Phytochemical composition, safety and *in vitro* anthelmintic activity of *Ziziphus mucronata* barks extract against *Haemonchus contortus*

NGARADOUM OLIVIER

MB300-0013/12

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DECLARATION

I do hereby declare that this thesis is my original work and has not previously been submitted anywhere else.

Signature: ..... Date: .....

NGARADOUM OLIVIER

MB300-0013/12

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

Signature: ..... Date: .....

Dr JOHN KAGIRA, Department of Animal Science, Jomo Kenyatta University of Agriculture and Technology

Signature: ..... Date: .....

Prof SIMON KARANJA, Department of Public and Community Health, Jomo Kenyatta University of Agriculture and Technology

DEDICATION

I dedicate this work to God, my Lord and Saviour and my first priority.

Mummy Amayan Esther Kladet and Daddy Bokade Ngaradoum Paul Ngarossoh, this thesis is the motive of your pride and upbringing.

My son Bokade Badjim Carlos who always promised me assistance of God in my studies.

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ABBREVIATIONS AND ACRONYMS

AE	Aqueous Extracts
ALT	Alanine transaminase
ANOVA	Analysis Of Variance
AST	Aspartate Transaminase
AWMA	Adult Worms Mortality Assay
BUN	Blood Urea Nitrogen
EC_{50}	Effective Concentration at fifty percent
EHIA	Egg Hatch Inhibition Assay
GCMS	Gas Chromatography Mass Spectrometry
GHS	Globally Harmonized System
IC_{50}	Inhibition Concentration at fifty percent
JICA	Japan International Cooperation Agency
JKUAT	Jomo Kenyatta University of Agriculture and Technology
LC_{50}	Lethal Concentration at fifty percent
LD_{50}	Lethal Dose at fifty percent
LCMS	Liquid Chromatography Mass Spectrometry
LMA	Larval Mortality Assay
ME	Methanolic Extracts
OECD	Organization for Economic Cooperation and Development
PAUISTI	Pan African University Institute for basic Science, Technology and Innovation

PBS	Phosphate Buffer Saline
SD	Standard Deviation
SPSS	Statistical Package for Social Scientist
TLC	Thin Layer Chromatography
UNDP	United Nations Development Programme
WAAVP	World Association for the Advancement of Veterinary Parasitology
WHO	World Health Organization

ABSTRACT

Helminthic infections are some of the most prevalent diseases affecting livestock and human beings in developing countries. In Chad, nematode infections are considered as a major health problem for the animal and human population. The current drugs of choice against these nematodes are known to have some limitations including toxicity, high cost and emergence of anthelmintic resistance. In Chad, the barks of *Ziziphus mucronata* are commonly used for treatment of helminthosis, although its activity has not been authenticated. Therefore, the current study aimed at determining the phytochemicals, safety and anthelmintic activity of *Z. mucronata* barks against *Haemonchus contortus*. *Z. mucronata* barks were obtained from Deli in Chad and extraction undertaken at JKUAT, Kenya. Qualitative phytochemical screening for the aqueous and methanolic extracts of *Z. mucronata* barks was done using standard methods. The *in vitro* anthelmintic activity of the plant extracts against *H. contortus* was evaluated using the egg hatch assay, larval mortality assay and adult worm mortality assay according to World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines. In accordance with the Organization of Economic Co-operation and Development (OECD) guidelines, the safety of the plant extracts was assessed in mice by acute oral toxicity test in dosages ranging from 500 to 2000mg/kg body weight. Probit analysis, paired-samples t-test and ANOVA using Turkey post hoc tests were used to determine whether there were significant differences. Phytochemical screening of *Z. mucronata* barks extracts showed the presence of saponins, tannins, glycosides, flavonoids and steroids. Anthelmintic activity of methanolic extract in egg hatch assay showed a higher activity with IC_{50} value of 3.9 mg/ml as compared with aqueous extract which had an IC_{50} value of 14.7 mg/ml. Albendazole exhibited IC_{50} value of 0.05 mg/ml in egg hatch assay. There were significant ($P<0.05$) differences in egg hatch inhibition of extracts compared to Albendazole. Results of larval mortality assay showed that a

concentration of 2.6 mg/ml of methanolic extract and 7.5 mg/ml of aqueous extract inhibited 50% (EC_{50}) of infective larvae of *H. contortus*. The EC_{50} value against larval mortality by Albendazole was 0.04 mg/ml. For larval mortality assay, the EC_{50} of the extracts were significantly ($P<0.05$) lower compared to that of Albendazole. However, for adult worms mortality assay, a maximum concentration of 2.4 mg/ml of methanolic extract, 5.0 mg/ml of aqueous extract and 0.04 mg/ml of Albendazole was required to induce mortality of 50% (EC_{50}) of adult *H. contortus* worms after 24 hours post exposure. For adult worms assay, the EC_{50} of the extracts were significantly ($P<0.05$) lower compared to that of Albendazole. Acute oral toxicity test revealed that the mean body weight of mice treated with *Z. mucronata* extracts did not differ significantly ($p>0.05$) from those of control mice. The mean values of Alanine transaminase (ALT) (in mice treated with methanolic extracts) and blood urea nitrogen (BUN) (of treated mice with both extracts) did not differ significantly ($P>0.05$) with those of the normal mice. In conclusion, this study demonstrated that methanolic extracts of *Z. mucronata* barks at dosages used were not toxic when used in the mice model and thus could be safe in mammalian hosts. Further, both extracts had an anthelmintic activity against all stages of *H. contortus* and can thus be considered for further development as an anthelmintic. The study recommends that quantification of the phytochemicals, identification and isolation of the bioactive compounds responsible for anthelmintic activity and chronic toxicity of the extracts of *Z. mucronata* should be undertaken.

CHAPTER ONE

1. 0- INTRODUCTION

1. 1- Background information

Helminthic infections of livestock and man are some of the most prevalent diseases in developing and developed countries (Krogstad and Andengleberg, 1998). Globally, an estimated two billion people are infected by gastrointestinal nematodes (Wen *et al.,* 2008). Human helminthosis in developing countries cause serious public health problems and contribute to malnutrition. Although the majority of infections due to worms are generally limited to tropical countries, they can occur in travellers who have visited those areas and some of them can occur in temperate climates (Bundy, 1994). Helminthosis affects most population in endemic areas with major economic and social consequences. Helminthosis in the livestock sector causes high economic losses through lowered fertility, reduction of food intake, reduced weight gain, lower milk production, treatment costs and mortality in severely parasitized animals (Zeryehun, 2012).

The helminths can be classified as nematodes, cestodes and trematodes. In humans, the commonest nematodes include *Ancylostoma duodenale* and *Ascaris lumbricoides*. *Taenia solium* and *T. saginata* are examples of cestodes common in human beings while *Schistosoma mansoni* and *S. haematobium* are the common trematodes. *Haemonchus contortus* is the common nematode of domestic animals in Africa. In both livestock and human beings, control of these parasites relies on modern anthelmintics and other strategies such as improvement of sanitation and public health education. Ideally an anthelmintic agent should have broad spectrum of action, high percentage of cure, be safe to the host and should be cost effective (Liu and Weller, 1996). However, most of the synthetic drugs do not meet these requirements. Resistance of the parasites to the existing drugs (Piyush *et al.,* 2013) and

their high cost warrants the search for newer anthelmintic molecules. In this regard, herbal drugs are perceived to be low cost, safe, and efficacious and are available to vulnerable sections of human populations in developing countries such as Chad (Sandhya and Kumar, 2010; Valiathan, 1998).

Due to the above mentioned challenges in modern drugs, most of the population in the world uses traditional medicinal remedies. Indeed, medicinal plants are used as anthelmintic agents by majority of the people in tropical developing countries (Satyavati, 1990). Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, particularly in the developing nations (WHO, 2002). It is also important to note that some of commonly used drugs were originally sourced from medicinal plants.

In Africa, *Z. mucronata* plant is used for treatment of variety of conditions including gastro-infections, schistosomiasis, boils, chronic cough, infertility, oedema, pneumonia, snake bite, toothache, venereal diseases and wounds (Palmer and Pitman, 1972; Hutchings *et al.,* 1996; Maroyi, 2013). A study done by Mølgaard *et al.,* (2001) showed that extracts from *Z. mucronata* have anti-schistosomiasis properties. In Zimbabwe, the leaves and fruits are sources of feed for small ruminants (Munodawafa, 2012).

1. 2- Problem statement

Most helminthic infections occur in tropical countries or developing countries where risk factors for helminthosis including poor sanitation, poor personal hygiene among others abound. Billions of people around the world are infected with helminths which cause serious morbidities and mortalities (Wen *et al.,* 2008). In tropical countries, gastrointestinal nematodes are responsible for a 23-63% reduction in growth, up to 25% of pre-weaning mortality and 24-47% of reductions in annual off take from small ruminants (Kumar *et al.,* 2010). The resistance has caused great losses to the poor farmers in both tropical and sub-

tropical countries due to the cost of treatment and mortality of their livestock (Siddiqui, 2009). Helminthiasis has been shown to be one of the most common setbacks in production and reproductive performance of livestock (Agaie and Onyeyili, 2007). A highly pathogenic nematode, *H. contortus*, is capable of causing acute disease and high mortality in all classes of stock and is the most prevalent and economically important parasite (Githiori, 2004; Eguale *et al.,* 2006).

However, the main limitation for anthelmintics use has been emergence of resistance in most of the livestock helminthes against the current drugs (Piyush *et al.,* 2013). The side-effects, the high cost and unavailability of anthelmintic drugs in some areas limit the use of anthelmintics especially in developing countries. From the foregoing, there have been calls for development of new drugs, chiefly from plants. This is also due to the availability of the medicinal plants and common usage in the treatment of parasites. However, the current use of herbal preparations is has not been well investigated and thus there is no precise dosage and their toxicity has not been tested. In Chad, the barks of *Z. mucronata* are commonly used for treatment of helminth infections (Personal communications, Herbalist, Deli in Chad), whereas in other African countries it is used for treatment of various ailments (Maroyi, 2013). However, the plant's anthelmintic activity has not been validated.

1. 3- Justification for the study

Many diseases caused by helminths are chronic, debilitating in nature, they probably cause more morbidity, greater economic and social deprivation among humans than other parasites. It has been estimated that about half of the world's population suffers from helminthosis. Helminthosis is not only limited to tropical countries but is also endemic in many regions because of poor sanitation, poor family hygiene, malnutrition and crowded living condition (Mohammed *et al.,* 2005).

Livestock is a major source of food and direct income from sale of the animals and animal products like meat, milk, wool, hides and skins. Livestock has traditionally been considered as a source of wealth to the pastoral communities in Africa. Loss of livestock due to helminths and helminths-related ailments has contributed to community conflicts as they try to restock their lost livestock. As a direct consequence of these losses, the family fabrics are weakened due to reduced income and food. Anthelmintic drugs became available a long time ago. However the high costs of these modern anthelmintic have limited the effective control of the parasites (Barar, 2000). In Chad, the estimated population is 12,830,000 with poverty rate 62.9%. The per capita income of Chad is $ 876 and Human Development Index 0.372 according to report of United Nations Development Programme (UNDP) in 2013. This situation shows that majority of Chadian people are poor and cannot afford costly conventional drugs. For these reasons, people in Chad commonly use the barks of *Z. mucronata* for treatment of helminth infections, although the usage of the plant has not been investigated scientifically. The proposed study aimed at investigating the safety and the *in vitro* activity of this medicinal plant against gastrointestinal nematode infections.

