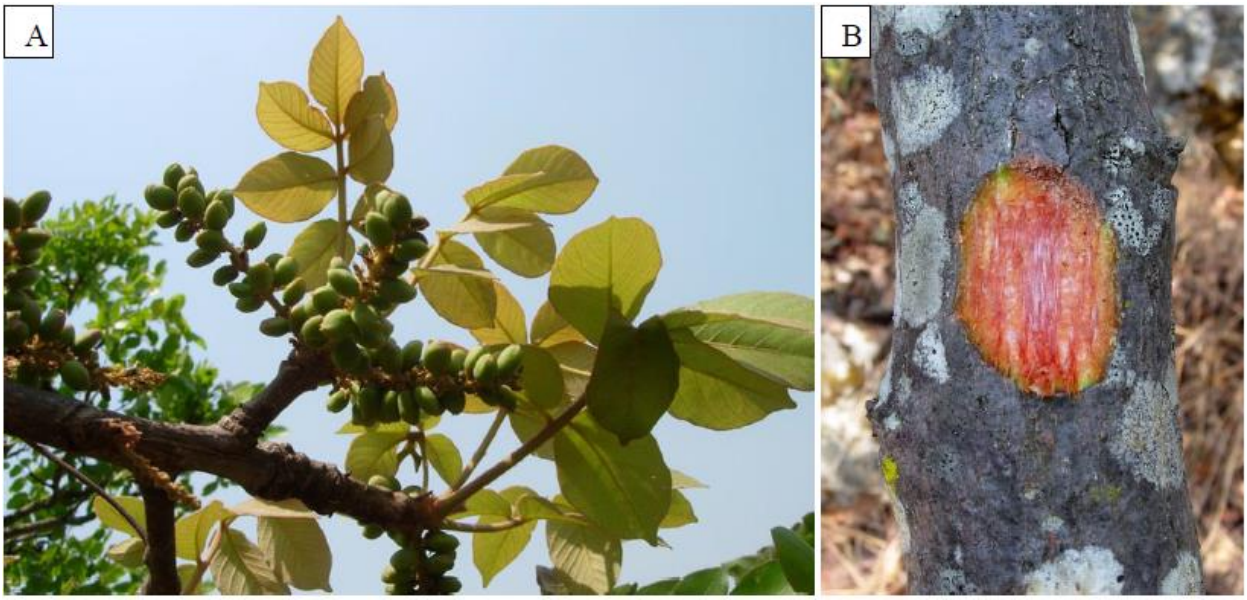


Figure 1: (A, B) Leafs pattern, fruits and stem of *Lannea schimperi*

(http://tropical.theferns.info/image.php?id=Lannea+schimperi)

The genus *Lannea* has been used for many years in different societies to manage mental disorders, gastrointestinal disorders, bacterial infections, viral infections, fungal infections, fever, as well as used as anti-bilharzia (Maroyi, 2011; Okoth, 2014).

Lannea schimperi in particular is used traditionally in some Tanzanian communities for the treatment of NIDDM (Non-insulin dependent diabetes mellitus), especially by managing some of the key symptoms manifested on diabetic patients such as polyuria, polydipsia, excessive thirst and sweating (Moshi and Mbwambo, 2002). Further, *Lannea schimperi* is used for the management of opportunistic diseases associated with HIV which includes tuberculosis, skin rashes, herpes zoster, herpes simplex and chronic diarrhea (Kisangau *et al.,* 2007; Chinsembu and Hedimbi, 2010). Moshi and coworkers (2006) reported ethno medicinal importance of *Lannea schimperi* for the treatment of malaria and epilepsy (Moshi *et al.,* 2006). In some Kenyan societies *Lannea schimperi* is broadly employed in management of diarrhea, stomachache and chest problems (Jeruto *et al.,* 2008). Regarding the ethno botanical survey conducted prior to the commencement of the present study, it was revealed that *Lannea schimperi* is utilized in tradition setting for the management of schistosomiasis particularly in Manyara region, Tanzania.

Methanolic leaf extract of *Lannea schimperi* has shown significant anti-nociceptive, anti-inflammatory and local anesthetic potentials (Egbe *et al.,* 2016; Mikail *et al.,* 2016). In addition, the methanolic extract from *Lannea schimperi* has shown high radicals scavenging activity and significant cytotoxicity on Chinese hamster ovarian mammalian cell-line (Sherfi, 2016; Okoth, 2014). Meanwhile, in some other studies, extracts from *Lannea schimperi* expressed anti-fungal and sparingly anti-microbial activity on gram negative bacteria (Kisangau *et al.,* 2009; Haule *et al.,* 2012; Ekuadzi *et al.,* 2016). Nevertheless, it has demonstrated protective potential against gastric ulceration during an *in vitro* study on sprague dawley rats (Haule *et al.,* 2012).

Phytochemical evaluation of the extracts from *Lannea schimperi* have revealed the presence of glycoside, triterpene, steroids, cardiac glycosides, flavonoids, phenolic glycosides, alkaloids, tannins, condensed tannins and saponins (Haule *et al.,* 2012; Egbe *et al.,* 2016; Sherfi, 2016).

2.1.2 *Rhus/Searsia*

Rhus/Searsia genus belongs to the family *Anacardiaceae* and it comprises more than 250 flowering plant species which are widely and abundantly distributed in temperate and tropical regions (Djakpo and Yao, 2010; Miller *et al.*, 2013). *Searsia longipes* fall under this genus; briefly it appears as shrub or small tree and possesses stems and branches which are long and drooping. The leaves are trifoliolate and bear obovate-elliptic leaflets. The leaflets are surface covered with veins which are conspicuous. Flowers are small, owning axillary and terminal inflorescences which are yellowish-green in color and fruits are small and red coloured.

Figure 2: (A, B) Leafs pattern and the fruits of the *Searsia longipes*

Generally, in folk medicine extracts from various parts of the *Searsia* plants are used to prevent and cure an array of health conditions. Ethno-botanical surveys have attributed the potentials of *Searsia* plants decoction in management of toxins (depurative), hemoptysis, inflammations, laryngitis, stomachache, traumatic fractures, spermatorrhea, snake bite, diarrhea, coughs (antitussive), dysentery, fever, jaundice, hepatitis, helminthic infections, rheumatism as well as for stimulating blood circulation (Duke and Ayensu, 1985; Kuo *et al.*,

1990; Ouyang, 2008; Abbasi *et al.,* 2009; Djakpo and Yao, 2010; Abbassi *et al.,* 2012; Kuma *et al.,* 2015; Abbassi *et al.,* 2012). In addition, Ethno-botanical survey conducted prior to the commencement of this study has revealed the ethno medicinal use of *Searsia longipes* for the management of schistosomiasis particularly in Manyara region.

Regarding some of the recently undertaken ethno botanical studies, *Searsia longipes* in particular is used traditionally in Nigeria for management of some health conditions such as asthma, cancer and malaria infection (Olorunnisola *et al.,* 2017). Nonetheless, roots from this plant are used in some African societies particularly in Zimbabwe to treat infertility in women and to dilate birth canal (Gelfand *et al.,* 1985; Maroyi, 2011).

Searsia plants have been reported to poses hypoglycaemic activity through inhibition of the alfa amylase enzyme (Giancarlo *et al.,* 2006). Hepatoprotective, anti-diarrheal, anti-viral, anti-cancer, anti-oxidant, broad spectrum ant microbial, and anti-fungal activities were also significantly divulged in biological activity studies of some *Searsia* plants (Kossah *et al.,* 2011; Abbassi *et al.,* 2012). For *Searsia longipes* in particular, study conducted to validate the acute toxicity and anti-oxidant activity of this plant has revealed the anti-oxidant potential of its acetone leaf extract with LD_{50} above 5000 mg/kg bwt (Olorunnisola *et al.,* 2017).

Searsia plants contain varieties of secondary metabolites in abundance, of which some of them have ethno medicinal imperatives. Phytochemical studies have identified the presence of flavonoids, volatile oils and fatty acids in some plants which belongs to *Searsia* genus (Özcan and Haciseferogullari, 2004; Anwer, 2013; Abu-Reidah *et al.,* 2015). To the best of my knowledge, *Searsia longipes* has never been evaluated for schistosomacidal activity, henceforth, presents a gap for scientific research to be done to investigate its anti-schistosomal potential.

2.2 Schistosomiasis

Schistosomiasis (Bilhazia) is the parasitic disease, caused by trematode worms of the genus *schistosoma*. It is an endemic disease in some countries with high prevalence in tropical and subtropical regions (WHO, 2010). Human being and other susceptible animals get infection through exposure to infected water. Briefly, in fresh water cercariae sheds from the snails which are the intermediate host of this parasite. Following release and upon contact with susceptible host cercariae enters the host body via skin. In the process of penetrating the susceptible host body, cercariae loses the tail and transformed to schistosomula (Gryseels *et*

al., 2006). Afterward schistosomula migrate through blood capillaries and lymphatic system to the mesenteric and portal veins or venous plexus in urinary bladder depending with the schistosome specie involved. In the aforementioned residence sites, they colonies and mature to adult worms. Following maturation, adult schistosome laid eggs of which some migrates to the small intestine or urinary bladder again depending with the schistosome specie. Some of the eggs invade vital organs such as liver and spleen (Allam, 2007).

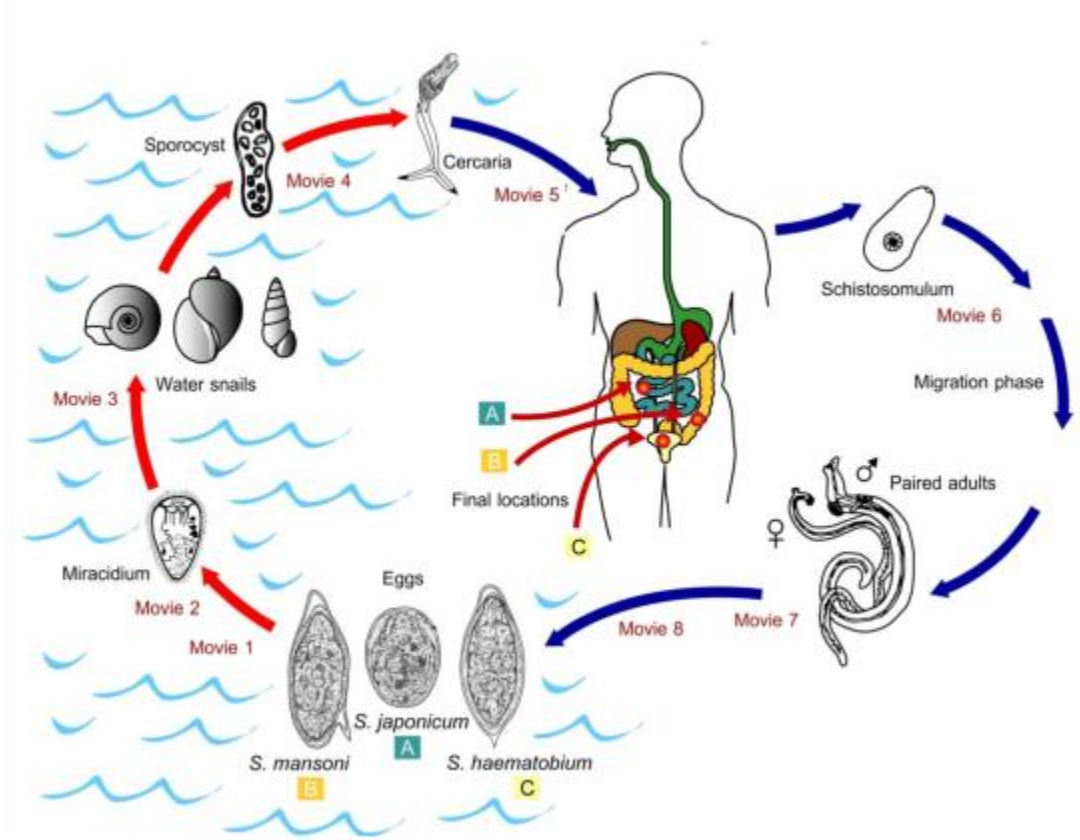


Figure 3: Diagrammatic presentation of the schistosome life cycle

(https://www.cdc.gov/parasites/images/shistosomiasis/Schistomes_LifeCycle.gif)

Eggs in the spleen and liver forms white spots namely granuloma as the result of microphage attack, which in turn result into splenomegaly, portal vein hypertension and hepatomegaly in chronic cases (David *et al.*, 1980; Boloukhere *et al.*, 1993; Brito and Borojevic, 1997; Andrade, 2009). Ascites fluid formation, renal problems and esophageal varices are also observed in chronic schistosomiasis. In the acute cases, disease conditions such as dermatitis, fever, malaise, urticarial, lymphadenopathy, edema, arthralgia and diarrhea are manifested (Chang *et al.*, 2006). These acute effects are due to body immune responses resulted from cercariae penetrations and schistomula migrations.