1. 4- Null hypothesis

Ziziphus mucronata barks extracts are unsafe in Swiss white mice model and ineffective against *Haemonchus contortus.*

1. 5- Main objective of the study

To determine the phytochemicals, safety and *in vitro* anthelmintic activity of *Ziziphus mucronata* barks against *Haemonchus contortus* using *in vitro* tests and animal models.

1. 6- Specific objectives

1) To determine the phytochemical constituents of *Ziziphus mucronata* barks extract

2) To determine the *in vitro* anthelmintic activity of *Ziziphus mucronata* barks extract against *Haemonchus contortus*

3) To determine the oral acute toxicity of *Ziziphus mucronata* barks extract in Swiss white mice

1. 7- Research questions

1) What are the major phytochemical constituents of *Ziziphus mucronata* barks extract?

2) What is the *in vitro* anthelmintic activity of *Ziziphus mucronata* barks extract against *Haemonchus contortus*?

3) What is the safe dose level of *Ziziphus mucronata* barks extract in Swiss white mice?

CHAPTER TWO

2. 0- LITERATURE REVIEW

2. 1- Epidemiology of human helminthosis

Helminthic infections affect a large part of the population in the world, particularly in the tropical and subtropical countries in which the sanitation conditions and the water supplies are poor. The prevalence of nematodes infections worldwide is high and varies from one country to another (Beyrer *et al.*, 2007). The estimated burden of nematode infections in the world in 2005 according to report of World Health Organization (WHO) was Ascariasis: 0.807 – 1.221 billion persons, Trichuriasis: 604 – 795 million while hookworm infection accounted for 576 – 740 million cases (Hökelek, 2011).

2. 2- Classification of helminths

Helminths are divided into three groups based on their body segmentation: trematodes (flukes), nematodes (roundworms) and cestodes (tapeworms) **(Figure 2.1)**. Helminths have multi-cellular body and complex life cycle involving maturation in a host organism.

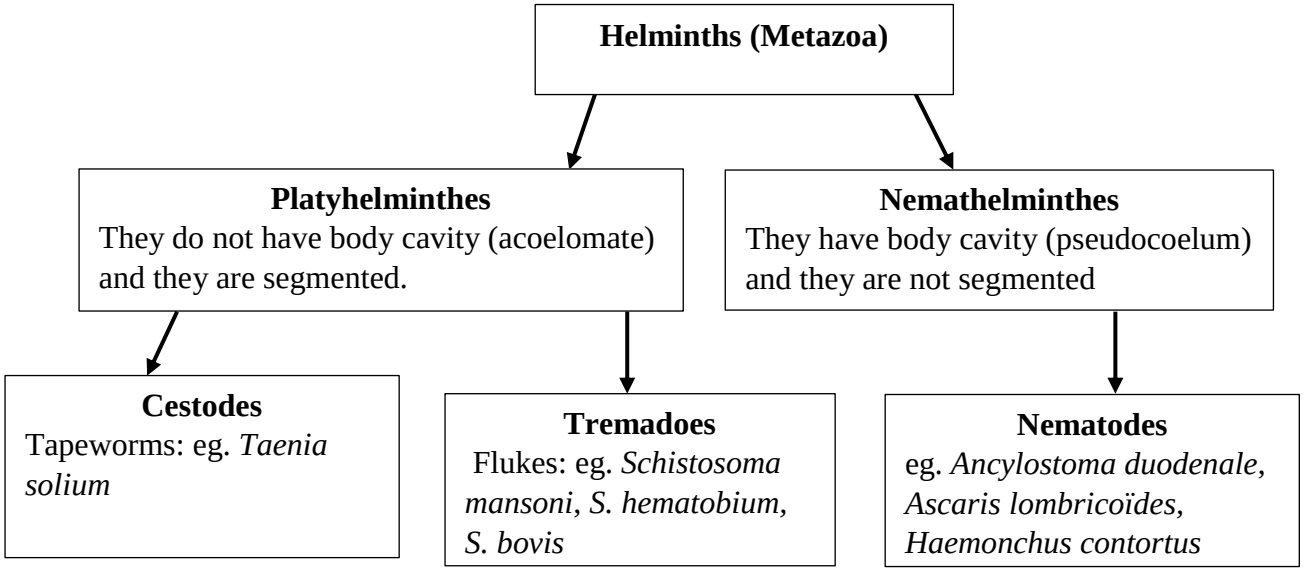

Figure 2.1: Classification of helminths

2. 3- Morphology and life cycle of human nematodes

Ascaris lumbricoides is one of the most common nematodes in man, affecting 2 billion people in the world. The adult Ascaris male measures 15-30 cm with a diameter between 3-4 μm and the female is 20-35 cm long with a diameter of 5μm. Both are creamy white. The adult worms live in the lumen of the intestine where the female produce approximately 200,000 eggs per day, which are passed out with the feces. The unfertilized eggs cannot undergo biological development while the fertilized eggs embryonate and become infective after several weeks. After infective eggs are swallowed, the larvae hatch, invade the intestinal mucosa. They are carried via the blood circulation to the lungs. The mature larvae penetrate the alveolar walls and reach the small intestine where they develop into adults (Michael *et al.,* 2014).

Ancylostoma duodenale and *Necator americanus* are two independent species of nematode hookworms. But they have similar morphology and measure, female 10-13 μm and male 8-11 μm. The buccal cavity of *Ancylostoma duodenale* bears two hooks like teeth on the top and two triangular cutting plates on the bottom. While the mouth of *Necator americanus* has four cutting plates, two on the ventral and two on the dorsal surfaces.

Both live in the small intestine, fixed to the mucous membrane and feed on blood and tissue. The adult females produce up to 20,000 eggs which are passed out in the feces. The released rhabditiform larvae grow and become filariform larvae that are infective. On contact with the human host, these infective larvae penetrate the skin and are carried through the veins to the heart and then to the lungs. They penetrate into the pulmonary alveoli, ascend the bronchial tree to the pharynx, and are swallowed then reach to the small intestine where they reside and mature into adults (Michael *et al.,* 2014).

2. 4- *Haemonchus contortus* as a model system in anthelmintic discovery and resistance research

Haemonchus contortus is a parasitic nematode of small ruminants and is of major socio-economic importance in many regions of the world (Urquhart *et al.,* 1996). The larvae of stage four (4) and adult stages reside on the surface of the host's abomasal (gastric) mucosa and feed on blood. The blood loss associated with parasite feeding and the haemorrhage from the associated mucosal lesions results in haemorrhagic anaemia. The severity of this is related to worm burden but it is often serious and not infrequently fatal (Urquhart *et al.,* 1996). Resistance of nematodes to commercial anthelmintics, mainly in the tropical regions, initiated intensive research on the search of novel anthelmintic lead compounds.

The effects of several plant extracts on *H. contortus* and other important gastrointestinal nematodes using egg hatch and larval Mortality tests have been examined by many authors (Gabino *et al.,* 2010, Aroche *et al.,* 2008). *Haemonchus contortus* has a very high propensity to develop resistance to anthelmintics and is the parasite species which has developed resistance most rapidly and in which resistance is most widespread (Kaplan, 2004). Partly because of this, and partly because of its economic importance and experimental tractability, it is the helminth species for which we have the greatest understanding of mechanisms, genetics, diagnosis and management of anthelmintic resistance (Gilleard, 2006). It is also one of the parasites for which we understand most about the modes of action of anthelmintics (Saunders *et al.,* 2013). Consequently, it already provides a framework for much of the anthelmintic resistance and drug discovery research occurring in other parasite species. *H. contortus* has also been an extremely important organism in anthelmintic discovery research within the pharmaceutical industry for many years. An excellent recent example of the value of *H. contortus* for drug discovery and mode-of-action studies has resulted in the

development of a new anthelmintic monepantel (Kaminsky *et al.*, 2008). The Novartis Animal Health group were exploring amino-acetonitrile derivatives (AAD) in their search for new anthelmintics. Six hundred AAD analogues were screened using an *H. contortus in vitro* larval development assay followed by *in vivo* validation in gerbils infected with *H. contortus* and *Trichostrongylus colubriformis*. Promising lead compounds were then tested by *in vivo* efficacy tests on *H. contortus* in sheep before other ruminant gastrointestinal nematodes were tested in the later stages of the development process. Most studies have recommended that *H. contortus* can be used in parallel with *Caenorhabditis elegans* to study the mechanisms of drug action and resistance and represents a notable success story in anthelmintic discovery and development.

2. 5- *In vitro* anthelmintic activity trials

Several *in vitro* anthelmintic activity tests have been developed and proposed for the screening of drug resistant strains of nematodes and anthelmintic discovery and development (Várady *et al.* 2009), which target either the eggs (egg hatch test), the larval stages (larval development assay, larval migration inhibition assay, larval mortality assay), or the adult stage (adult worms mortality assay). Some of these bioassays are employed in screening of nematodicidal effects of plants.

2. 6- Pathology caused by helminths

Helminths worms derive nutrients from their host leading to several pathophysiological sequelae. By consuming nutrients from their host, worms cause malnutrition in which result physical development and retard growth. Other consequences include: anaemia due to the iron deficiency, retardation of cognitive development, abdominal pains, malnutrition and others health problems of nematode infections such as decrease immune status of host (Crompton and Nesheim, 2002; Kirwan *et al.*, 2009). The decreasing host immune status as

result of nematode infections, therefore increases susceptibility to others pathogens to the host (Brooker *et al.,* 2006; Borkow and Bentwich, 2006). Others symptoms associated with intestinal helminths infection are insomnia, weakness, intestinal obstruction, stomach pains and vomiting (John and William, 2006). The increasing prevalence of helminths infections and treatment failure is a major problem facing human health in most African countries (James and Davey, 2007, 2009).

2. 7- Conventional anthelmintic drugs

Mebendazole, Albendazole, Pyrantel and Levamisole are the four major anthelmintic drugs used for the treatment of gastrointestinal nematode infections **(Table 2.1)**. These drugs are classified into two groups:

a) Group 1: Benzimidazoles are composed of Mebendazole and Albendazole which are broad spectrum drugs that inhibit microtubule synthesis in nematodes and irreversibly block glucose uptake. This leads to the lack of glycogen and decrease of production of adenosine triphosphate which is needed for survival of the parasites and their reproduction. Due to inhibition of microtubule synthesis the worms are immobilized leading to death (Jay and John, 1979).

b) Group 2: Tetrahydropyrimidines/Imidazothiazoles includes Levamisole and Pyrantel. Levamisole is an anthelminthic active against nematodes infection through stimulating and blocking the neuromuscular junctions. This causes paralysis of parasites, which are eventually excreted through faeces (Rang *et al.,* 2003). Pyrantel works by depolarizing the neuromuscular junction of helminths, thus inducing paralysis. It is particularly effective against *Ancylostoma duodenale*. Pyrantel also, has some anticholinesterase activity (Rang *et al.,* 2003).

Table 2.1: Anthelminthic drugs currently used against infections with human gastrointestinal nematodes and their percentage cure rates

Anthelminthic	**Hookworms**	***Ascaris lumbricoides***	***Trichuris trichiura***	***Enterobius vermicularis***	***Strongyloides stercoralis***
Mebendazole	95-100%	95-100%	45-100%	96%	44%
Albendazole	33-95%	67-100%	10-77%	40-100%	17-95%
Levamizole	66-100%	86-100%	16-18%	-	-
Pyrantel	37-88%	81-100%	0-56%	>90%	-
Thiabendazole	-	-	-	-	89-100%
Piperazine	-	74-94%	-	90%	-

(Gillian Stepek *et al.,* 2006), (Horton, 2000), (Albonico M. *et al.,* 1999)

In general, anthelmintics are well tolerated and rarely cause serious side effects. The most common side effects are cramps, diarrhea, headache and feeling sick. The use of Albendazole in the treatment of gastrointestinal infections produces few side effects such as nausea, vomiting and headache (Horton, 2000). While Mebendazole produces rarely allergic reactions and hypospermia. It is advised that Mebendazole should not be taken by pregnant women and children less than 2 years of age, because it has been shown to be a potent embryotoxin and teratogen in laboratory animals (Horton, 2000). Headache, rash and fever are sometimes observed in Humans during the use of Pyrantel pamaote. Recently the emergence of resistance of parasites to the synthetic anthelmintics that are used currently has been notified. Also, the use of anthelmintics produces toxicity in human beings (Piyush *et al.,*

2013). Hence the development of new substances as anthelmintics from plants which are considered to be the best source of bioactive substances will solve the problem of anthelmintic resistance.