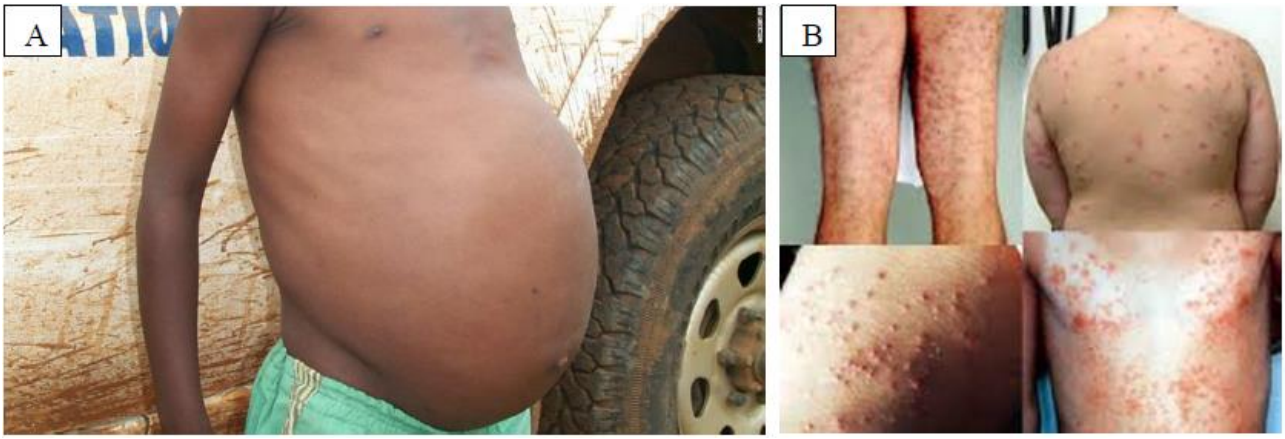


Figure 4: (A) Hepatospleenomegaly in chronic schistosomiasis, (B) swimmer itches (Dermatitis) in acute schistosomiasis

(https://www.thelifeyoucansave.org/portals/0/Images/charities/sci-profile-1.JPG),

(https://www.omicsgroup.org/articles-admin/disease-images/schistosomiasis-1471.jpg)

Societies which highly depend on fishing activities and agricultural activities particularly through irrigation farming are at high risk of acquiring the disease (Doenhoff and Pica-Mattoccia, 2006). Moreover, children's under the age of 10 and women's of reproductive age are reported to be the most vulnerable groups to schistosomiasis infection (WHO, 2017). Whereby, forty million women's of this age are estimated to be infected and 10 million amongst them are pregnant African women's (WHO, 2017; Friedman *et al.,* 2017). Due the aforementioned effects, there is an urgent need to discover new and best strategy to control this disease. Currently, chemotherapy is viewed as the best strategy to manage schistosomiasis particularly through mass administration of the drugs to the most vulnerable groups. Therefore, investigations of new drugs that will complement the available drugs to manage this disease are highly needed.

2.3 Trends on Schistosomiasis Management and Diagnosis

2.3.1 Past Trends on Schistosomiasis Management

Various strategies such as snails control, environmental management, chemotherapy, health education and sanitation have been employed to control and manage schistosomiasis problem at different scales (Doenhoff and Pica-Mattoccia, 2006). The use of chemotherapy has been viewed as the only way of treating persons suffering from schistosomiasis. Antimonial

compounds and emetine were used as chemotherapy for decades but were phased out from use due to their toxicity (Pica-mattoccia and Archer, 1995). Whereby, antimonial compounds were reported to have side effects such as diarrhea, myalgia, colic, skin rushes and arthralgia.

In 1960s, metrifonate which is an organophosphorous compound was introduced for management of schistosomiasis particularly the one caused by *Schistosoma haematobium.* The drug did not last for long time due to the shortfall such as the need of repeated dosage, that in turn lead to lack of patients compliance. Nonetheless, it has many side effects such as fatigue, tremors, muscular weakness, sweating and diarrhea. The aforementioned effects are due to the compound impact on the cholinesterase enzyme (Pica-mattoccia and Archer, 1995). In a very same era, other compounds namely niridazole and lucantrone were also introduced for schistosomiasis treatment, however, were facing challenges of slow absorption, first pass metabolism as well as the need of multiple dose to efficiently cure the disease.

In 1970s, oxamniquine and praziquantel were discovered; the former has activity only against *Schistosoma mansoni* whilst other schistosome species are resilient to the drug (Pica-mattoccia and Archer, 1995). Nonetheless, it has exerted minute activity against East African strain of *Schistosoma mansoni* (Pica-mattoccia and Archer, 1995). Thus, praziquantel became the drug of choice hitherto due to its low toxicity and effectiveness to all species of schistosome that can potentially affect human (Doenhoff and Pica-Mattoccia, 2006; Vale, 2017). However, it is currently facing challenges of drug resistance and selective efficacy (only effective against adult worms) (Botros and Bennett, 2007; Aly *et al.*, 2010). It is therefore necessary to search for drugs that will be effective to all stages of the parasite, in order to compliment praziquantel in treatment of schistosomiasis.

2.3.2 Current Trends on Schistosomiasis Management

Nanotechnology is an emerging technology and has recently portrayed imperatives in many fields including medical field. Regarding nanotechnology implication in medical field, it has currently demonstrated the potential of restoring the potency of many drugs which have lost their effectiveness. Through this technology, challenges such as toxicity, water solubility and bioavailability that encounters anti-schistosomal drugs can be addressed (Frézard *et al.*, 2005). Nano-constructs containing anti-schistosomal agents such as nanoemulsions, liposomes and nanoparticles have successively demonstrated the ability to address the aforementioned challenges. Thus, efficiently reducing the worm load as compared to free

drugs during an *in vitro* and *in vivo* study in animal models (Frézard *et al.,* 2005; Mainardes *et al.,* 2006; de Araújo *et al.,* 2007; Xie *et al.,* 2010; Souza *et al.,* 2014; Dkhil *et al.,* 2015). However, no studies have being conducted to evaluate the effectiveness of these nano-constructs to human subjects. Nevertheless, no study has being done to evaluate the efficacy of these nano-constructs on resistant strains of schistosome. Hence, further studies must be conducted to evaluate whether this paziquantel encapsulated nano-construct can also solve the challenge of drug resistance.

Indeed there are no vaccines for many parasitic diseases including schistosomiasis. Whilst there are many factors for that, among which lack of appropriate *in vitro* method for propagation of parasites and the parasite ability to modulate host immune response, thus, prevents or delay their clearance (Morrison *et al.,* 2016). However, nanotechnology has currently demonstrated the potential of introducing vaccine for schistosomiasis, whereby Mbanefo and coworkers (2015) have introduced a DNA vaccine which is currently under investigation. In this case, nano-construct containing gene from schistosome was engineered to target the immune cells during an *in vivo* study in animal model. This vaccine demonstrated the ability to boost immune response particularly through increasing T cell cytokines, antibodies and interferon gamma production upon introduction to mice. Nevertheless, it divulged anti-fecundity effect to the females worms, however did not reduce the worm burden (Mbanefo *et al.,* 2015). Therefore, this vaccine must be further evaluated for its safety and immediately employed in schistosomiasis endemic areas in compliment with chemotherapy to prevent the spread of schistosomiasis.

2.3.3 Past Trends on Schistosomiasis Diagnosis

Diagnosis is an important step towards effective treatment of diseases, because failure to detect the disease timely and correctly leads to severity of the disease and therefore complicated treatment process. For schistosomiasis in particular, there have being limited number of diagnostic test of which almost all are facing challenges. Microscopic examinations of the urine (Urine filtration technique) and fecal samples (Kato katz) for detection of eggs of different species of schistosome is one of the diagnostic tests (Gray *et al.,* 2011; Hawkins *et al.,* 2016). It has being employed for years but it has low sensitivity, meaning that it cannot certainly detect eggs from a sample taken from a patient with minute infection (Hawkins *et al.,* 2016). For this kind of test, it's therefore necessary to analyze several samples collected at different time before concluding patient schistosomiasis status.

Further, serological tests were also used and still employed hitherto for diagnosis of this disease particularly by detecting antibodies that are produced as the result of schistosomiasis infection. However, this kind of test is also facing the challenge of failure to discriminate the antibodies produced in current infection with those produced in previous infection. This is simply because when a patient had acquired an infection previously, the body responds by producing specific antibodies which in turn persist in the body for a while even after treatment. Henceforth, when a patient gets infected for the second time, this test will not be able to distinguish whether the antibodies detected are from the previous infection or current infection (CDC, 2016). Moreover, along with the aforementioned tests, other tests such as biochemical test to detect albumin level and total cell count to detect eosinophil level are used to suggest the presence of this disease.

Molecular diagnostic techniques were used and still used to detect the disease by using blood or urine samples. Contrary to microscopic examination and serological test, this technique has very high sensitivity and specificity. However, it encounters number of shortfalls among which lack of good infrastructures, financial incapability and lack of molecular experts (Pica-mattoccia and Archer, 1995). Radiography, abdomen and pelvic ultra sound are also employed for schistosomiasis diagnosis particularly for the detection of neural schistosomiasis and spleen hepatomegaly respectively. Nevertheless, despite their huge contribution to schistosomiasis diagnosis, the aforementioned tests detect the disease few weeks post infection. Therefore, it is of high imperative to investigate new diagnostic method that can detect the disease at very early stage in order to facilitate timely treatment and complete elimination of the disease.

2.3.4 Current Trend on Schistosomiasis Diagnosis

Nanotechnology as an imaging technology has demonstrated various potentials in medical field including introduction of the potent drugs and better diagnostic tools. Helminthex, is a new diagnostic tool for diagnosis of various types of the schistosomes of medical imperative (Fagundes *et al.,* 2007; Candido *et al.,* 2015). It employs magnetic iron oxide particles for detection of the schistosome eggs in the light of their affinity. Afterward, the Iron particles eggs complexes are isolated from the fecal material by using magnetic field gradient (Candido *et al.,* 2015). This method has demonstrated high sensitivity up to 100% eggs burden in 1.3 g of the fecal material. Further, Caldeira and coworkers (2012) have reported

the sensitivity of the helminthex to be higher than that of the Kato Katz in a comparative study conducted in Brazil (Caldeira *et al.,* 2012).

Additionally, circulating antigen detection test is another imaging diagnostic method that portrayed high efficacy. Briefly, it employs an up-converting phosphor-lateral flow (UCP-LF) technique. In this case, schistosome specific excreted antigens namely circulating anodic antigen and cathodic circulating antigen are detected by their respective monoclonal antibodies even when they exist at very low level (Utzinger *et al.,* 2015). Moreover, serological test for diagnosis of acute schistosomiasis was also developed. The test detects immunoglobulin G specific to antigen on the schistosomula tegument wall. In a comparative study done in Brazil by Grenfell and coworkers (2013), this test demonstrated heightened sensitivity when compared to ELISA-SWAP (Grenfell *et al.,* 2013).