2. 8- Herbal plants used as anthelminthic drugs in Africa

Some of the common conventional anthelminthics have side effects and are contraindicated in pregnancy. Some of the factors which have led to increased usage of herbal plants as anthelmintics include:

- Medicinal plants are safe to ingest and directly kill the parasites
- The anthelminthic activities of herbal plants are consistent
- They are broad-spectrum
- The traditional method of preparation anthelmintic plants is simple
- The herbal drugs have few side effects (Piyush *et al.*, 2013).

Determination of the efficacy and the toxicity of the herbal plants are necessary to validate these medicinal plants as the local anthelmintics used in the treatment of gastrointestinal nematode infections.

2. 8. 1- *Albizia anthelmintica*

Albizia anthelmintica is a deciduous shrub of Mimosaceae family which grows up to 8m tall. It has gray, smooth, bark and bipinnate leaves. The flowers are pale greenish and usually appear when the shrub is almost leafless. The root bark concoction is used to treat intestinal worms infection. It is a common shrub in dry bushland and in Maasai-land it is the most effective medicine against intestinal worms. Saponins from its bark have been reported in South West Africa (Kareru, 2008; Githiori, 2004). Saponins were first reported to kill worms as early as 1962, using an extract from *Albizia anthelmintica*. Phytochemical studies using

extracts from *A. anthelmintica* has shown presence of triterpenes, saponins, tannins and anthraquinones (bound) (Kareru, 2008). Sesquiterpene and kosotoxins have also been isolated from the bark and root bark extracts of *Albizia anthelmintica* (Husain *et al.,* 2008).

2. 8. 2- *Prosopis juliflora*

Prosopis juliflora is an evergreen tree with a large crown and an open canopy and can grow to a height of up to 14 m. Its stem is greenish-brown, sinuous and twisted with axial and strong thorns. The barks are reddish-brown and rough and the root system has a deep tap root that allows the tree to reach deep water tables. The leaves are compound, dark bluish-green and are high in tannin. Its fruits are pods, which are green when immature and yellow when mature and are high in sugar content (Matthews and Brand, 2004). The tree is native to South America, Central America and the Caribbean (Pasiecznik *et al.,* 2004). It was introduced in Kenya in the early 1970s from Latin America and is locally known as "Mathenge" (Maghembe *et al.*, 1983). In Kenya, it is considered as a noxious weed.

2. 8. 3- *Azadirachta indica A. Juss.*

This plant is commonly known as neem, neem-tree, Indian lilac, or white cedar. It is a hardy tree growing to a height of 15 to 20 m, with a dense leafy, oval-shaped canopy. The bark is rough, pale grey-brown in colour. The plant has shiny compound leaves, with 5 to 8 pairs of leaflets, crowded towards the end of branches. The flowers are scented, small creamy white and hang down in long sprays, while the fruits are oval and yellow when ripe, yielding aromatic oil. It is found in arid and semi-arid areas of Eastern Africa (Dharani, 2002). The plant is used in various ailments including helminth parasites (Githiori, 2004).

2. 8. 4- *Carica papaya Linn.*

The tree is distributed worldwide. The active ingredient is known as benzyl isothiocyanate. The benzyl isothiocyanate exerts its action by inhibiting energy metabolism and hence affecting motor activity of the parasite (Mali and Mehta, 2008).

2. 8. 5- *Salvadora persica L.*

This is widely distributed in Africa and Asia. In Tanzania it is found in every district and has different uses (Mbuya *et al*., 1994). Various plant parts are edible. It is believed to possess antimicrobial and other medicinal properties and it is important in dental hygiene thus the plant has been used as toothbrush for centuries (Ruffo *et al*., 2002).

2. 8. 6- *Calotropis procera*

The plant belongs to the family *Asclepiadaceae*. It is commonly used for the treatment of syphilis, eczema, and leprosy. It is effective against *H. contortus* where it acts by decreasing the number of larvae and eggs produced. The plant also is effective against larvae of *Ostertagia, Fasciola, Nematodirus, Taenia* and *Dictyocaulus* (Mali and Mehta, 2008).

2. 8. 7- *Terminalia arjuna*

Terminalia arjuna is used as an antimicrobial and antiviral agent (Kusumoto *et al.,* 1995). Bark of the some species of genus *Terminalia* like *T. macroptera, T. superba* and *T. vorensi* have also been reported for their use as antidiarrheal, antidysentric (Alawa *et al.,* 2002) and trypanocidal (Adewunmi *et al.,* 2001). Decoction prepared from the bark of *T. arjuna* is used as an anthelmintic both in man and animals in Pakistan. However, there is no scientific evidence for the anthelmintic effects of *T. arjuna* bark. In 2009 a work done by Bachaya *et al.,* (2009) was carried out to validate the anthelmintic activity of *T. arjuna* bark. In that

study, crude methanolic extracts of *T. arjuna* exhibited inhibitory effects on eggs hatching and lethal median concentration (LC_{50} values) was determined graphically from the regression equation after correcting from negative control. The calculated LC_{50} values of *T. arjuna* and positive control (oxfendazole) were respectively 645.65 and 1.9 µg mL-1. For larval development, test crude methanolic extracts of *T. arjuna* exhibited inhibitory effects on larval development and LC_{50} was determined graphically from the regression equation after correcting from negative control. The calculated LC_{50} values of *T. arjuna* and positive control (levamisole) were 295.12 and 0.222 µg mL-1, respectively. In adult motility assay, efficacy of the extract was evident by the mortality of *H. contortus* at different hours post exposure. *In vivo* results revealed maximum (87.3%) egg count percent reduction in sheep treated with crude methanolic extracts at 3 g kg-1 body weight on day 11 post-treatment. *Terminalia arjuna* bark exhibited anthelmintic activity both in vitro (eggs, larvae and adult of *H. contortus*) and *in vivo* studies against mixed gastrointestinal nematodes of sheep.

2. 8. 8- *Ziziphus mucronata*

Ziziphus mucronata belongs to the family of *Rhamnaceae* and is a shrub or medium-sized tree up to 9 m tall with a trunk that is frequently crooked; branches spreading, often drooping, branching well above ground or near the base. Bark grey-brown and smooth when young, but becoming darker brown and fissured with age. Biologically, *Z. mucronata* is a hermaphroditic species found in south, central, east, and west of Africa. *Ziziphus mucronata* grows in areas dominated by thorny vegetation in both temperate and tropical climates. Also found in open scrubland, woodland, forest margins and riverine vegetation. It is a very hardy species, most common in dry areas and is resistant to both frost and drought (Orwa *et al.*, 2009).

In Chad, barks of *Z. mucronata* are traditionally used as local anthelmintic in the treatment of gastrointestinal infections. The drying leaves are boiled and drunk or mixed with food and eaten. However, the usage of plant has not been investigated scientifically. The current study evaluated the safety and efficacy of this medicinal plant.

2. 9- Phytochemical screening of medicinal plants

Medicinal plants are rich source of novel drugs that forms the ingredients in traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles and lead compounds for many synthetic drugs (Ncube *et al.,* 2008).

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. They are natural bioactive compounds found in different plant parts and components (barks, leaves, roots, flowers) or other parts of plants that interplay with nutrients and dietary fiber to protect them. Recent research demonstrates that phytochemicals can protect humans against diseases as well as, in the respiratory system, urinary tract, gastrointestinal, biliary systems and on the skin (Rìos and Recio, 2005; Adekunle and Adekunle, 2009). Some phytochemicals have been demonstrated to have anthelmintic activity. For instance, alkaloids possess anti-oxidating effects, thus reduce nitrate generation which is useful for protein synthesis, and suppress transfer of sucrose from stomach to small intestine, diminishing the support of glucose to the helminths. Tannins increase supply of digestible proteins by animals by forming protein complexes in rumen, interfere with energy generation by uncoupling oxidative phosphorylation and cause a decrease in gastrointestinal metabolism. However, saponins lead to vacuolization and disintegration of teguments (Prashant *et al.,* 2011).

2. 9. 1- Tannins

Tannins are naturally occurring polyphenolic compounds with high molecular weight. They are of two groups which are based on their structural types, hydrolysable tannins and condensed tannins (FAO/IAEA, 2000**) (Figure 2.2)**. Condensed tannins are polymeric flavonoids which are most common in forage legumes, trees and shrubs (Min and Hart, 2003). Condensed tannins are relatively stable in the digestive tract of the animal and rarely have toxic effects (Githiori, 2004).

An *in vitro* study of anthelmintic activity of *Baliospermum montanum* by Mali and Wadekar, (2008) concluded that there was a possibility that tannins were responsible for the anthelmintic activity displayed against *Pheretima posthuma* and *Ascaridia galli*. The anthelmintic activity of condensed tannins against *H. contortus* has been evidenced by inhibited egg hatching (Zafar *et al.*, 2007).

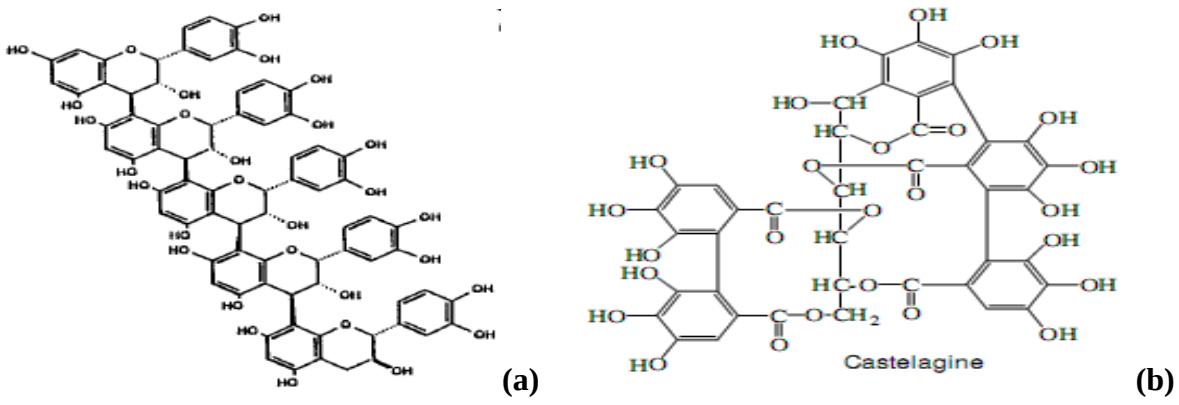

Figure 2.2: Condensed tannins (a) and hydrolysable tannins (b)

(FAO/IAEA, 2000)

2. 9. 2- Saponins

The name saponin was derived from soapwort plant, whose root was traditionally used as soap. Saponins are glycosides of triterpenes, streroids and sometimes alkaloids and occur but

not exclusively in plants. Glycone parts of saponins are generally oligossccharides. Aglycone part of saponin is referred to as a sapogenin. Many of the plants contain little or no saponins while others, the triterpenoid saponins predominate **(Figure 2.3)**. The oleanane-type of triterpene is the base structure found in the largest variety of medicinal plants (Kareru, 2008).

Saponins have been reported to have anthelmintic properties. Saponins were first reported to kill worms as early as 1962 using an extract from *Albizia anthelmintica*. Other studies have been done to characterize other plants believed to have anthelmintic effects. Work done by Ajayi *et al.,* (2008) through larval survival assay revealed that *Aframomum danielli* have anthelminthic activity against gastrointestinal nematodes. A study done by Deore and Khadabadi, (2010) was to prove the efficacy of *Chlorophitum borivilianum* root tuber against selected worms. From the results, which confirmed the presence of saponins from the TLC analysis of the crude extracts, they concluded that tubers could be used as anthelmintics and this further lead to confirmation that anthelmintic activity of *C. borivilianum* was due to presence of saponins.

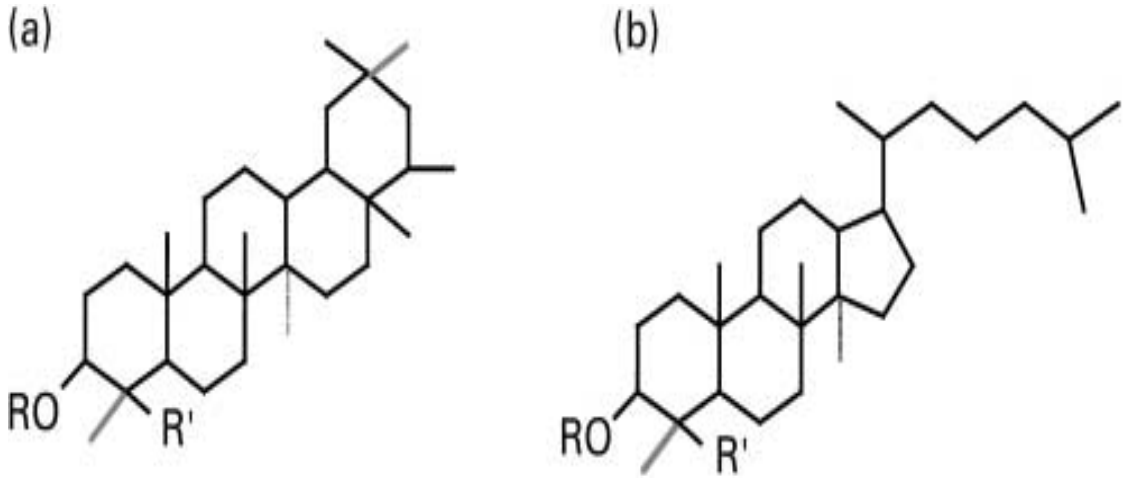

Figure 2.3: Structures of saponins (a) triterpenoid and (b) steroid

(Kareru, 2008)

2. 10- Testing for anthelmintic activity of the medicinal plants

In vitro and *in vivo* studies are usually done to assess anthelmintic activities of the medicinal plants. *In vivo* test, involves animal models such as mice, rats, ruminant animals which are infected with helminths. The infected animals are treated with herbal extract followed by monitoring helminths eggs in the animal faeces over time after the drug is administered. Reduction of faecal egg counts with time is an indication of efficacy of the plant extract (Githiori, 2004; Agaie and Onyeyili, 2007; Krimpen *et al.*, 2008; Burke *et al.*, 2009; Deore and Khadabadi, 2010).

In vitro studies involve culturing helminth egg and larvae from animal faeces either at room temperature or in an incubator and monitoring of death of adult worms. The effects of herbal products are performed by egg hatch inhibition, larval mortality and adult worm mortality assays. The percentage of egg hatch inhibition and mortality of the larvae are then determined. For adult worm mortality assay, the time of the dead adult worm is recorded (Zafar *et al.*, 2007; Kareru, 2008; Sujon *et al.*, 2008; Jesupilla and Palanivelu, 2009).

In Ethiopia, the ED_{50} of egg hatching of *H. contortus* for the aqueous extracts of *Acacia nilotica*, *Cyperus macrostachyus* and *Elegia capensis* was shown to be 0.87, 0.10 and 0.06 mg/ml, respectively (Egual et al., 2006). In Brazil, Maciel *et al.*, (2006) found 50% inhibition of *H. contortus* egg hatching using *Melia azedarach* ethanol extracts of leaves at 2.2 mg/ml concentration. Another study done by Kareru *et al.*, (2012) revealed that methanol extracts of *E. leptostachya* and *R. rhododendroides* had anthelmintic activity against *H. contortus* adult worms with mortalities of 77% and 54%, respectively.

2. 11- Testing for safety of the medicinal plants

The original report of the toxicity of herbal plants originated from Galen who was a Greek pharmacist and physician and showed that herbs do not contain only medicinally beneficial constituents, but may also be constituted with harmful substances (Cheng and Zhen, 2004).

By 2003 in the United States, over 1500 herbal products sold were nutraceuticals which are exempt from extensive preclinical efficacy and toxicity testing by the U.S. Food and Drug Administration (Bent and Ko, 2004). This increased usage, which has been reported in other countries, has led to increase concerns about potential harmful effect of these plant products, which has resulted in efforts to globally harmonize standards of toxicity testing methods. Methods used for herbal medicine toxicological characterization include tests for acute high dose exposure effects, chronic low dose toxicity tests and specific cellular, organ and system based toxicity assays.

The main objective of toxicological assessment of any herbal medicine is to identify adverse effects and to determine limits of exposure level at which such effects occur. Two important factors which are taken into consideration in evaluating the safety of any herbal drug are the nature and significance of the adverse effect and in addition, the exposure level where the effect is observed. Toxicity testing can reveal some of the risks that may be associated with use of herbs especially in sensitive populations. An equally important aim of toxicity testing is the detection of toxic plant extracts or compounds derived thereof in pre-clinical and clinical stages of drug discovery and development from plant sources. This facilitates the identification of toxicants which can be discarded or modified during the process and create an opportunity for extensive evaluation of safer, promising alternatives (Gamaniel, 2000).

Acute toxicity analysis of many medicinal plants extracts has been researched to obtain safer drugs. Because many herbs contain pharmacologically active compounds, some may cause

severe side effects through excessive biological activities. Therefore, the need to determine the safety of the plants extracts is important.

2. 12- Animal models for testing safety of the herbal medicine

In gastrointestinal nematode infections research, animal models have been extensively used. The Wistar rat developed at the Wistar Institute in 1906 for use in biological and medical research served as a model organism (Clause, 1998). Currently Wistar albino rats and Swiss white albino mice are the most popular animal models used for anthelmintic drugs toxicity test by laboratory research. In most research, these animal models are used for physiological purposes. Mostly the toxicity of plant extracts as an anthelmintic are determined in accordance with Organization for Economic Co-operation and Development (OECD) guidelines.

CHAPTER THREE

3. 0- MATERIAL AND METHODS

3. 1- Study area

The present study was undertaken in Chad and Kenya. The plant samples were collected from Chad. Deli locality of south region of Chad was the source of *Z. mucronata* plant. Deli is located in Soudanian zone, region of Moundou in Chad with tropical and subtropical climate, longitude 16°04′59″ east, latitude 8°34′00″ north and temperature 25 – 38 ° C. Extractions, qualitative phytochemicals screening, *in vitro* efficacy and toxicity trials were undertaken in Kenya at the research laboratories of Jomo Kenyatta University of Agriculture and Technology.

3. 2- *Ziziphus mucronata* and sample collection

Ziziphus mucronata belongs to the family *Rhamnaceae* and order *Rosales. Ziziphus mucronata* tree is a thorny, medium-sized up to 9 metres tall with a trunk that is frequently crooked; branches spreading, often drooping, branching well above ground or near the base. The bark is grey-brown and smooth when young (often spiny), but becomes darker brown and fissured with age **(Plate 3.1)**. Leaves are ovate to broadly ovate, shiny, and densely hairy to quite smooth (Orwa *et al.,* 2009).

Barks (500 grams) of *Z. mucronata* (local name: Ngohkroh) were collected from Deli in Chad during the month of April 2015. The barks of *Z. mucronata* were authenticated by herbalist and botanist from Herbal Treatment Center in Deli. The barks were then dried under shade and powdered using pestle and mortar **(Plate 3.2)**. The powdered bark was packaged in an envelope and transferred to Kenya. Extraction was done at Biochemistry and Chemistry Laboratories of Jomo Kenyatta University of Agriculture and Technology.

Plate 3.3: *Ziziphus mucronata* tree and barks collection process

Plate 3.4: Fresh collected barks and drying process

3. 3- *Z. mucronata* selection criteria

The selection of plant was based on the common use of the plant in the treatment of helminthosis by Chadian population (Personal communications, Herbalist, Deli in Chad). Herbalists in Chad also use *Z. mucronata* barks for treatment of helminthosis in humans.

3. 4- Study design

The study design was an experimental research which evaluated the qualitative phytochemicals composition of *Z. mucronata* barks and also assessed the activity and toxicity of the extracts of the medicinal plant in Laboratories at Jomo Kenyatta University of Agriculture and Technology. This study was conducted from February to November 2016.

3. 5- Extraction of *Ziziphus mucronata* barks

Extraction of the plant product was carried out using distilled water and methanol as solvents.

3.5.1. Aqueous extraction

For the aqueous extraction, the powdered plant material was prepared according to the standard methods described previously (Onyeyili *et al.,* 2001). Briefly 200 g of the powdered bark was mixed with 1000 ml of distilled water at a ratio of 1:5 (w/v) in a flask. The mixture was boiled for one and half hours using hot plate. The mixture was cooled and filtered using Whatman N^{o} 1 filter paper. The extracts were then dried using freeze dryer (Christ Alpha 1-4 LD, SciQuip Ltd, Newtown UK). The obtained extracts were weighed and the percentage yield was determined then stored at 4°c.

3.5.2. Methanolic extraction

Methanolic extraction of powdered bark was prepared according to the method described by Saidu *et al.,* (2013). Two hundred grams (200 g) of the plant sample was mixed with 1000 ml of analytical grade methanol (Sigma-Aldrich, USA) at a ratio of 1:5 (w/v) in a flask and shaken vigorously. The mixed solution was allowed to stand for 72 hours to allow extraction to take place. The mixture was filtered using Whatman N^{o} 1 filter paper **(Plate 3.3)**. The extracts were concentrated using rotary evaporator (BUCHI R-200, Labortechnik AG CH-9230 Flawil Switzerland). The concentrated extracts were dried in an incubator at 50^{o} C **(Plate 3.4)**. The final extracts obtained were weighed to obtain the percentage yield then stored at 4^{o} C in the refrigerator until use.