However, despite the promising advantages of the new schistosomiasis diagnostic test and their potential contributions towards schistosomiasis elimination, there are some challenges that prevent their accessibility. Lack of funding, company's commercial interest and obtaining official approval from regulatory authority are some of the challenges that hinder timely accessibility of the diagnostic tests (Utzinger *et al.,* 2015). Henceforth, awareness should be raised so that companies and government authorities can understand their importance on contributing to schistosomiasis elimination particularly through funding and timely provision of the approval of the new diagnostic tools.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Reagents

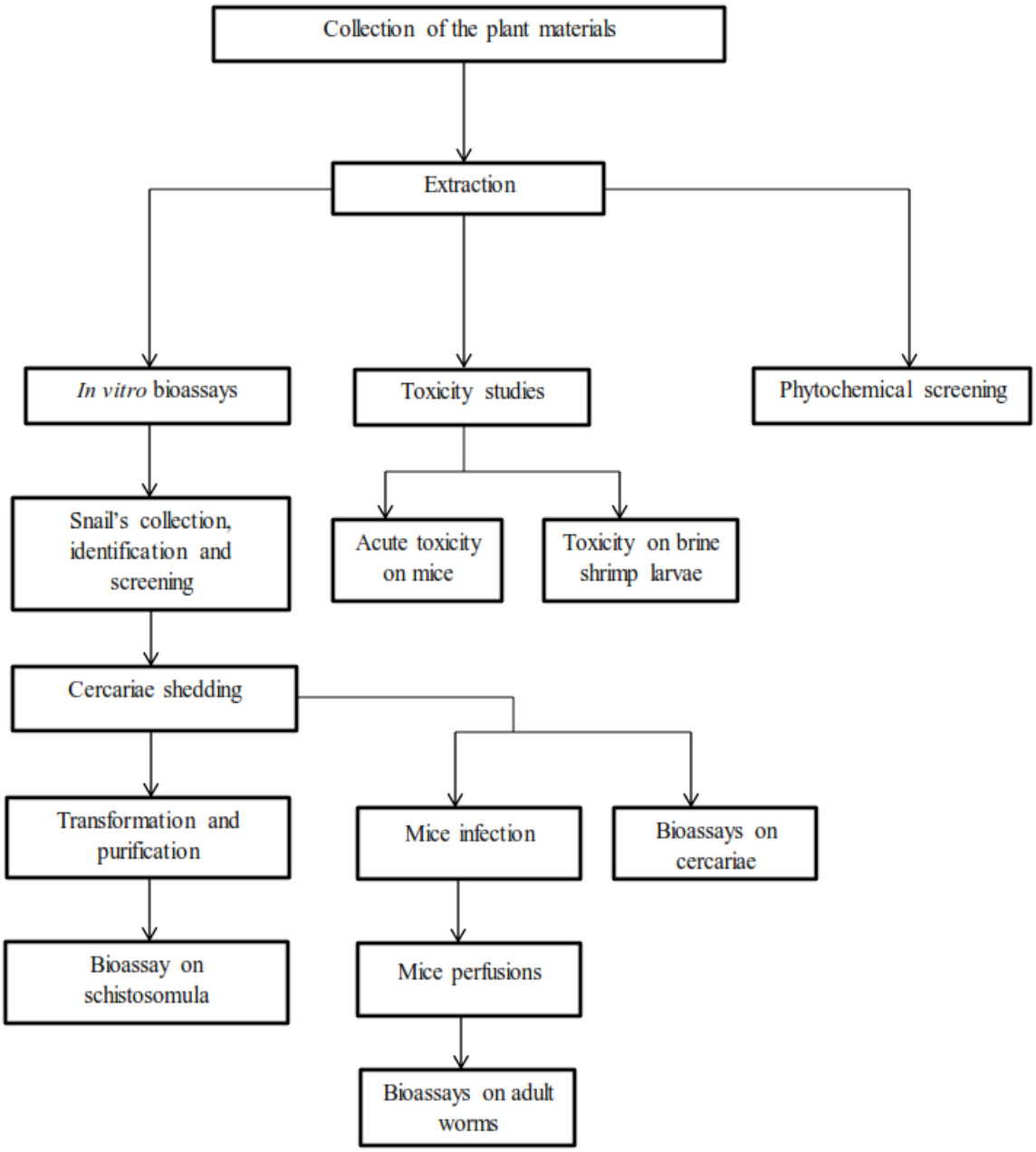
(FBS) Fetal bovine serum (SIGMA), RPMI 1640 media (SIGMA), analytical grade methanol, antibiotics (Penicillin & streptomycin) (SIGMA), sodium pentobarbital, distilled water, citrate saline (Sodium chloride and Sodium citrate), brine Shrimps eggs (Grahamstown 6140, South Africa), sea salt and dimethyl sulfoxide (DMSO).

3.2 Research Design

The present study was experimentally designed and piggy back rode on a Kenya Medical Research Institute approved protocol, KEMRI/SERU/CBRD/PROP164/3406.

3.3 Experimental Plan

Scheme 1 displays the overall experimental plan in which the present study based on.



Scheme 1: Experimental plan for the entire research work

3.4 Sample Collection, Preparation and Extractions

3.4.1 Sample Collection

Lannea schimperi and *Searsia longipes* stem barks were collected from Endasaki ward in Manyara region with geographical coordinate of 4° 25' 0" S, 35° 31' 0" E. Prior to sample collection, the aforementioned plants were identified by botanists from TPRI. Thereafter, voucher specimens were prepared and preserved in NHT (National Herbarium of Tanzania) with voucher specimen number NM 01 and NM 02 for *Lannea schimperi* and *Searsia longipes* respectively.

3.4.2 Sample Preparation and Extractions

Collected stem bark samples were pulverized at Nelson Mandela African Institution of Science and Technology natural product laboratory and thereafter dried under shade for two weeks. Maceration method was used for extraction as described by Azwanida (2015). Briefly, the dried plant materials were grinded by laboratory mill to get fine powder. Approximately, 1 kg of *Lannea schimperi* and *Searsia longipes* powder were measured respectively by using weighing balance and soaked in 2.5 litters of methanol each. Extraction was maintained for 48 hours, and afterward extracts were filtered and concentrated under vacuum by using rotary evaporator. Following extraction process 35 g and 45 g of *Searsia longipes* and *Lannea schimperi* crude extracts were obtained respectively. Extracts were thereafter stored in laboratory refrigerator at - 4 ^{0}C.

3.5 Snail's Collection and Screening

Snails were collected from two areas namely Dunga and Car wash, located along the shores of Lake Victoria in Kisumu, Kenya (Fig. 5 B). Briefly snail's collection was done by using standard scoop (Odiere *et al.*, 2011) and about 699 snails of different species were obtained. Following collection, *Biomphalaria pfeifferi* snails were identified based on guideline described by PAHO (Pan American Health Organization, 1968) and thereafter screened for cercariae shedding (Fig. 5 A). Screening was done under inverted microscope after exposing snails plated on 24 well plates to the direct sun light for 1 hour. Snails exposure were scheduled between 12:00 pm to 1:00 pm since is the pick shedding time for the snails. Positive snails particularly those which were shedding *Schistosoma mansoni* cercariae were selected for use in bioassay (Frandsen and Christensen, 1984). Moreover, three days after the

first screening snails which were negative during an initial screening were rescreened to identify the positive ones which were in prepatent stage during the first screening.

Figure 5: (A) Snails collection and (B) plating in flat bottomed 24 well plates for screening

3.6 Cercariae Shedding and Transformation

Shedding of cercariae was done by exposing *Biomphalaria pfeifferi* snails to light for one hour at KEMRI Schistosome laboratory. Thereafter, portion of the obtained cercariae were transformed to schistosomula and other portion were used to infect mice and for plants extract bioassay against cercariae stage. In a nutshell, transformation was done by using slightly modified mechanical method prescribed by Ramalho-Pinto *et al.* (1967). Whereby, the obtained cercariae were chilled on ice for 45 minutes, afterward cercariae suspension was centrifuged for 5 minutes at 1500 RPM. Supernatant was discarded and the pellet was suspended in RPMI 1640 media. Following resumption, cercariae were mechanically transformed through vigorous agitation for two minutes by using vortex machine. After transformation, the obtained schistosomula were purified by using simple swilling method (Marxer *et al.*, 2012).

Figure 6: Snail exposition to direct sun light and microscopic observation to ensure accurate transferring of the *Schistosoma mansoni* cercariae

In this method of purification, transformed mixture was poured in the glass petri dish and the latter was gently swirled for about 15 rounds. Following gentle swirling, schistosomula accumulated at the center of the petri dish. Thereafter, schistosomula were transferred to a graduated eppendorf tube by using micropipette. This process was repeated three times to ensure no schistosomula has remained. The obtained schistosomula were enumerated and maintained in RPMI 1640 media supplemented with 10% FBS and 1% penicillin and streptomycin ready for bioassay.

3.7 Preparation of the Adult *Schistosoma mansoni*

3.7.1 Mice Infection

Adult worms used in the present study were obtained from Swiss albino mice pre infected with *Schistosoma mansoni* cercariae through abdominal percutaneous exposure (Lewis, 1998). Briefly, 20 albino mice were anaesthetized with 0.2 ml of 5 mg/ml sodium phenobarbital and shaved at the abdominal part. Wet towel was used to rub the shaved part of the mice to remove shaved hairs. Thereafter, each mouse was infected with about 200 *Schistosoma mansoni* cercariae. Infection was done by pouring the suspension containing the aforementioned number of the cercariae in special metal rings placed at shaved abdominal part of the mice (Fig. 7). The suspension was maintained for 1 hour to allow penetration of the cercariae into the mice body. Following infection, mice were maintained for 8 weeks to allow the development of the schistosomula to an adult schistosome worms.

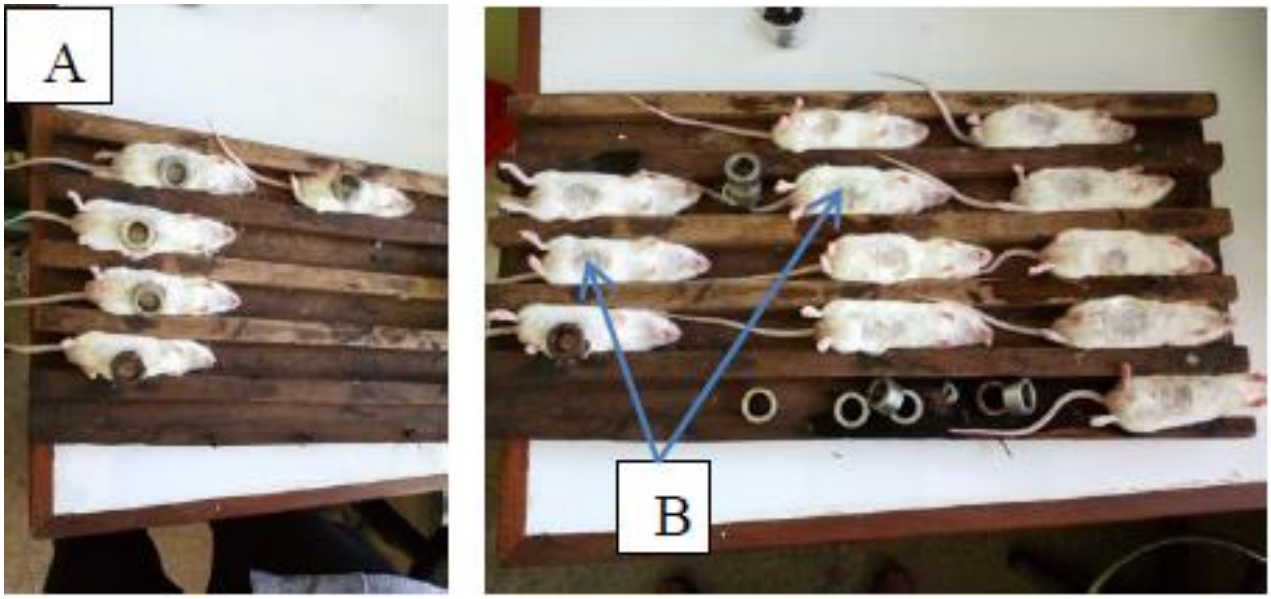


Figure 7: (A) Mice infection with aproximatelly 200 *Schistosoma mansoni* cercaria, (B) shaved abdomen that facillitate cercariae penetration

3.7.2 Mice Perfusion

Mice perfusions were done to isolate adult worms from the mesenteric portal system, whereby 20 infected mice were perfused 8 weeks post infection. Prior to mice perfusion, two liters of the perfusion solution were prepared (0.85% Sodium citrate and 0.15% Sodium chloride) and approximately 100 ml was used for each mouse (Duvall and DeWitt, 1967). Additionally, 4 ml euthanasia solution constituted with sagital and 900 I.U heparin solutions was also prepared. The role of heparin in the aforementioned solution was to prevent blood clotting during perfusion process.