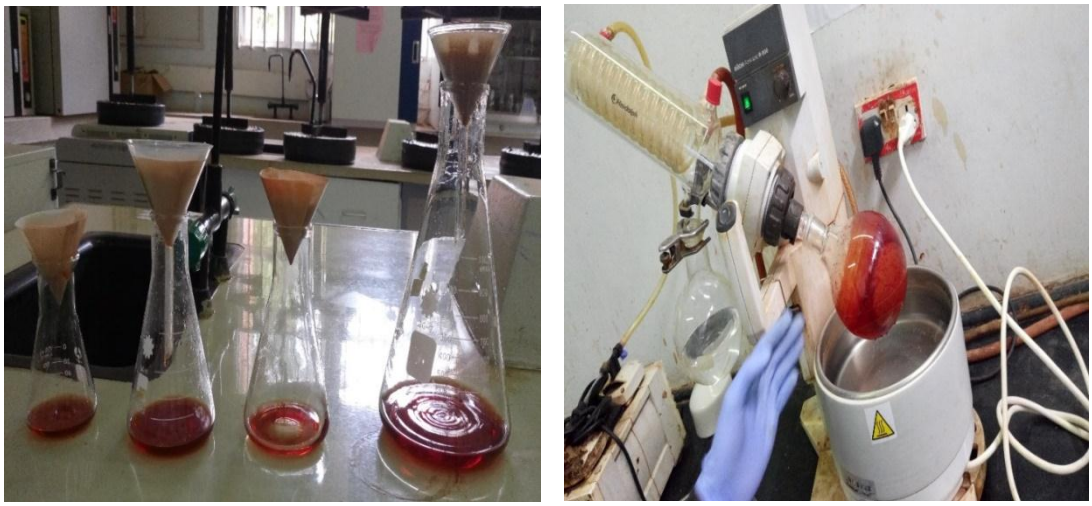

Plate 3.3: Filtration and concentration process

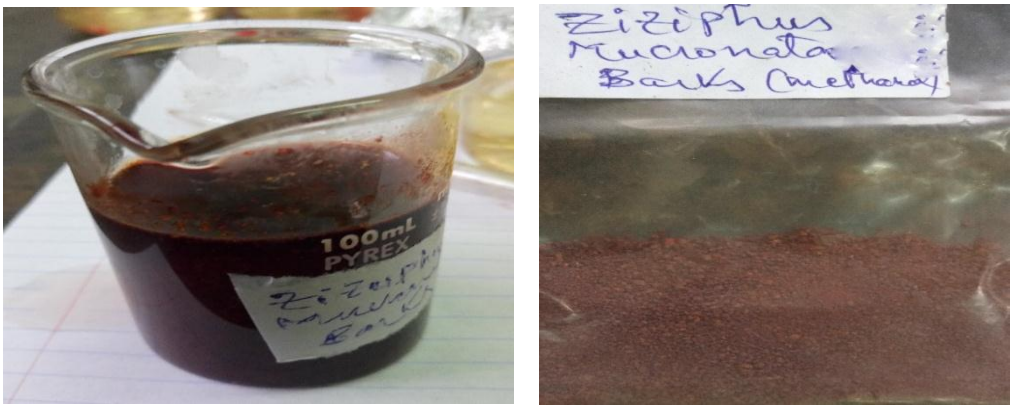

Plate 3.4: Concentrated and powdered Extract

3. 6- Determination of phytochemicals from *Ziziphus mucronata* barks

Plant extracts were subjected to qualitative phytochemicals screening as described previously (Harborne, 1998). The extracts were tested for saponins, tannins, alkaloids, glycosides, flavonoids and steroids as described below:

3. 6. 1- Test for saponins

Two milliliters (2 ml) of crude extract was mixed with 5ml of distilled water in a test tube and shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins **(Plate 3.5)**.

3. 6. 2- Test for tannins

Two milliliters (2 ml) of crude extract was mixed with 2ml of 2% solution of ferric chloride ($FeCl_{3)}$. A blue-green or black coloration indicated the presence of tannins **(Plate 3.5)**.

3. 6. 3- Test for alkaloids

Presence of alkaloids was tested by Dragendorff's test by adding 1 or 2 drops of the reagent to 1 ml of the sample and formation of a prominent yellow precipitate indicated the presence of alkaloids.

3. 6. 4- Test for glycosides

Two ml of crude extract was mixed with 2 ml of chloroform. Thereafter, 2 ml of concentrated sulfuric acid ($H_2SO_{4)}$ was added carefully and shaken gently. A reddish brown ring at interphase indicated the presence of glycosides.

3. 6. 5- Test for flavonoids

To 1 ml of crude extract, 3 drops of ammonia solution were added and thereafter 0.5 ml of concentrated hydrochloric acid (HCL) was added. The formation of pale brown coloration indicated the presence of flavonoids **(Plate 3.5)**.

3. 6. 6- Test for steroids

Two milliliters of crude extract was mixed with 2 ml of chloroform and evaporated to dryness. To this, 2 ml of concentrated sulfuric acid (H_2SO_4) was added and heated for about 2 minutes. A red coloration indicated the presence of steroids.

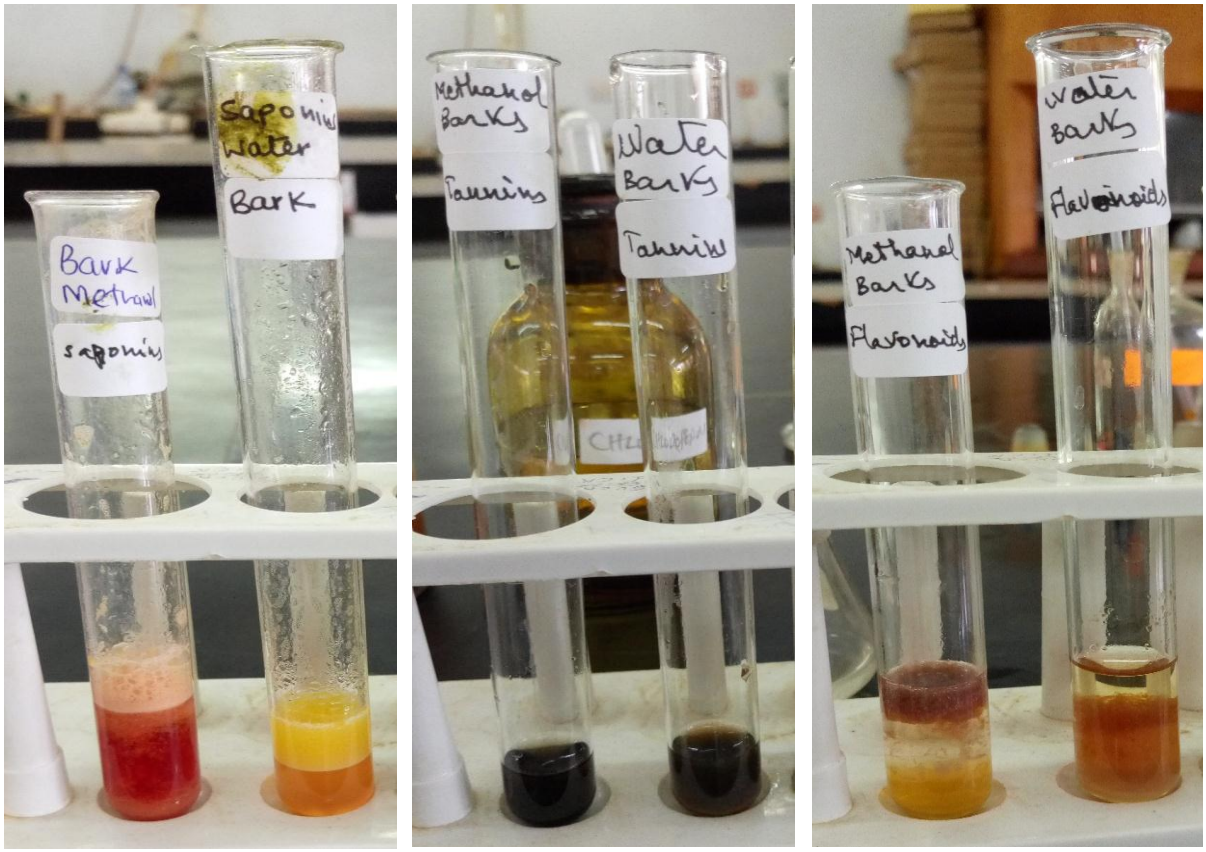

Plate 3.5: Positive test of saponins, tannins and flavonoids

3. 7- *In vitro* anthelmintic activity of *Ziziphus mucronata* barks extract

In vitro anthelmintic activity was conducted according to World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles *et al.*, 1992) with slight modification on parasite collection, eggs preparation, egg hatch inhibition, larval mortality and adult worms mortality assays as described below:

3. 7. 1- Parasite collection and eggs preparation

Mature adult parasites of *H. contortus* were collected directly from abomasum of slaughtered sheep from a local slaughter house in Ruiru, Kenya. The worms were transported to the laboratory in Phosphate Buffer Saline (PBS, pH 7.4). Identification and separation of adult mature female were done using a microscope as described by Hansen and Pery, (1994). They were washed using PBS and crushed using pestle and mortar in 5 ml PBS to liberate eggs. McMaster slide technique, as described by Urquhart *et al.*, (1996), was used to estimate the concentrations of egg in aliquots of 200 µl.

3. 7. 2- Egg Hatch Inhibition Assay (EHIA)

For each extract, a stock solution of 4 mg/ml was taken as the initial concentrations and serial (1:2) dilutions of 0.0625 to 4 mg/ml prepared in phosphate buffered saline. Approximately 100 *H. contortus* eggs in 200 µl of egg suspension were pipetted into each well of 96 well micro titre plates. Two hundred µl of *Z. mucronata* extracts were then added into each well. Albendazole (Albendazole, Sigma-Aldrich) was used as positive control. Three replicates for each concentration of both aqueous and methanolic extracts of *Z. mucronata* and control were performed. The plates were labelled and incubated for 48 hours at 27^{o}C and 70% relative humidity **(Plate 3.6)**. After 48 hours of incubation, hatched larvae (live or dead) and unhatched eggs were counted using a compound microscope at X40 magnification with the

help of a counter (Rechab *et al.*, 2014; Cheruiyot *et al.*, 2015). The egg-hatch inhibition rates assessed were calculated by the following formula:

$$\textbf{Egg hatch inhibition}\ (\%) = \frac{\textbf{Total number eggs} - \textbf{number hatched larvae}}{\textbf{total number of eggs}} \times \textbf{100}$$

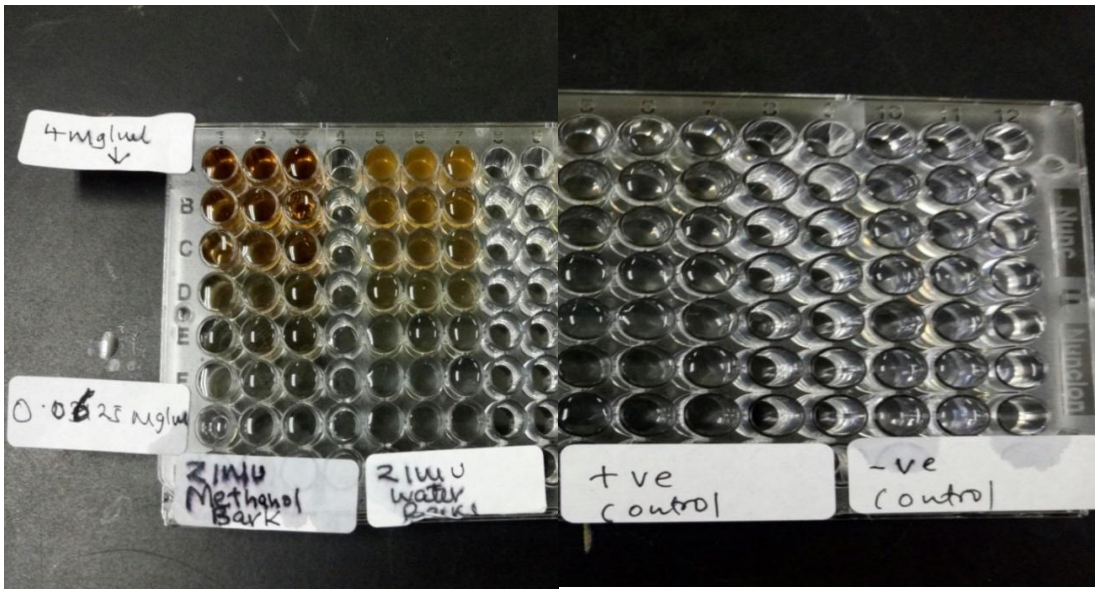

Plate 3.6: Incubated eggs with different concentration of plant extract

3. 7. 3- Larval Mortality Assay (LMA)

Larval mortality assay were performed according to Wabo *et al.*, (2006). After collection of adult parasite, the female *H. contortus* was identified and separated from male as described above. Eggs were recovered by grinding the female with pestle and mortar in 5 ml PBS. One hundred eggs in 180 µl of egg suspension were put into each well of 96 well micro titre plates. A 20 µl of nutritive media (comprising of 1g yeast in 90 ml of normal saline and 10 ml Earle's balanced salt) was added into each well. The plates were then incubated under humidified condition at ambient temperature for 48 hours. After 48 hours of incubation, 200 µl of *Z. mucronata* extracts in concentrations ranging from 0.0625 to 4.0 mg/ml were added to respective plates. Albendazole was used as positive control. There were three replicates for each extract concentration and control. The plates were further incubated for 24 hours (total

of 3 days). Counting of all larvae in each well was done under an inverted microscope. The percentage of mortality of the larvae was determined using the following formula:

$$\textbf{Larval mortality}\ (\%) = \frac{\textbf{Number of dead larvae}}{\textbf{Number of larvae in culture}} \times \textbf{100}$$

3. 7. 4- Adult Worms Mortality Assay (AWMA)

Adult worm mortality assay was conducted following the technique described by Bachaya *et al.,* (2009). Briefly, mature live *H. contortus* worms were collected from abomasum of freshly slaughtered sheep in a local abattoir at Ruiru in Kenya. The worms were kept in PBS at pH 7.4 and transported to Zoology laboratory of Jomo Kenyatta University of Agriculture and Technology. Five worms were exposed in triplicate to each of the following treatments in separate Petri dishes **(Plate 3.7)** at laboratory temperature (25–30◦C):

1. Methanolic extract diluted in PBS at concentrations 0.25, 0.5, 1, 2 and 4 mg/ml.
2. Aqueous extract diluted in PBS at concentrations 0.25, 0.5, 1, 2 and 4 mg/ml.
3. Albendazole was used as a positive control at the same concentrations 0.25, 0.5, 1, 2 and 4 mg/ml.

The inhibition of motility and/or mortality of the worms subjected to the above treatments were used as the indicator for anthelmintic activity. Motility of worms was observed on different time intervals 0, 1, 2, 4, 8 and 24 hours post exposure and on every observation motile worms were counted. Worms not showing any motility were picked out and were exposed to lukewarm PBS for 5 to 10 minutes and in case of revival in motility, the observed worms were counted as alive, otherwise dead. The percent mortality of worms was calculated for each extract concentration using the formula:

$$\textbf{Mortality}\ (\%) = \frac{\textbf{Number of dead worms}}{\textbf{Number of worms in culture}} \times \textbf{100}$$

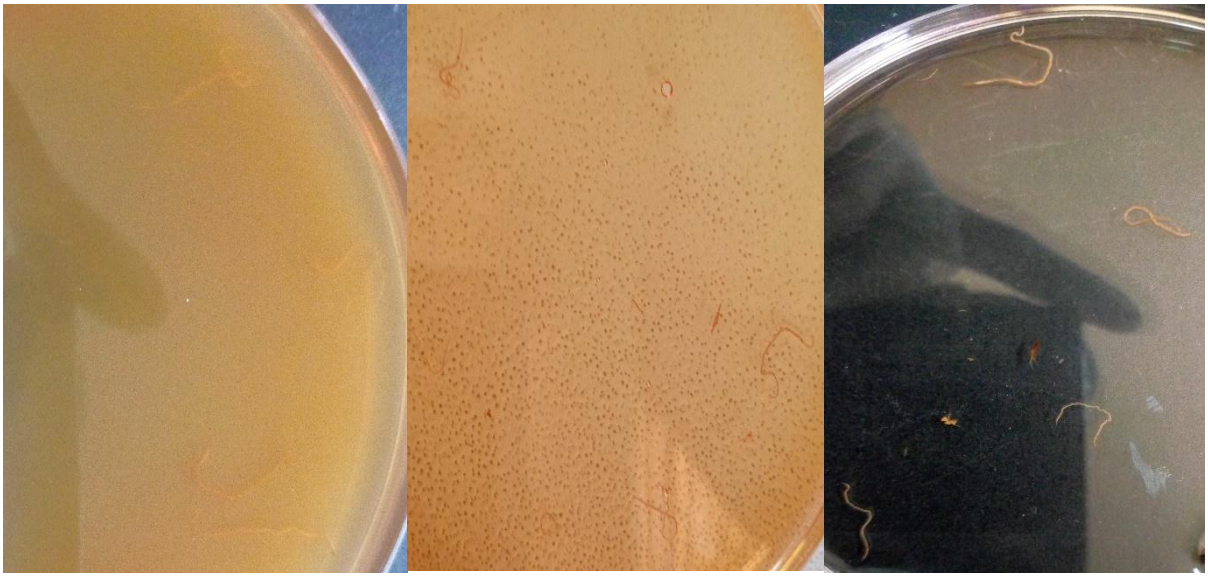

Plate 3.7: Live adult *Haemonchus contortus* exposed in different concentration of plant extract

3. 8- Acute toxicity study

3. 8. 1- Laboratory animals

Forty (40) male adult Swiss albino mice weighing 20-30 g were used in this study**.** They were purchased from Institute of Primate Research, Kenya, carried in mice cages in group of 5 mice each cage (8 cages)**.** At JKUAT, they were acclimatized for 2 weeks before the start of experiment.

3. 8. 2- Housing of the mice

Mice were housed in 8 aluminium cages that consisted of 5 mice per group each cage and clearly labelled**.** Each cage had 5 mice. The mice were fed mice pellets (Unga Ltd, Kenya) and water was provided ad libitum. The cages contained the sufficient quantity of wood shavings to allow good housing mice and their well living. The aluminium cages were placed in a well-ventilated house at 23 ± 1°C and humidity of 40-45%. All the mice had free access

of water and mice pellets**.** The use of mice and experimental protocols was approved by the JKUAT Animals Ethics Committee and adhered to international standards for care and use of laboratory animals.

3. 8. 3- Acute toxicity test

Acute toxicity test was performed according to the Organization of Economic Co-operation and Development (OECD) guidelines (OECD, 2001)**.** Thirty five **(**35) mice were randomly divided into 7 groups consisting of each 5 mice per group. Mice were weighed using a weighing balance and fasted overnight with only free access to water.

1. Single oral gavage dose of the 3 chosen doses (500, 1000, 2000 mg/kg body weight) of the methanolic extracts of *Z. mucronata* barks was administered to mice in groups 1, 2 and 3 **(Plate 3.8)**.
2. Single oral gavage dose of the 3 chosen doses (500, 1000, 2000 mg/kg body weight) of the aqueous extracts of *Z. mucronata* barks was administered to mice in groups 4, 5 and 6 **(Plate 3.8)**.
3. Mice in Group 7 received only distilled water as negative control.

All mice per groups were weighed on days 0, 3, 5, 7, 10, 14 and checked for signs of toxicity on daily basis up to 14 days. Mortality of mice was checked also on daily basis up to 14 days. At 14 days post treatment, the mice were euthanized using concentrated carbon dioxide to collect blood, liver and kidney. Blood sample was collected via cardiac puncture by use of a syringe and needle **(Plate 3.9)**. The needle has been removed from the syringe and the blood was placed into labelled Eppendorf tubes to allow for serum separation. The serum was then centrifuged at speed of 1,300 rpm for 10 minutes. Serum samples were kept at -20° and analysed immediately using automatic clinical chemistry analyser, Reflotron^{R} biochemical

analyser, (Roche Diagnostic Gmbh, Germany). Biochemical parameters assayed were ALT (enzyme for liver function test) and BUN (kidney function test) as described in Appendix 1.

Plate 3.8: Identification of mice and administration of extract

Plate 3.9: Collection of blood for analysis of biochemical parameters

3. 9- Statistical analysis

Descriptive statistics were used to analyse data from experimental groups treated and from the control groups untreated. The data output included mean, standard deviation and standard error of mean using Microsoft Excel and SPSS version 22. Probit analysis, paired-samples t-test and ANOVA were used to determine whether there were any significant differences within the treated groups when compared to the untreated control groups. A value of $P<0.05$ was considered statistically significant.

CHAPTER FOUR

4. 0- RESULTS

4. 1- Percentage yield of *Ziziphus mucronata* barks extract

The results showed that the highest (4.5%) yield was recorded for methanol extraction and lowest (2.6%) percentage yield for aqueous extraction **(Table 4.1)**. However, there were no significant (P>0.05) differences between the yields of the two solvents.

Table 4.1: Solvent extraction yield (Mean ± SD) of *Ziziphus mucronata* barks

Solvent	Amount of barks used (g)	Volume of solvent used (ml)	Extract concentrated (g)	Solvent extraction yield (Mean ± SD)	P-value
Methanol	200	1000	8.97	4.5 ± 0.21	0.067
Water	200	1000	5.10	2.6 ± 0.50	

4. 2- Qualitative determination of phytochemicals

The screening of *Z. mucronata* barks showed the presence of the following phytochemicals: saponins, tannins, glycosides, flavonoids and steroids (**Table 4.2)**.

Table 4.2: Qualitative phytochemicals screening results of *Ziziphus mucronata* barks

Phytochemicals	Aqueous extraction	Methanolic extraction
Saponins	+	+
Tannins	+	+
Alkaloids	-	-
Glycosides	+	+
Flavonoids	+	+
Steroids	+	+

(+) signifies presence of phytochemicals and (-) signifies absence of phytochemicals.

4. 3- *In vitro* anthelmintic activity of *Ziziphus mucronata* barks extract

4. 3. 1- Egg Hatch Inhibition Assay (EHIA)

Different concentrations of aqueous and methanolic extracts of *Z. mucronata* barks in concentrations ranging from 0.0625- 4mg/ml were tested for their anthelmintic activity. The higher drug concentration resulted in higher egg hatch inhibition compared with lower concentration suggesting a concentration dependent response (**Figure 4.1)**. At all extracts concentrations, the egg hatch inhibition was highest in eggs exposed to Albendazole, followed by methanol and aqueous extracts. The mean percentage egg hatched inhibition for both extracts was significantly ($P<0.05$) lower than that of Albendazole.