Afterward, each mouse was euthanized by using 0.2 ml of the previously prepared euthanasia solution administered through peritoneal route as depicted in Fig. 8(A) and perfusions were done under perfusion kit as per protocol described by Lewis (1998). Briefly, mice were dissected to open the abdomen and the thorax cavity and then clipped on the perfusion kit in such a way that the head of the mouse is pointing on the right and the tail on the left. Thereafter, the hepatic portal vein was cut to make a slit and the left ventricle of the heart was punctured by a needle connected to 60 ml syringe filled with perfusion solution. Then, hepatic portal system was gradually flushed by applying pressure on the syringe to release perfusion solution which in turn released the adult worms (Fig. 8(B)). Intestines of the mice were also washed by using perfusion fluid while gently massaged to release the worms.

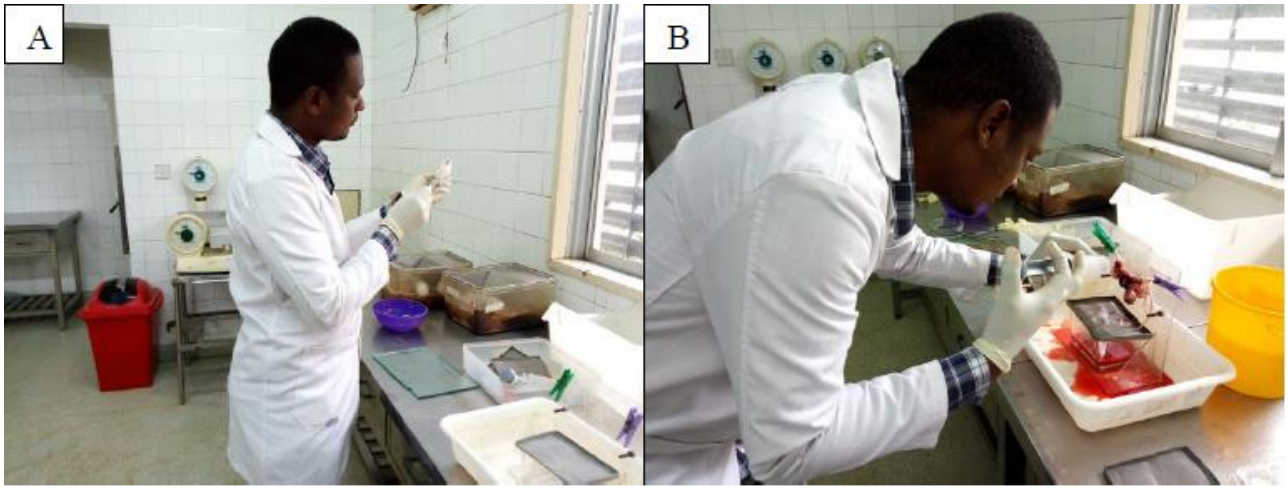


Figure 8: (A) Anesthetization of infected mice, (B) mice perfusion to harvest adult worms

Following perfusion, obtained adult schistosome worms were washed two times by using perfusion solution and RPMI 1640 media consecutively to remove mice blood. Undamaged pairs were sorted out and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin ready for bioassay.

3.8 Bioactivity Assay of the Plants Extracts against Different Stages of the *Schistosoma mansoni*

3.8.1 Media Preparation

Complete media used for schistosomula and adult worms culturing was prepared under aseptic condition in a safety hood by using RPMI 1640, antibiotics (penicillin and streptomycin) and fetal bovine serum (FBS). RPMI 1640 constituted 89% of the media meanwhile antibiotics and fetal bovine serum contributed 1% and 10% respectively. FBS was heated at 56^0C for 30 minutes to inactivate complement proteins. A total of 500 ml of the complete media was prepared and afterward sterilized by using 0.2 membranes (micron micro filter) and stored in refrigerator at – 4^0C.

Figure 9: (A) Aseptic preparation of complete media, (B) RPMI 1640 media and other supplements

3.8.2 *In vitro* Plants Extracts Bioassays against Cercariae Stage

Bioassays were conducted on 24 multi well plates to examine the activity of the extracts against cercariae stage of the *Schistosoma mansoni* by using methods described by Aziz *et al.* (2011) and Simões *et al.* (2015) with modifications. In this case, cercariae were subjected to different concentrations of the methanolic plants extracts namely *Lannea schimperi* and *Searsia longipes*. Briefly, 4 mg/ml stock solutions of the aforementioned extracts were prepared after dissolving an appropriate amount of the crude extracts with distilled water, and the dissolubility was facilitated by warming the suspension at 37^0C for 15 minutes.

From the stock solutions different subsequent concentrations of 2 mg/ml, 1 mg/ml, 0.25 mg/ml, 0.05 mg/ml and 0.025 mg/ml were prepared on the plates following dilution with distilled water. A well contain only distilled water was used as the control. Approximately 20 cercariae were subjected in each concentration and each well contained a final volume of 1 milliliter. Afterward, cercariae were monitored from 0 to 6 hours and bioactivities were assessed based on the loss of motility, mortality and structural deformation including loosing of the tail. All tests were done in duplicate and at least two tests were perfumed at different time in KEMRI laboratory, Kenya.

3.8.3 *In vitro* Plants Extracts Bioassays against Schistosomula

Following transformation of the cercariae stage of *Schistosoma mansoni* to schistosomula stage, bioassays were performed on 24 multi well plates to examine the activity of the plants extracts against the latter stage. In this case, schistosomula were subjected to different

concentrations of the methanolic plants extracts as previously described in cercariae bioassay. Briefly, 4 mg/ml stock solutions of the plants extracts were prepared after dissolving 20 mg of the crude extract in approximately 5 ml of the RPMI 1640 (SIGMA) media.

Thereafter, five different concentrations were prepared on the plates following further dilution of the stock solutions with the RPMI 1640 (SIGMA) media supplemented with 10% fetal bovine serum and 1% streptomycin and penicillin. Whereby, 2 mg/ml was the highest limiting concentration used in this test for each extract, followed by other four subsequent concentrations similar to above described in cercariae bioassay protocol. Following dilution, approximately 20 schistosomula were subjected in each concentration and each well contained a final volume of 1 milliliter. A well containing complete media were used as the control. Afterward, schistosomula were monitored from 0 to 6 hours while incubated at 37^0C and 5% CO_2. Bioactivities were assessed based on the loss of motility, mortality, structural deformation, shortening of the body and increase in body opacity. All tests were done in duplicate and at least two tests were perfumed at different time.

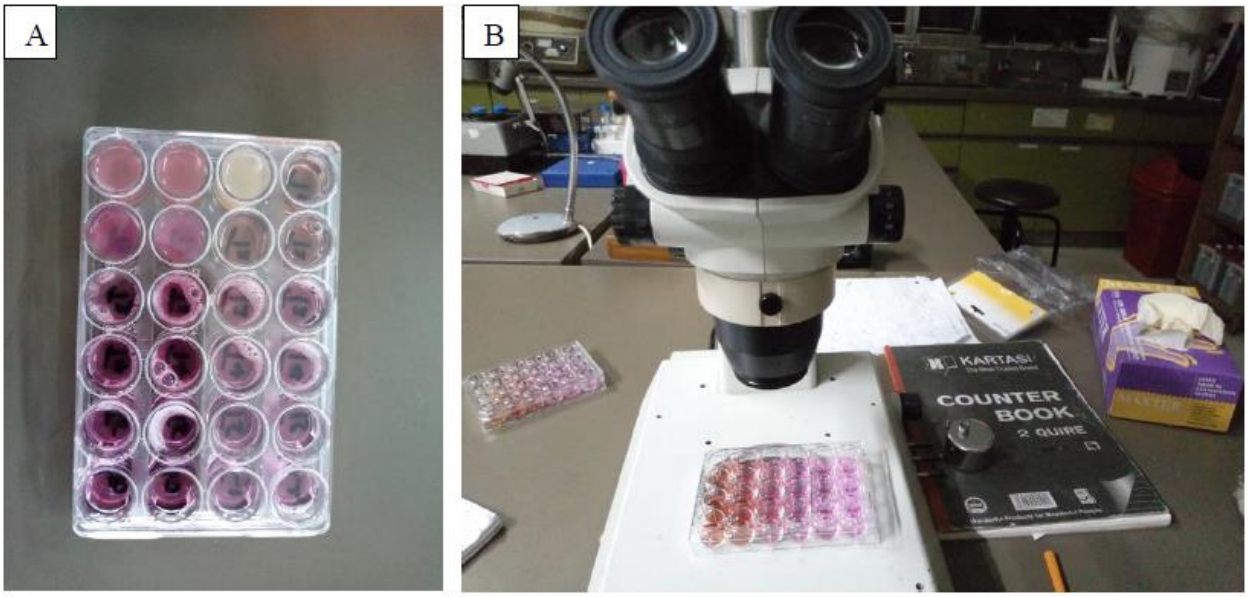


Figure 10: (A) Bioassay on 24 well plate, (B) microscopic examination under dissection microscope (Olympus)

3.8.4 Plants Extracts Bioassay against Adult Worms

This test was performed to evaluate the activity of *Lannea schimperi* and *Searsia longipes* methanolic extracts against adult stage of the *schistosoma mansoni*. In this case, previously perfused adult worms of both sexes were subject to different concentrations of the *Lannea schimperi* and *Searsia longipes* methanolic extracts. In a nutshell, for each extract 8 mg/ml stock solution was prepared in a graduated eppendorf tube. Afterward, from the stock solutions 6 serial concentrations of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.05 mg/ml and 0.025 mg/ml were prepared in the 6 well plates in duplicate. Roswell park memorial institute 1640 (SIGMA) media supplemented with 10% FBS, 1% Penicillin and streptomycin was used for dilution as well as culture media. Additionally, wells containing only RPMI 1640 and 0.01 mg/ml praziquantel were used as negative and positive control respectively. Thereafter, 10 adult worms (5 females and 5 males) were placed in each well and each well contained a final volume of 5 milliliter. Monitoring was done from 0 to 48 hour in 24 hours interval. Henceforth, activity of the plants extracts was assessed under dissection microscope (Olympus) based on loss motility, mortality and structural changes (Xiao *et al.*, 2007).