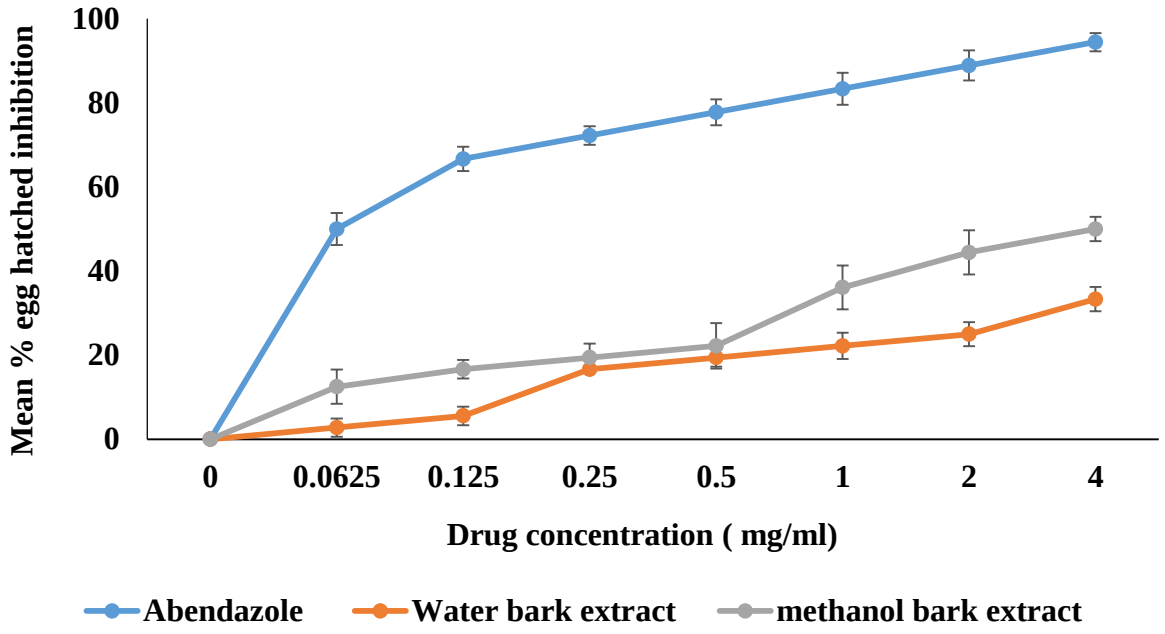

Figure 4.1: Mean hatch inhibition for eggs of *Haemonchus contortus* exposed to *Ziziphus mucronata* extracts and Albendazole

The concentration required to inhibit 50% of egg hatched (IC_{50}) of both extracts and Albendazole are presented in **Table 4.3**. Methanolic extract showed a significantly ($P<0.05$) higher activity with IC_{50} value of 3.9 mg/ml as compared with aqueous extract with IC_{50} value of 14.7 mg/ml.

Table 4.3: IC_{50} values of methanol and aqueous extracts of *Ziziphus mucronata* barks and positive control (Albendazole) exposed to eggs of *Haemonchus contortus*

95% confidence limits for concentration (mg/ml)			
Sample type	**IC_{50} values (mg/ml)**	**Lower Bound**	**Upper Bound**
Aqueous extract	14.683	6.896	55.000
Methanol extract	3.932	2.416	8.282
Albendazole	0.050	0.024	0.079

4. 3. 2- Larval Mortality Assay (LMA)

Aqueous and methanolic extracts of Z. *mucronata* in different concentration showed variable effect on mortality of larvae of *H. contortus*. At the maximum tested concentration of 4 mg/ml, the highest (53.3%) larval mortality was from methanolic extracts with while the least (43.3%) was from aqueous extract. Albendazole required a maximum concentration of 0.5 mg/ml to induce 100% larval mortality **(Figure 4.2)**. The mean percentage larval mortality for both extracts was significantly (P<0.05) lower than that of Albendazole.

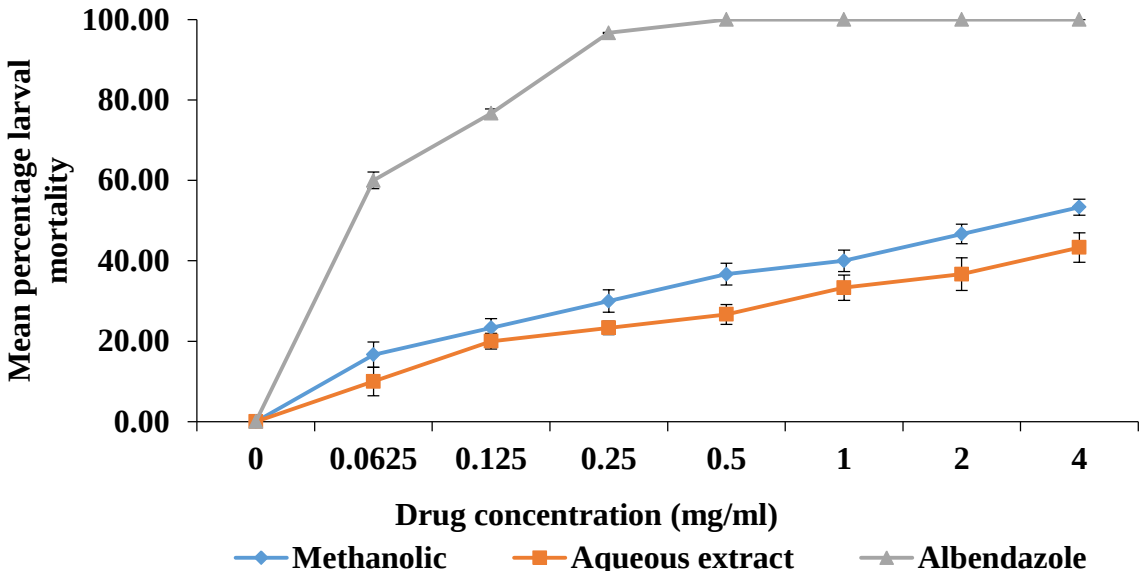

Figure 4.2: *In vitro* larvicidal activity of methanolic and aqueous extracts of *Ziziphus mucronata* barks and Albendazole against *Haemonchus contortus*

The highest EC (Effective Concentration)$_{50}$ value against larval mortality was by Albendazole (0.045 mg/ml), followed by methanolic extract (2.7 mg/ml) and the least was aqueous extract (7.5 mg/ml). The EC_{50} of Albendazole was significantly ($P<0.05$) different from those of the extracts **(Table 4.4)**.

Table 4.4: EC_{50} of methanolic and aqueous extract of *Ziziphus mucronata* and positive control (Albendazole) exposed to larval stages of *Haemonchus contortus*

95% confidence limits for concentration (mg/ml)			
Sample type	**EC_{50} values (mg/ml)**	**Lower Bound**	**Upper Bound**
Aqueous extract	7.516	3.638	28.032
Methanol extract	2.646	1.593	5.929
Albendazole	0.045	0.031	0.057

4. 3. 3- Adult Worms Mortality Assay (AWMA)

Different concentrations of the extracts and Albendazole that caused paralysis and mortality of the tested worms are shown in **Figures 4.3, 4.4** and **4.5**. Albendazole had a significantly ($P<0.05$) higher percentage mean adult mortality and EC_{50} than both extracts **(Table 4.5)**. However, there were no significant ($P>0.05$) differences in the percentage mean adult mortality and IC_{50} of both extracts **(Table 4.5)**.

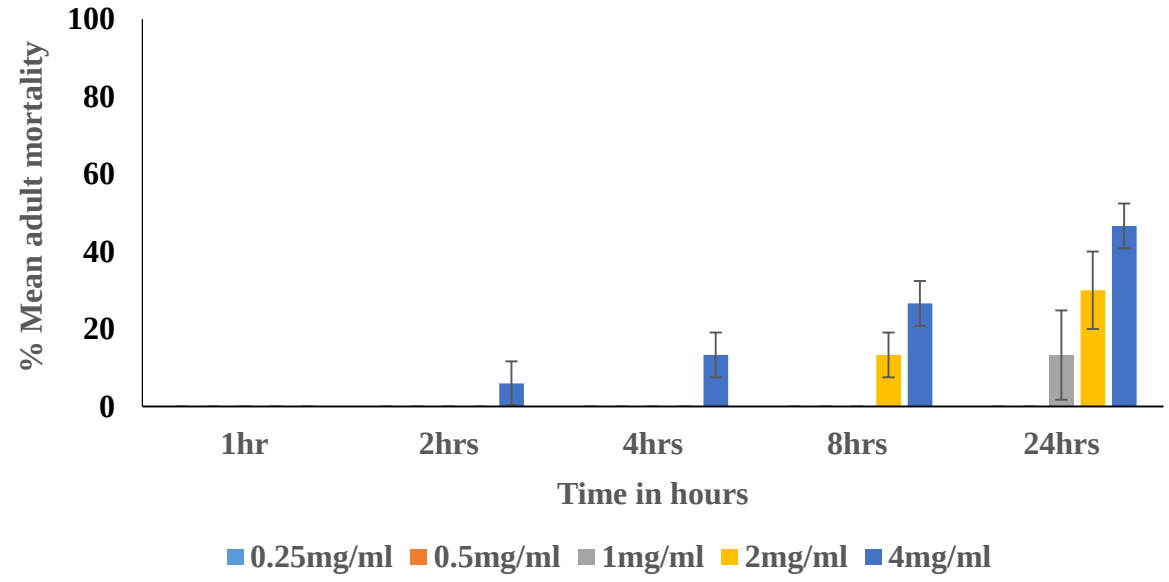

Figure 4.3: Mean adult mortality of *Haemonchus contortus* exposed to aqueous extract of *Ziziphus mucronata* barks

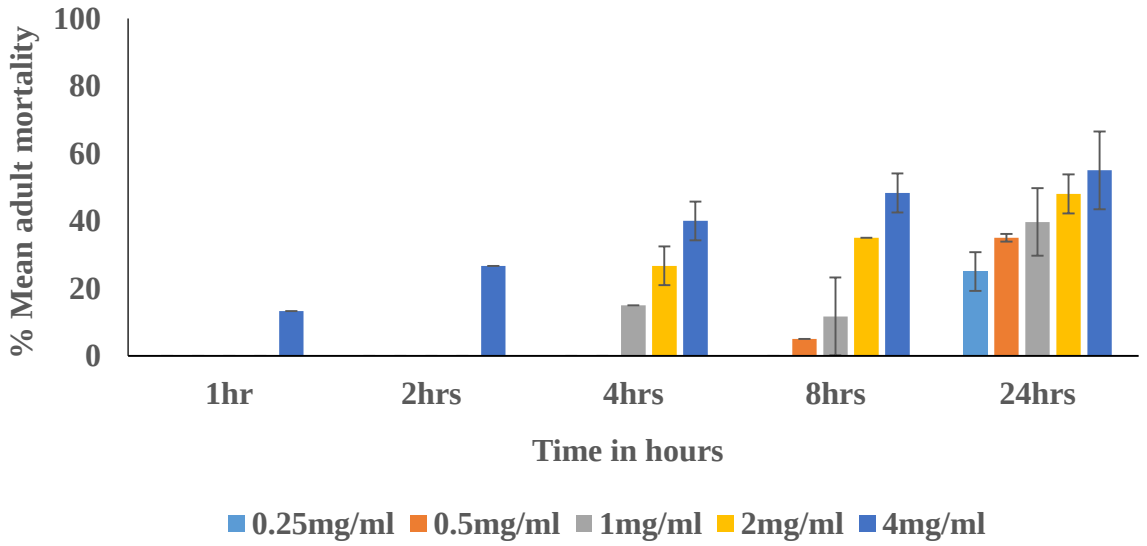

Figure 4.4: Mean adult mortality of *Haemonchus contortus* exposed to methanolic extract of *Ziziphus mucronata* barks