3.9 Acute Toxicity Study

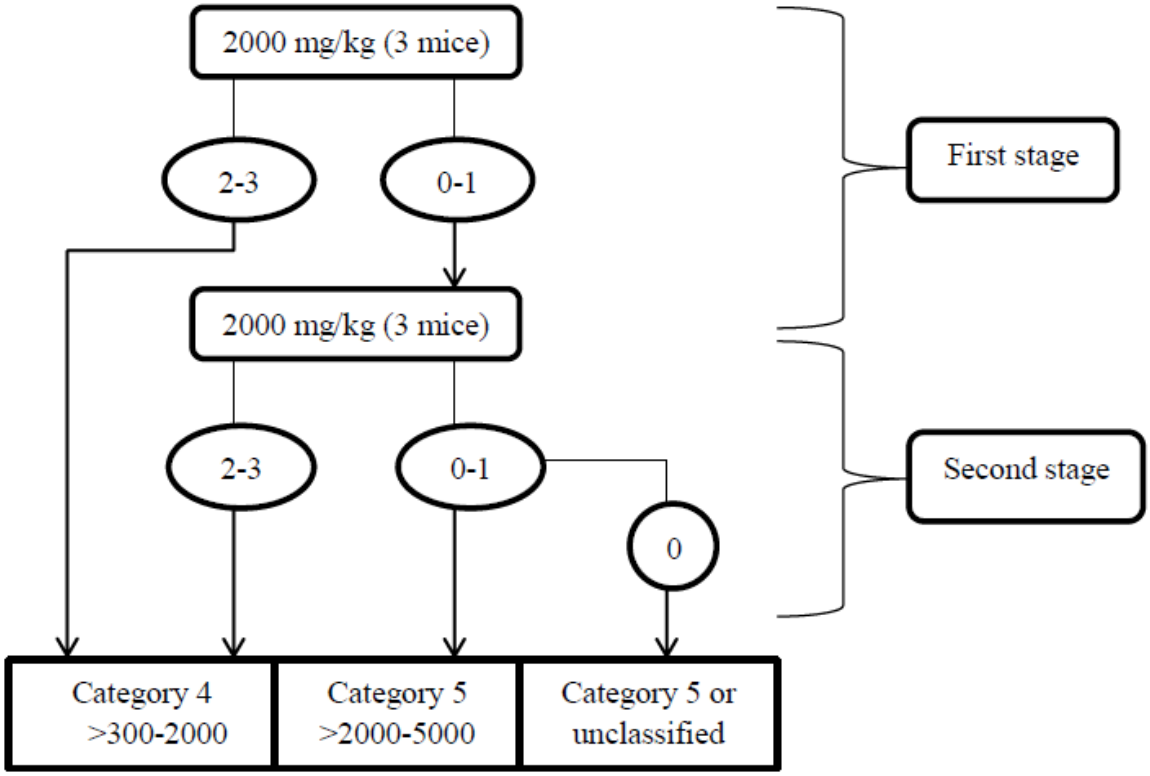
3.9.1 Preparation of Laboratory Animals for Acute Toxicity Study

Laboratory animals used in this study were Swiss albino mice from Sokoine University of Agriculture animal house. A total of 12 females mice of 10 to 12 weeks and 28 to 30 kg body weight were purchased and thereafter transported to Nelson Mandela African Institution of Science and Technology. In Nelson Mandela African Institution of Science and Technology, animals were maintained in environment of not less than 70% humidity and light condition of 12 hours dark and 12 hours light. Animals received standard laboratory animal food and water *ad libitum* (OECD, 2000).

3.9.2 Acute Toxicity Test of the Extracts on Mice

Acute toxicological study was performed to evaluate immediate toxic effects of the methanolic extracts of *Searsia longipes* and *Lannea schimperi* to experimental animals. Study was conducted in abidance to the steps stipulated in OECD guideline number 427. Briefly, two groups of mice each containing 3 female mice were selected and kept for 5 days before dosing for acclimatization to the new laboratory environment. One group was used for

Lannea schimperi extract and other group for *Searsia longipes* extract. On the fifth day, mice were fastened overnight with only supply of water *ad libitum*. Thereafter, mice in each group were administered their respective extract dose of 2000 mg/kg body weight. Distilled water was used as the diluent solution to prepare the aforementioned dose and each animal received 0.5 ml. Following drug administration, water and food were further withheld for 1 hour and afterward animal were observed for 24 hour with much attention given on first four hours. Death and other significant changes such as coma, convulsion, tremors, salivation and respiratory problems were carefully examined and recorded (OECD, 2000). Additionally, observation was prolonged for 14 days to observe any delayed response and this test was done twice as illustrated in Scheme 2.



Scheme 2: Diagrammatic presentation of the acute toxicity test of the extracts on mice as stipulated on OECD guideline number 423

Three female mice were used and was expected that if 2-3 mice would have died following the administration of 2000 mg/kg bwt dose, the lower limiting concentration would thereafter be used as stipulated in OECD guideline number 423. And if 0-1 mouse died, the procedure

in first stage would be repeated and if the result remained the same, would therefore concluded that the lethal dose $_{50}$ of the extract is > 2000 mg/kg body weight.

3.10 Cytotoxicity of the Extracts on Brine Shrimp Larvae

3.10.1 Media Preparation

Media were used to mimic the natural environment suitable for the survival of the Brine shrimp larvae. Media were prepared on the basis of the protocol prescribed by Meyes and coworkers (1967). Briefly, Sea salt was obtained after evaporation of sea water collected from Indian Ocean at Dar es Salaam coast. Artificial seawater was thereafter prepared at the concentration of 3.8 g/L by diluting the obtained sea salt with distilled water. Following preparation, artificial sea water was filtered and afterward transferred into a sterilized tank that has been divided into two compartments by perforated polythene wall. One compartment of the tank was large and covered meanwhile the other compartment was small and not covered. Shrimp eggs (500 mg) were sprinkled into the large and covered compartment of the tank and a lamp was illuminated on the uncovered part in order to attract the hatched shrimps. The mature nauplii were collected between 24 and 36 hours of hatching. Hence, the obtained brine shrimp larvae were used as indicator organisms for general cytotoxicity assessment (Meyer *et al.,* 1982).

3.10.2 Cytotoxicity Assays

Lannea schimperi and *Searsia longipes* extracts were dissolved in dimethyl sulphoxide (DMSO) to make a stock solution of 40 mg/ml each. From the stock solution, Seven different concentrations of 4 µg/ml, 8 µg/ml, 24 µg/ml, 40 µg/ml, 80 µg/ml, 120 µg/ml and 240 µg/ml were tested in duplicate. Cyclophosphamide drug and DMSO were used as positive and negative control respectively (Meyer *et al.,* 1982). In every tested concentration including positive and negative control, 10 brine shrimp larvae were used. Following 24 hours of larvae subjection to different concentrations of the extracts, number of viable nauplii which were motile and didn't sediment to the bottom of the test container were counted under the illumination condition. Regarding the Meyers *et al.* (1982) report, cytotoxicity (LC_{50}) of the extracts below 1000 µg/ml was considered to be potent.

3.11 Qualitative Phytochemical Screening

Phytochemical screening was performed to identify groups of compound present in the *Searsia longipes* and *Lannea schimperi* methanolic extracts. In this regard, a total of five groups of compounds namely terpenoids, glycosides, flavonoids, tannins and saponins were analyzed by using qualitative analytical methods described by Gul *et al.* (2017).

3.11.1 Terpenoids

One gram of the crude extracts (*Lannea schimperi* and *Searsia longipes*) were dissolved in 10 ml of distilled water each and thereafter warmed until were completely dissolved. Solutions were filtered by using whattman filter paper number one. Afterward, two (2) ml of the extracts aqueous solutions were placed on the test tubes and then 2 ml of the acetic acid and H_2SO_4 were added. Hence, formation of reddish brown coloration indicated the presence of the terpenoides.

3.11.2 Glycosides

Two milliliter of the extracts aqueous solutions were measured and dispensed in test tubes. One milliliter of the acetic acid was added, followed by addition of 1 to 2 drops of $FeCl_3$. Afterward, 1 ml of the concentrated sulfuric acid was also added. Consequently, formation of violet ring indicated the presence of the glycosides.

3.11.3 Tannins

Two milliliter of the filtered aqueous solutions of the extracts were measured and poured into the test tubes. Thereafter, 2 ml of the 5% $FeCl_3$ were added and then observed for the formation of yellow precipitate which portrayed the presence of the tannins.

3.11.4 Saponins

Two grams of the plants crude extracts were dissolved in 10 ml of the distilled water and then warmed until dissolved completely. Solutions were filtered and thereafter 5 ml of the filtrates were placed in test tubes. Extracts in the tubes were shaken vigorously until formation of the stable persistent froth. Afterward, 2 to 3 drops of olive oil were added and again the solutions were shaken vigorously and then observed for the formation of the emulsion. Hence, emulsion formation portrayed the presence of the saponins.

3.11.5 Flavonoids

Two grams of the crude extracts were dissolved in 10 ml of the distilled water and then heated until they dissolve completely. The solutions were filtered and 2 ml of the filtrates were dispensed in the test tubes. Two to three drops of HCL and magnesium turning were added and thereafter solutions were observed for the formation of the pink precipitate which indicates the presence of flavonoids.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Snails Collection

After snail's collection and identification, a total of 699 snails of 3 different genera namely *Ceratofallus, Bulinus* and *Biomphalaria* were obtained, from which 587 were *Biomphalaria* species. Following the first and the second screening, 10 positive *Biomphalaria* snails (snails which were shedding *Schistosoma mansoni* cercariae) were obtained as shown in Table 1.

Table 1: Summary of the collected species of snails and number of the positive snails

Collection sites	Snails	Positive	Negative
Car wash	*Ceratofallus* spp	0	22
	Bulinus spp	0	22
	Biomphalaria spp	8	394
Dunga	*Ceratofallus* spp	0	64
	Bulinus spp	0	4
	Biomphalaria spp	2	183
Total		**10**	**689**

4.1.2 *In vitro* Bioassay of the Extracts against Cercariae

For *in vitro* bioassay against cercariae stage, both extracts exhibited significant activity manifested through reduction of the cercariae viability after six hours of exposure. Whereby, overall significant different between viability means of the treatments were observed following statistical analysis using one way ANOVA at p value < 0.05. Further, multiple comparison between treatments and control were done by using Tukey Kramer test at p value < 0.05 and statistically significant differences on viability means were observed up to the lower concentration of 0.05 mg/ml for *Searsia longipes* extract. Whilst for *Lannea schimperi* extract, significant differences in viability means were observed up the lowest concentration used. However, the highest activities of the extracts were observed at the concentrations range of 1 to 2 mg/ml and 2 mg/ml for *Lannea schimperi* and *Searsia longipes* respectively, where 100% reduction of cercariae viability was divulged (Table 2). Furthermore, in all

tested plants extracts concentration based reduction of cercariae viability were observed, particularly after 6 hours of exposition as depicted in Fig. 11 and 12. Meanwhile, concentration and time dependent cercariae motility reduction were also manifested as shown on Table 3. Nevertheless, at the concentrations below 0.5 mg/ml, *Lannea schimperi* was able to induce tail detachment.

Table 2: Percentage viability of cercariae at different concentrations of *Searsia longipes* and *Lannea schimperi* extracts

Concentrations (mg/ml)	Number of cercariae (per well)	Incubation time (Hours)	Viability (Mean ± SE, %)	
			LSM	SLM
2	20	0	100±0	100±0
		1	100±0	100±0
		3	92.5±0.5	100±0
		6	0±0	0±0
1	20	0	100±0	100±0
		1	100±0	100±0
		3	100±0	100±0
		6	32.5±3.5	0±0
0.25	20	0	100±0	100±0
		1	100±0	100±0
		3	95+1	100±0
		6	60±1	2.5±0.5
0.05	20	0	100±0	100±0
		1	100±0	100±0
		3	100±0	100±0
		6	77.5±1.5	7.5±1.5
0.025	20	0	100±0	100±0
		1	100±0	100±0
		3	100±0	100±0
		6	97.5±0.5	52.5±0.5
Control[a]	20	0	100±0	100±0
		1	100±0	100±0
		3	100±0	100±0
		6	100±0	100±0

[a]Distilled water

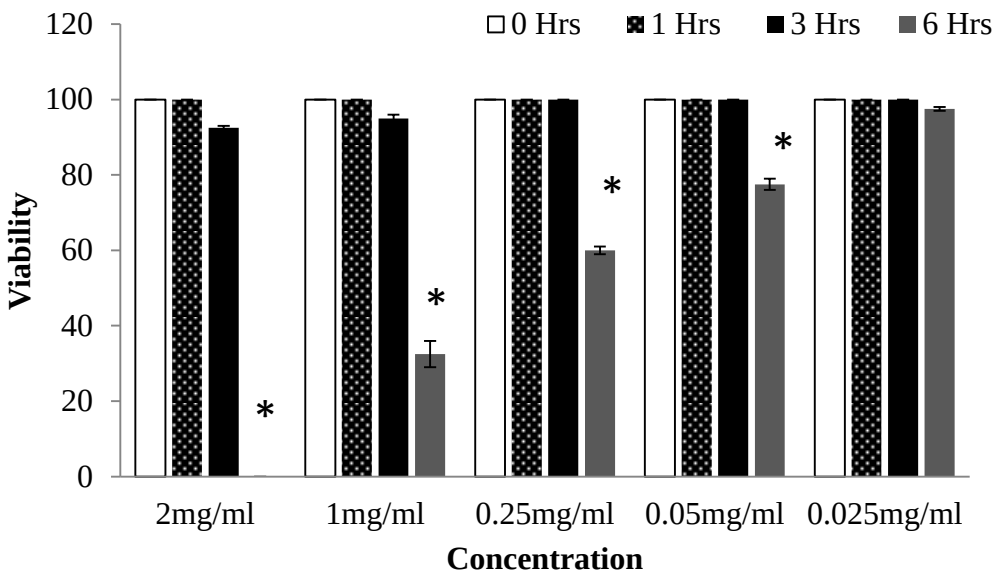


Figure 11: Viability of cercariae at different time and concentrations of *Searsia longipes* extract

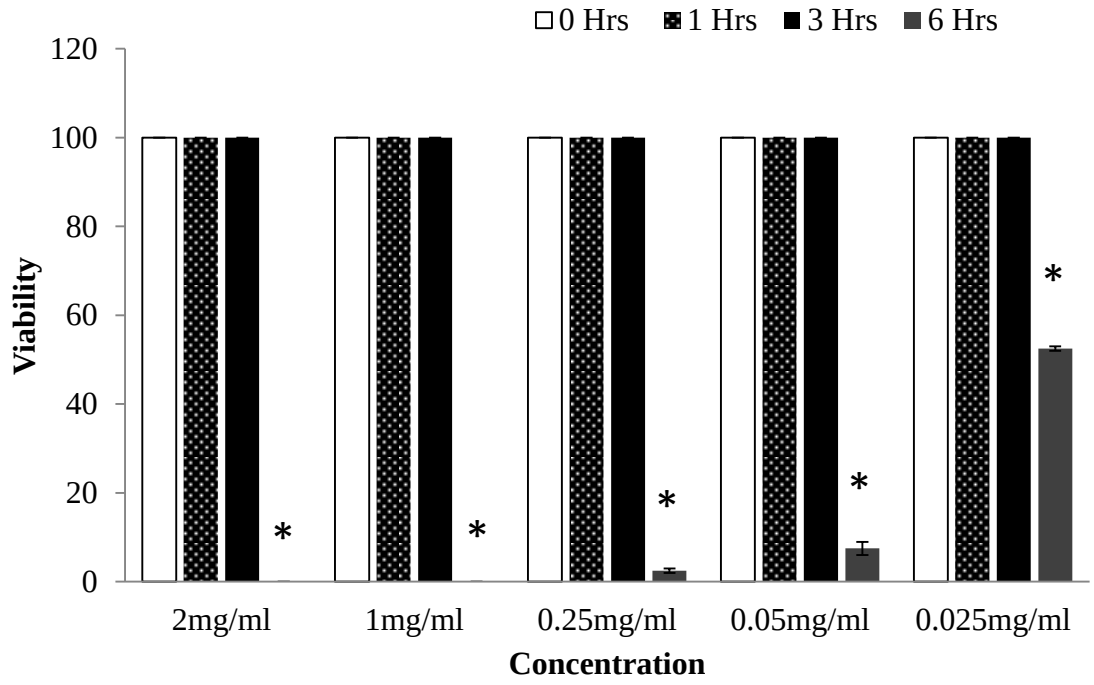


Figure 12: Viability of cercariae at different time and concentrations of *Lannea schimperi* extract

Table 3: Motility assessment of the live cercariae following exposition to different concentrations of extracts

Concentration (mg/ml)	0 Hrs		1 Hrs		3 Hrs		6 Hrs	
	RLM	LSM	RLM	LSM	RLM	LSM	RLM	LSM
2	+++	+++	++	++	+	+	-	-
1	+++	+++	++	++	++	++	+	-
0.25	+++	+++	++	++	++	++	+	+
0.05	+++	+++	++	+++	++	++	++	+
0.025	+++	+++	+++	+++	++	++	++	+
Control[a]	+++	+++	+++	+++	+++	+++	+++	+++

[a]RPMI 1640, +++ = High motility, ++ = Moderate motility, + = Low motility and – = No motility

4.1.3 In vitro Bioassay of the Extracts against Schistosomula

Following *In vitro* bioassay on schistosomula stage of the *Schistosoma mansoni*, both plants exhibited significant anti-schistosomal activity at all tested concentrations. Additionally, both extracts were able to exhibit 100% mortality at concentrations range of 0.25 mg/ml up to 2 mg/ml (Table 4), whereby completely loss of motility was observed following 6 hours of schistosomula exposition. Regarding statistical analysis done using one way ANOVA at p value of < 0.05, overall significant differences between tested concentrations were observed. Further, statistically significant differences were also divulged when multiple comparison of the viability mean of each treatment were tested against that of the control by using Tukey Kramer test at p value of < 0.05. Moreover, time and concentration based reduction of schistosomula viability were also observed as depicted in Fig. 13 and 14.

Table 4: Percentage viability of schistosomula at different concentrations of *Searsia longipes* and *Lannea schimperi* extracts

Concentrations (mg/ml)	Number of Schistosomula (Per well)	Incubation time (Hours)	Viability (Mean ± SE, %)	
			LSM	SLM
2	20	0	100 ± 0	100±0
		1	0±0	0±0
		3	0±0	0±0
		6	0±0	0±0
1	20	0	100±0	100±0
		1	2.5±0.5	0±0
		3	0±0	0±0
		6	0±0	0±0
0.25	20	0	100±0	100±0
		1	12.5±0.5	5±0
		3	0±0	5±0
		6	0±0	0±0
0.05	20	0	100±0	100±0
		1	17.5±1.5	30 ±1
		3	12.5±0.5	12.5±0.5
		6	7.5±0.5	2.5±0.5
0.025	20	0	100±0	100±0
		1	35±1	30±1
		3	22.5±0.5	15±0
		6	17.5±0.5	10±0
Control[a]	20	0	100±0	100±0
		1	100±0	100±0
		3	100±0	100±0
		6	100±0	100±0

[a]RPMI 1640 (Supplemented with 10% FBS and 1% Antibiotics)

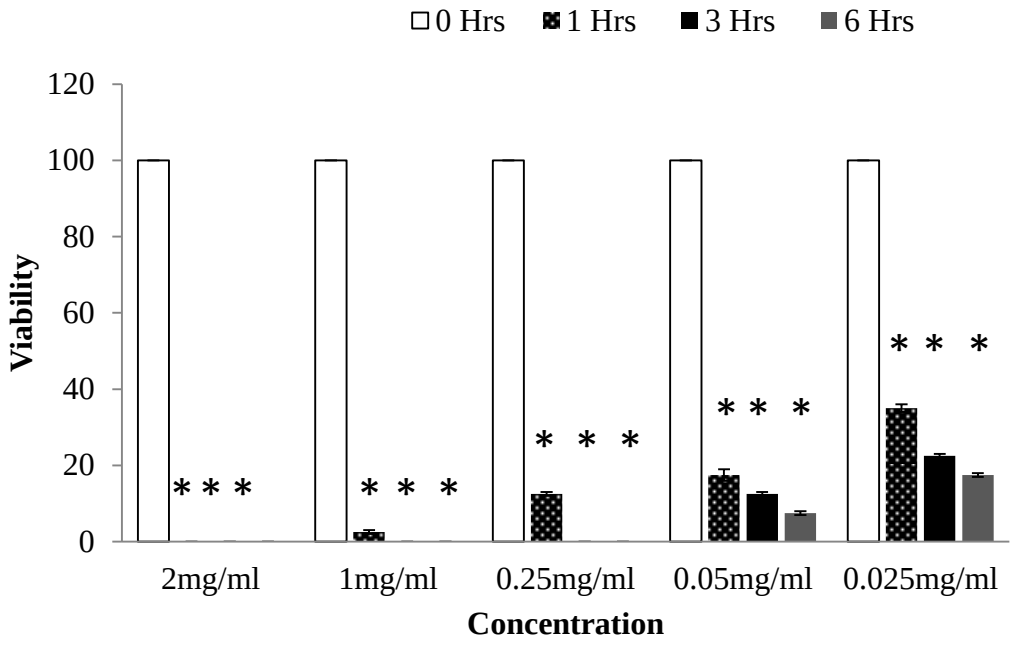


Figure 13: Viability of schistosomula at different time and concentrations of *Searsia longipes* extract

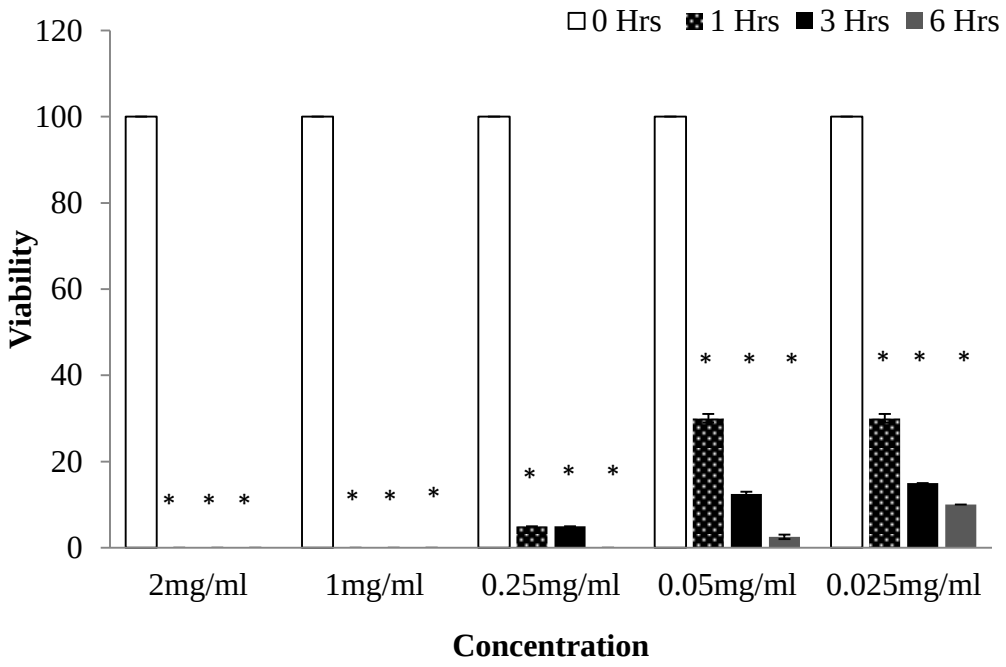


Figure 14: Viability of schistosomula at different time and concentrations of *Lannea schimperi* extract

4.1.4 *In vitro* Bioassay of the Extracts against Adult Worms

Searsia longipes methanolic extract (SLM) has divulged concentration based reduction of worm viability, where the viability reduction was observed to be high as concentration increased. At the three highest concentrations of 2 mg/ml, 1 mg/ml, and 0.5 mg/ml, worms viability was reduced to 0% after 48 hours of exposition as depicted in table 5. Despite the effect on the viability of the worms, the extract has also demonstrated the concentration dependent reduction of worms motility especially at the concentrations distal to the lethal concentrations. Meanwhile, *Lannea schimperi* methanolic extract (LSM) has exhibited 100% anti-schistosomal activity of all worms at two highest concentrations of 2 mg/ml and 1 mg/ml after 48 hours of exposition (Table 6). However at the concentration lower than 1 mg/ml the extract was observed to have no effect on the motility of the worms, hence the worms were highly motile even after 48 hours of exposure. Moreover, male's worms were observed to be more susceptible to all tested concentrations of the extracts than female, whereby 100% mortality were observes at the concentration range of 0.025 to 2 mg/ml and 0.05 to 2 mg/ml for *Searsia longipes* and *Lannea schimperi* respectively (Table 5 and 6). Additionally, dead male's worm were also demonstrated fragmentation and tightly coiled characteristic as depicted on Fig. 15. The viability of the control group subjected to RPMI 1640 only was 100% up to the end of the observation time.

Table 5: Viability of the adult schistosome worms from 0 to 48 hours of exposure to different concentration of *Searsia longipes* extract

Concentration	**0 Hrs**		**24 Hrs**		**48 Hrs**	
(mg/ml)	**M**	**F**	**M**	**F**	**M**	**F**
2	100	100	0	20	0	0
1	100	100	0	20	0	0
0.5	100	100	0	60	0	0
0.25	100	100	0	100	0	20
0.05	100	100	0	100	0	40
0.025	100	100	40	100	0	40
RPMI 1640[a]	100	100	100	100	100	100
PZQ[b]	100	100	0	0	0	0

[a]Negative control

[b]Positive control (0.01 mg/ml of Praziquantel)

Table 6: Viability of the adult schistosome worms from 0 to 48 hours of exposure to different concentrations of *Lannea schimperi* extract

Concentration (mg/ml)	0 Hrs		24 Hrs		48 Hrs	
	M	F	M	F	M	F
2	100	100	0	80	0	0
1	100	100	20	100	0	0
0.5	100	100	20	100	0	40
0.25	100	100	20	100	0	40
0.05	100	100	40	100	0	60
0.025	100	100	40	100	20	80
RPMI 1640[a]	100	100	100	100	100	100
PZQ[b]	100	100	0	0	0	0

[a]Negative control

[b]Positive control (0.01 mg/ml of Praziquantel)

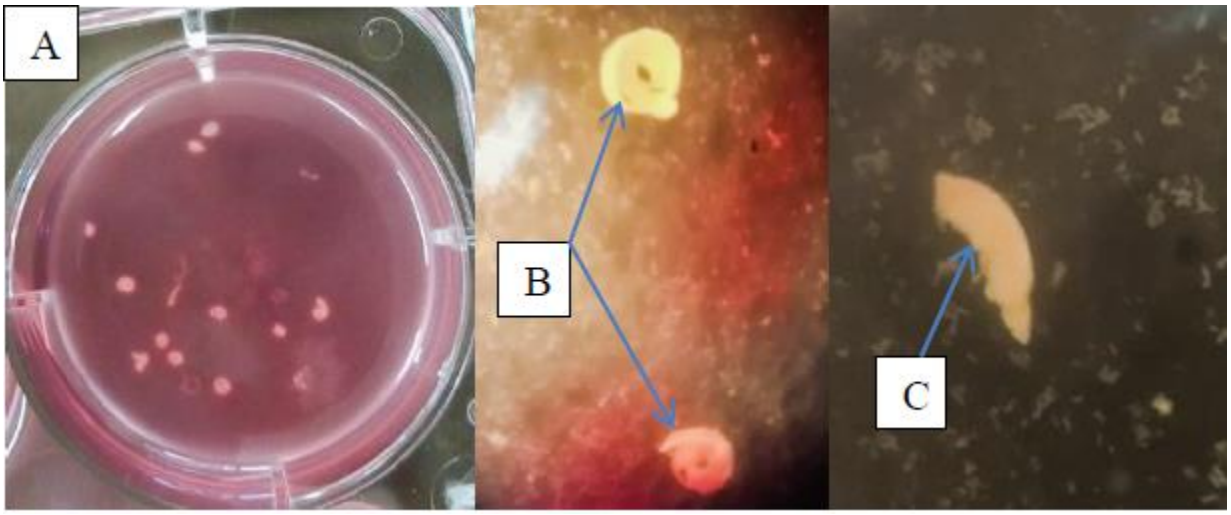


Figure 15: (A, B) Dead adult males worms displaying tight coiling characteristics after exposition to *Searsia longipes* and *Lannea schimperi* extracts, (C) worm fragmentation after been exposed to *Lannea schimperi* extract

4.1.5 Acute Toxicity of the Extracts

Following administration of 2000 mg/kg bwt of *Lannea schimperi* and *Searsia longipes* extracts, none of the treated mice have died and due to that the lethal dose 50 (LD_{50}) of the

extracts were regarded to be greater than 2000 mg/kg bwt (Table 7). Further, mice were observed individually for the behavioral and other changes, whereby *Lannea schimperi* seemed to induce tremor, sleepy and respiratory changes (Table 8). Meanwhile, *Searsia longipes* caused reduction of somatomotor activity and respiratory changes (Table 8).

Table 7: Lethal dose 50 of the extracts on mice

Extracts	Concentration (mg/kg bwt)	No of live mice	LD_{50} (mg/kg)	GHS
LSM	2000	3	> 2000	Category 5
SLM	2000	3	> 2000	Category 5

[a]Lethal dose 50

[b]Global harmonized classification system

Table 8: Mortality and behavioral assessment of mice following administration of 2000 mg/kg bwt of the extracts

	LSM			SLM		
Observation	**M1**	**M2**	**M3**	**M1**	**M2**	**M3**
Comma	No	No	No	No	No	No
Convulsion	No	No	No	No	No	No
Tremors	Yes	Yes	Yes	No	No	No
Salivation	No	No	No	No	No	No
Skin and fur	Normal	Normal	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal	Normal	Normal
Motor activity	Normal	Normal	Normal	Reduced	Reduced	Reduced
Lethargy	No	No	No	No	No	No
Diarrhea	No	No	No	No	No	No
Sleepy	Yes	Yes	Yes	No	No	No
Respiratory changes	Yes	Yes	Yes	Yes	Yes	Yes
Death	No	No	No	No	No	No

M1 = Mice 1, M2 = Mice 2, M3 = Mice 3

4.1.6 Cytotoxicity Test on Brine Shrimp Larvae

Both *Lannea schimperi* and *Searsia longipes* extracts demonstrated concentration dependent mortality of the brine shrimp larvae. Whereby, the viability seemed to decrease as the concentrations of the extracts increased (Fig. 16). Nonetheless, both plants were significant toxic to brine shrimp larvae and divulged lethal concentrations 50 (LD_{50}) of 150 and 280 µg/ml for *Lannea schimperi* and *Searsia longipes* respectively as depicted in Table 9.

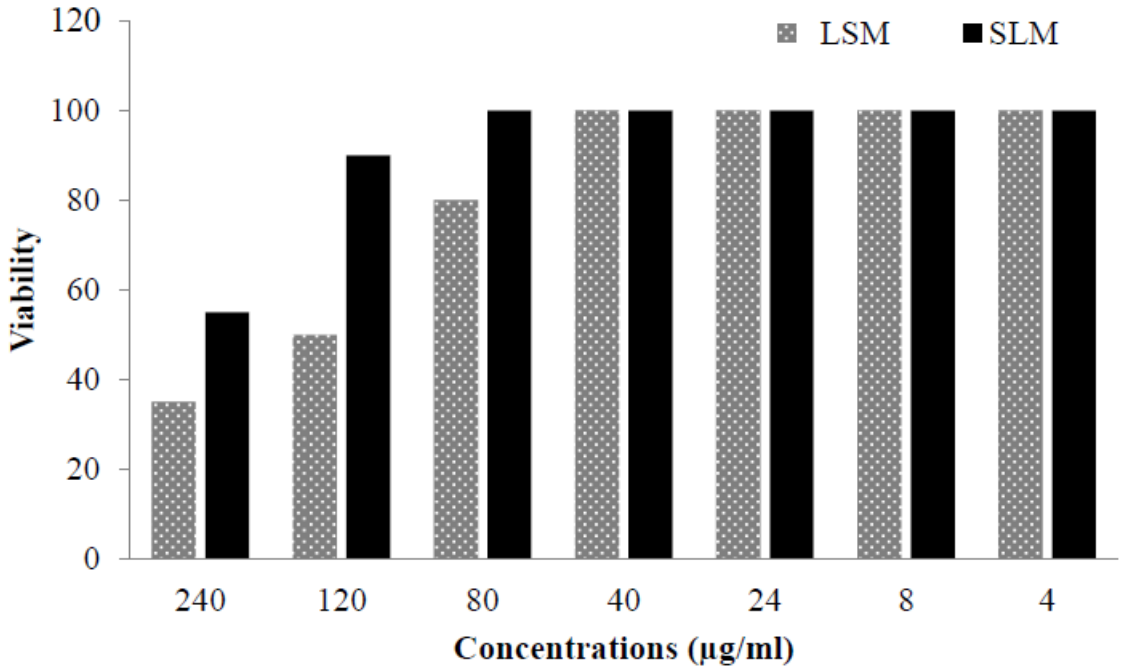


Figure 16: Viability of brine shrimpe larvae at different concentrations of *Lannea schimperi* (LSM) and *Searsia longipes* (SLM)

Table 9: Cytotoxicity of the extracts

Sample Code	[a]LC_{50} (µg/ml)	95% [b]CI; (µg/ml) Lower Limit – Upper Limit	Regression Equation	Retention Factor (R^2)
LSM	150.0478	118.0559 - 199.1434	Y = 87.647logx - 140.74	0.9542
SLM	280.7875	210 - 375.1040	Y= 96.664logx - 186.67	0.9745
[c]Cyclophosphamide	16.365	12.006 - 22.305	Y = 69.9680logx - 34.9360	0.994929

[a]Lethal Concentration 50 , [b]CI = Confidence Interval, [c]Positive control

4.1.7 Qualitative Phytochemical Screening of the Extracts

Methanolic extracts of *Lannea schimperi* and *Searsia longipes* were screened for five major groups of compounds namely terpenoides, flavonoids, glycosides, tannins and saponnins. Hence, following phytochemical screening both extracts were found to contain flavonoids, glycosides, tannins and saponnins as displayed in Table 10.

Table 10: Groups of compounds present in *Lannea schimperi* and *Searsia longipes* extracts

Phytochemical compounds	Extract	
	LSM	SLM
Terpenoids	-	-
Glycosides	+	+
Flavonoids	+	+
Tannins	+	+
Saponnins	+	+

+ = present, - = absent

4.2 Discussion

4.2.1 *In vitro* Schistosomacidal Assay

Schistosomiasis is termed as the neglected tropical disease of public health imperative, and it has ranked as second parasitic disease leading for causing death following malaria (Van Bogaert, 2011; Riveau *et al.*, 2012; Coli *et al.*, 2014). Moreover, there are only two drugs namely praziquantel and oxamniquine recommended by WHO (World Health Organization) for management of schistosomiasis. Despite being the only drugs available for the treatment of schistosomiasis, the aforementioned drugs are facing the challenge of resistance, whereby field resistance to these drugs were reported in Kenya, Senegal and Egypt (Pica-mattoccia and Archer, 1995; Cioli *et al.*, 2014; Vale *et al.*, 2017). Due to these reasons, searching for the new drug to combat this disease is therefore mandatory (Cioli *et al.*, 2014). Study on medicinal plant is an emerging field which has demonstrated the potential of solving this problem (Tekwu *et al.*, 2017). In this aspect, medicinal plants are investigated as the possible source of new therapeutic agents based on their ethno botanical uses (de Oliveira *et al.*, 2014).

Lannea schimperi and *Searsia longipes* are plants in the family *Anacardiaceae*. These plants are utilized ethno medically for management of an array of disease conditions including helminthic infections (Okoth, 2014). Further, in some bioactivity studies *Lannea schimperi* has demonstrated the broad spectrum anti-microbial, antifungal and anti-inflammatory activity (Kisangau *et al.*, 2009; Haule *et al.*, 2012; Egbe *et al.*, 2016). Aziz and coworkers (2011) have reported that, plants possessing the aforementioned activity are more likely to have anti-schistosomal activity. Therefore, these activities and ethno-botanical information

cumulatively have made these plants to be the possible candidates for evaluation of anti-schistosomal activity (Aziz *et al.*, 2011). Thus, in present study the *Lannea schimperi* and *Searsia longipes* were investigated for the *in vitro* schistosomacidal activity against three life stages of *Schistosoma mansoni* namely cercariae, schistosomula and adult worm. Since current drugs lack efficacy on the first two stages and considering their importance in pathophysiology of the disease, it is therefore necessary to test the extracts against these stages (de Oliveira *et al.*, 2014; Vale *et al.*, 2017).

In the current study, *in vitro* evaluations of both *Lannea schimperi* and *Searsia longipes* methanolic extracts against cercariae stage have exhibited significant anti-schistosomal activity. Whereby, *Searsia longipes* methanolic extract divulged 100% mortality of cercariae at the highest concentration of 2 mg/ml, meanwhile *Lannea schimperi* has demonstrated 100% reduction of cercariae viability at two higher concentrations of 2 mg/ml and 1 mg/ml. Further, concentration based reduction of cercariae viability were observed in both extracts, however *Lannea schimperi* was more efficacious compared to *Searsia longipes* extract.

Similar results were observed by Mohamed *et al.* (2005) and Tekwu *et al.* (2017) and reported the cercariacidal activity of *Nigella sativa* seeds and *Rauwolfia vomitoria* extracts respectively to be concentration dependent. The same observation were also reported by Kiros *et al.* (2014), whereas the cercariacidal activity of aqueous extract of *Glinus lotoides* fruits were reported to increase as concentration increased. Additionally, activity of both extract tested in the present study were observed to be time specific and in all tested concentration activity occurred after six hours of exposure.

Interestingly, *Lannea schimperi* methanolic extract has observed to induce tail detachment particularly at concentration below 0.5 mg/ml. The same observation was reported by Tekwu *et al.* (2017) during an *in vitro* evaluation of cercaricidal activity of *Rauwolfia vomitoria*. Ability to induce tail detachment might be of medical implication because tails play a vital role during infection particularly in identification of the susceptible host. Perrett and coworkers (1995) reported the cercariacidal activity of *Millettia thonningii* and isoflavonoid and alpinumisoflavone were anticipated to induce that activity. Since *Lannea schimperi* and plant from *Searsia* genus have also reported to possess flavonoids, therefore these compounds together with other related compounds may be responsible for the cercariacidal activity exhibited in present study.

Moreover, the *in vitro* evaluations of *Lannea schimperi* and *Searsia longipes* methanolic extracts against schistosomula stage have also demonstrated significant activity. Whereby, a concentration and time based reduction of the schistosomula viability were observed. Similar findings were reported by Aziz and coworkers (2011) during an *in vitro* evaluation of *Plectranthus tenuiflorus* extract on different life stages of *Schistosoma mansoni*, where the viability reduction was proportional to the increase of concentration. Further, extracts seemed to have an impact on the muscular function which in turn lead to the reduced and completely loss of motor activity. Contrary to Tekwu *et al.* (2017) and in harmony with Aziz *et al.,* (2011), in the current study, schistosomula were observed to be more susceptible to the extract than other life stages.

Nonetheless, *in vitro* evaluation of the extracts on the adult worms has also exhibited significant activity. Whereby, *Searsia longipes* extract was more efficacious, since it caused 100% mortality of the males worms up to the lowest concentration used in the present study and 100% mortality of all worms at the three higher concentrations after 48 hours of exposition. Whilst, *Lannea schimperi* extract induced 100% mortality of the males worms up to the second lower concentration of 0.05 mg/ml and 100% mortality of all worms at two highest concentrations. These observations indicated that, extracts are more potent to male's worms than female's worms. Henceforth, these observations agree with the Cioli and coworkers (1995) report on the anti-schistosomal activity of oxamniquine, whereby it was reported to have more potency to male's worms than females. Additionally, both extracts were able to induce dose dependent reduction of motility of the worms; similar result was reported by de Oliveira and cowekers (2014) when evaluating the activity of *Baccharis trimera* on adult *Schistosoma mansoni.* Motility reduction induced by these plants extracts may be related to the ability to cause effect on neural transmitters such as dopamine and acetylcholine. Meanwhile, both extracts were observed to induce worm coiling characteristics particularly on males; this may be related to what referred as contraction by de Oliveira *et al.* (2014). Worms subjected to positive control (Praziquantel) have also demonstrated coiling characteristic, however the coiling was not tight as that of the extracts. Tightly coiling/ contraction characteristic exhibited by treated worms may be due to induced calcium ion influx, resulted from the extracts impact on the receptors and channels such as FMRFamide-related peptide and serotonin that modulate Ca^{2+} ion level (Fetterer *et al.*,1980; Greenberg, 2005). Further, *Lannea schimperi* extract at concentration ranging from 2 mg/ml to 0.025 mg/ml was able to induce worm fragmentation. Since integument wall was reported to be the

main target for many anti-schistosomal agents as reported by Dias and coworkers (2017), thus worm's fragmentation may be related to the extract effect on the integument wall.

4.2.2 Acute Toxicity

Medicinal plants have being utilized for decades in different places of the world and particularly by economically disadvantaged societies as an alternative to the industrial made drugs for management of diseases (WHO, 2004). Beside the economical reason, preferability of the medicinal plants are due to accessibility and cultural acceptability (WHO, 2013). However, despite the huge contribution of the medicinal plants on sustaining the wellbeing of people in traditional settings, safety has becoming a major challenging issue (WHO, 2004; Ekor, 2014). This is due to the fact that, many medicinal plants are employed to human subjects without prior evaluation of their optimal doses (Ekor, 2014). Thus in turns leads to side effects resulted from drug overdose or interaction with other therapeutic agents (WHO, 2004). Henceforth, toxicity evaluations of the extract from herbal plants are very important since will reveal the toxicity profile of the plants as well as proper doses.

In this study, acute toxicity tests were performed to evaluated safety of *Lannea schimperi* and *Searsia longipes* on Swiss albino mice. Following administrations of 2000 mg/kg bwt doses of the extracts to the groups containing 3 mice, mortality and behavioral changes were assessed. For *Searsia longipes*, 30 minutes after dosing animals demonstrated respiratory problem and was manifested by rapid and existence of voice during breathing. This character disappeared after 4 hours of observation. Other changes such as reduced motor activity were also observed, however all of the aforementioned changes were disappeared after four hours. Regarding mortality rate, none of the treated mice was died after administration of 2000 mg/kg dose. Henceforth, the lethal dose $_{50}$ of the extracts are therefore regarded to be greater than 2000 mg/kg, thus it falls under category five (5) of the GHS as stipulated in OECD guideline number 423. This observation agrees with the Olorunnisola and coworkers (2017) report on the acute toxicity of the acetone leaf extract of *Searsia longipes*, whereby the latter was reported to have the LD $_{50}$ above 5000 mg/kg bwt. This finding portrayed that, *Searsia longipes* possesses good margin of safety since did not kill any of the tested animal at the highest limiting dose of 2000 mg/kg bwt.

Lannea schimperi in particular, has also divulged good margin of safety, since did not kill the mice following administration of limiting dose of 2000 mg/kg bwt. This result is in harmony with the Haule and coworkers (2012) report on the acute toxicity of the ethanolic extract of

the *Lannea schimperi*, whereby the LD_{50} was above 5000 mg/kg body weight. Moreover, the finding is in line with Dialo *et al.* (2009, 2010) report on the acute toxicity of *Lannea kerstingii* which shares similar genus with *Lannea schimperi*. Nonetheless, the plant extract seemed to induce tremors, respiratory changes, sleeping and reduced food and water intake.

4.2.3 Cytotoxicity

Brine shrimp lethality bioassay employs the larvae of the brine shrimp *Artemia salina* which belongs to family *Artemiidae* (Parra *et al.,* 2001). In recent years, it became the method of choice for evaluation of the general cytotoxicity of medicinal plants extracts, pesticides and heavy metal (Wu, 2014). Meanwhile, there are many reasons for the preferability of this test, among which low cost, shorter time and do not need sophisticated equipment's. Henceforth, in present study general cytotoxicity of *Lannea schimperi* and *Searsia longipes* were evaluated by using brine shrimp lethality assay. Whereby, both extracts from *Lannea schimperi* and *Searsia longipes* demonstrated concentration dependent mortality induction and significant cytotoxicity on brine shrimp larvae with the IC_{50} of 150 µg/ml and 280 µg/ml respectively. *Lannea schimperi* seemed to be more potent since it expressed lower IC_{50} than that of the *Searsia longipes*. The result agrees with that of Haule *et al.* (2012), who reported the cytotoxicity of the acetone extract from *Lannea schimperi* to be 128 µg/ml. The overall cytotoxicity findings are in line with Meyers and coworkers (1982) protocol on the brine shrimp lethality assay, which demonstrated the cytotoxicity of the plant extract below 1000 µg/ml to be significant active. In the light of correlation between brine shrimp lethality and cancer cell toxicity, therefore toxicity expressed by these plants may be related to their ability to kill cancer cells (Meyer *et al.,* 1982; Sánchez, 1993). Hence, further studies are warranted to evaluate the cytotoxicity of these plants on cancer cells.

Moreover, *in vitro* and *in vivo* toxicity result did not express correlation. This result is in harmony with that of the comparative study done by Sánchez and cowekers (1993). Meanwhile it disagrees with the findings of the comparative study done by Parra *et al.* (2001). Hence, based on current findings brine shrimp lethality assay should not replace toxicity studies in animal models, despite the advantages of the former in economic point of view.

4.2.4 Phytochemical Screening

Phytochemicals are the compounds produced and employed by plants for different proposes, among which controlling growth, reproduction and protection against short and long term threats such as competitor and pathogens (Molyneux *et al.,* 2007). Despite their imperatives to plants, some of these compounds are worth to human health since can cure an array of diseases. Hence, phytochemical screenings of medicinal plants along with bioactivity assays are vital because will reveal the plant active principles, which in turn will enhance new drugs development (Kubmarawa *et al.,* 2007).

Phytochemical screenings of the extracts from both *Lannea schimperi* and *Searsia longipes*, have exhibited the presence of flavonoids, tannins, saponnins and glycosides. These findings are in harmony with the Egbe and coworkers (2016) report, which demonstrated the presence of flavonoids, tannins, saponnins and glycosides in the acetone extract of the *Lannea schimperi*. For the *Searsia longipes* in particular, this is the first study to report its phytochemical constituents. However, results are in harmony with the findings from phytochemical screening of other plants under *Searsia* genus, which reported to poses flavonoids, saponnins and tannins (Gibr, 2015; Mtunzi *et al.*, 2017). Nonetheless, the two plants seemed to possess similar phytochemical compounds, and this may be due to close relatedness since they share a family as well as habitat from which they were collected.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Lannea schimperi and plants from *Rhus/Searsia* genus have being extensively evaluated for their activities against different disease conditions based on the ethno botanical information's. In the current study these plants were validated for their ethno botanical uses in management of helminthic infections, whereby both have exhibited significant activity on different life stages of the *Schistosoma mansoni*. This is the first study to evaluate and report the *in vitro* schistosomacidal activity of *Lannea schimperi* and *Searsia longipes* methanolic extracts. Hence, *in vivo* studies to validate schistosomacidal activity of the extracts is also warranted.

Both extracts from *Lannea schimperi* and *Searsia longipes* had similar results in term of the phytochemical compounds and exhibited presence of vast groups of compounds. Therefore, this study confirms previous reports on the phytochemical compounds of *Lannea schimperi*. Meanwhile, for the first time this study reports the presence of phytochemical compounds namely flavonoids, tannins, saponnins and glycosides in *Searsia longipes* extract.

Extract from *Lannea schimperi* and *Searsia logipes* exhibited good margin of safety on Swiss albino mice, hence can continuously being employed in tradition settings for management of diseases. However, further studies to evaluate toxic effect on the prolonged exposure to the extracts are also necessary. Moreover, both extracts have significant cytotoxicity on brine shrimp larvae. Since the latter has great correlation with cancer cell, thus evaluation of the anti-cancer activity of the extracts is warranted.

5.2 Recommendations

i. *In vivo* schisosomacidal evaluations of the extracts are necessary, since will provide an overview of the pharmacokinetic of the drug.
ii. Isolation and structural elucidation of the anti-schistosomal compound in *Lannea schimperi* and *Searsia longipes* extract is of high imperative.
iii. Further toxicity evaluation can be conducted to validate the effect of the extracts on prolonged exposure to the mice.
iv. Evaluation of anti-cancer activity of the extracts particularly through *in vitro* studies is also necessary.

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RESEARCH OUTPUT

Output one: A paper on "Schistosomacidal Activity of *Lannea schimperi* and *Searsia longipes* against Cercariae, Schistosomula and Adult Stage of *Schistosoma mansoni*".

Output two: A paper on "Acute Toxicity, Brine Shrimp Lethality and Phytochemical Screening of *Lannea schimperi* and *Searsia longipes*".