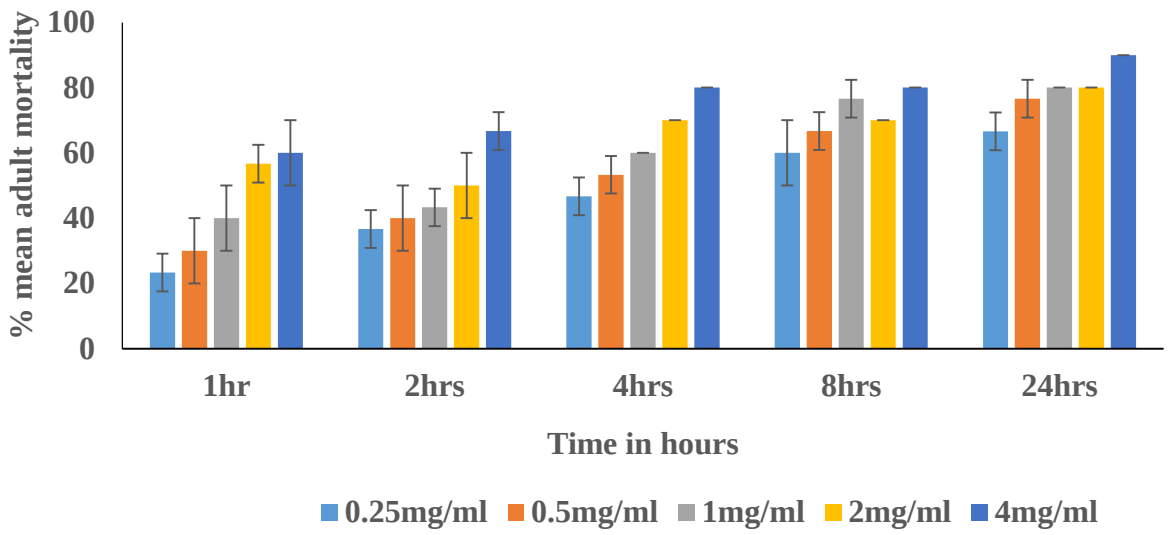

Figure 4.5: Mean adult mortality of *Haemonchus contortus* exposed to Albendazole

Table 4.5: EC_{50} values for adult mortality *a*ssay in different time interval

Time in hours	1 hr	2 hrs	4 hrs	8 hrs	24 hrs
Aqueous extract	-	7.8mg/ml	6.8mg/ml	6.1mg/ml	5.0mg/ml
Methanolic extract	6.2mg/ml	5.0mg/ml	4.7mg/ml	3.7mg/ml	2.4mg/ml
Albendazole	1.8mg/ml	1.3mg/ml	0.4mg/ml	0.05mg/ml	0.04mg/ml

4. 4- Acute toxicity test

The acute toxicity test revealed that administration of a single oral gavage dose of the chosen dosages of 500, 1000 and 2000 mg/kg body weight of the aqueous and methanolic extracts of *Z. mucronata* barks to the mice did not cause any signs of toxicity or mortality in treated animals during the 14 days observation period. The mean body weight of mice fluctuated during the first week of post treatment and increased during the second week (**Figures 4.6** and **4.7)**. There were no significant differences (P>0.05) in mean body weight of different dosages of the extracts compared to the control (distilled water).

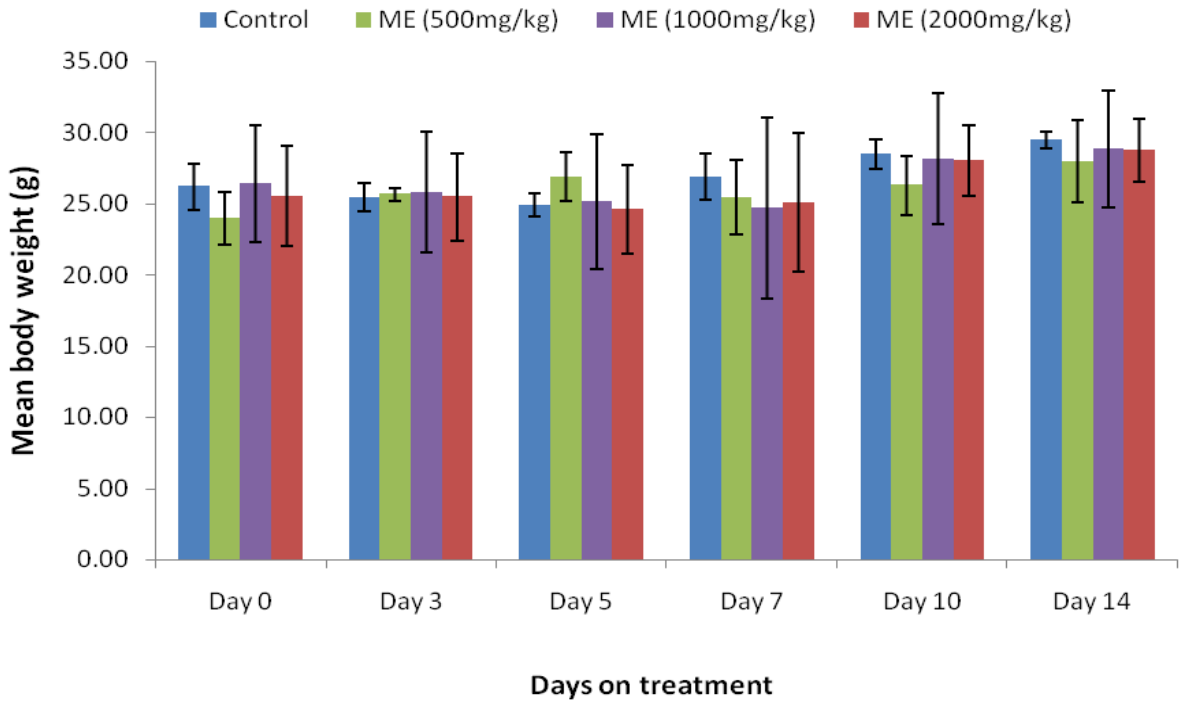

Figure 4.6: Body weight of mice (mean ± SD) treated with dosage of (500, 1000 and 2000 mg/kg) methanolic extracts (ME) of *Ziziphus mucronata* barks compared to the control (n=5)

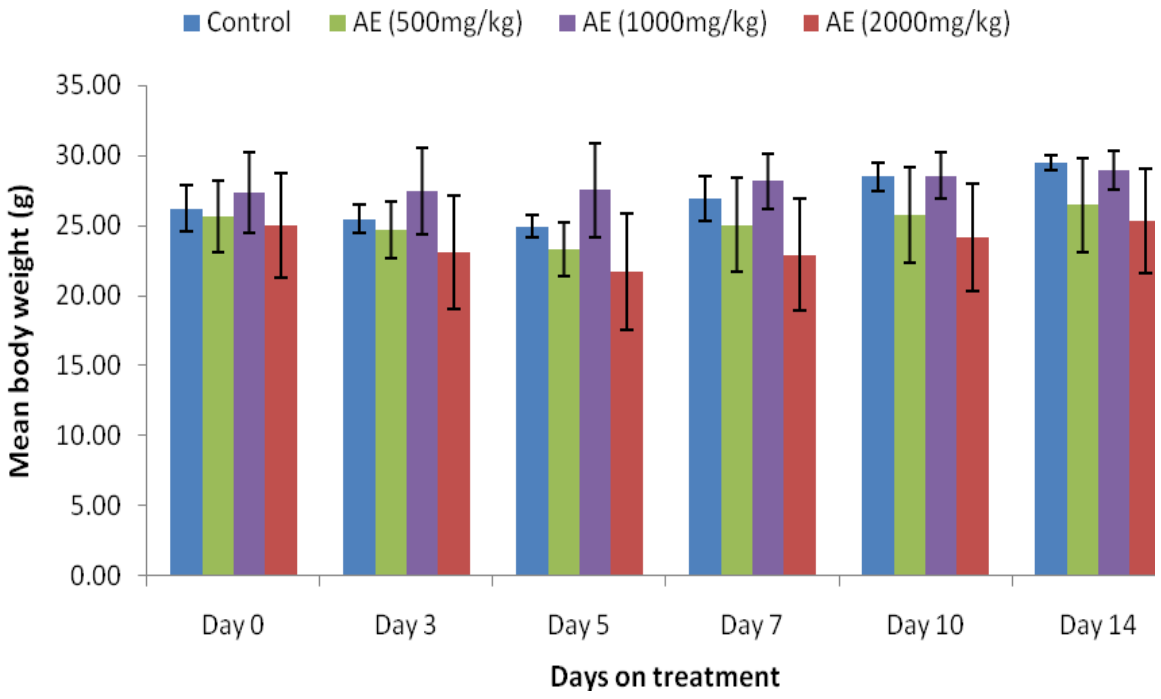

Figure 4.7: Body weight of mice (mean ± SD) treated with dosage of (500, 1000 and 2000 mg/kg) aqueous extracts (AE) of *Ziziphus mucronata* barks compared to the control (n=5)

The mean ALT levels of mice treated with different dosages (500, 1000 and 2000mg/kg body weight) of aqueous and methanolic extracts of *Z. mucronata* barks are presented in **Figure 4.8**. The mean values of ALT of mice treated with methanolic extracts did not differ significantly (P>0.05) compared those of the control mice. However, the mean values of ALT of mice treated with aqueous extracts at 1000mg/kg and 2000mg/kg was significantly (P<0.05) higher than that of control mice.

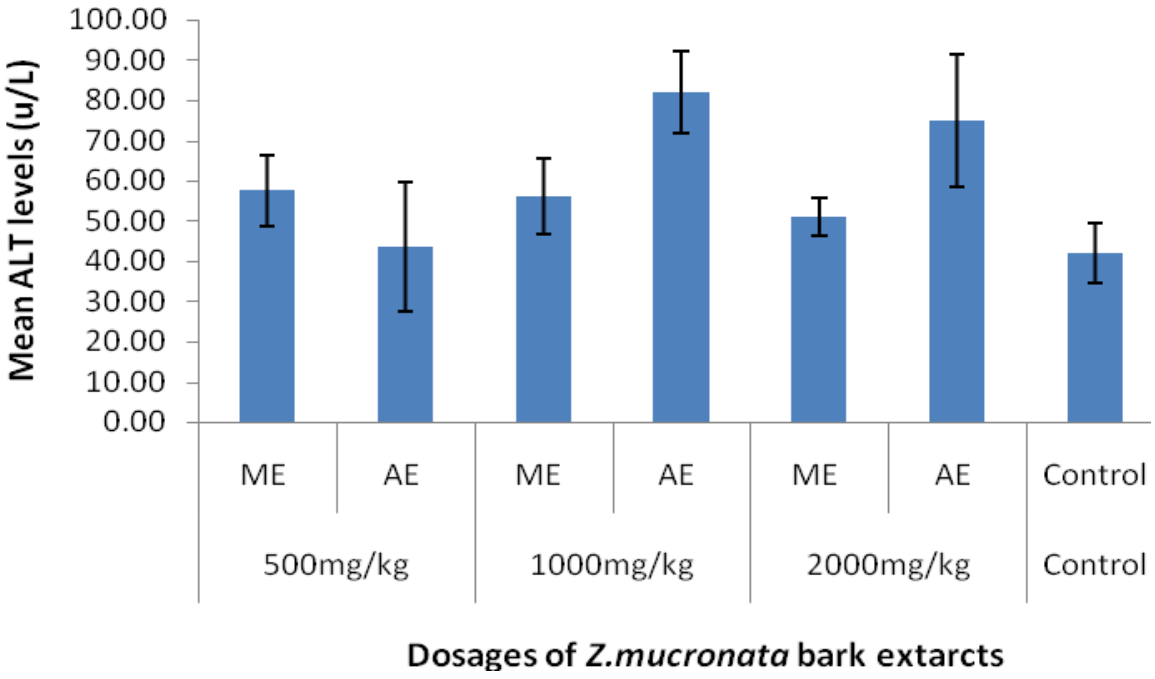

Figure 4.8: Alanine transaminase (ALT) of mice (mean ± SD) treated with dosage of (500, 1000 and 2000 mg/kg) methanolic extracts (ME) and aqueous extracts (AE) of *Ziziphus mucronata* barks compared to the control (n=5)

The mean BUN levels of mice treated with different dosages of aqueous and methanolic extracts of *Z. mucronata* barks are presented in **Figure 4.9**. The mean values of BUN of mice treated with either extracts did not differ significantly (P>0.05) compared those of the control mice.

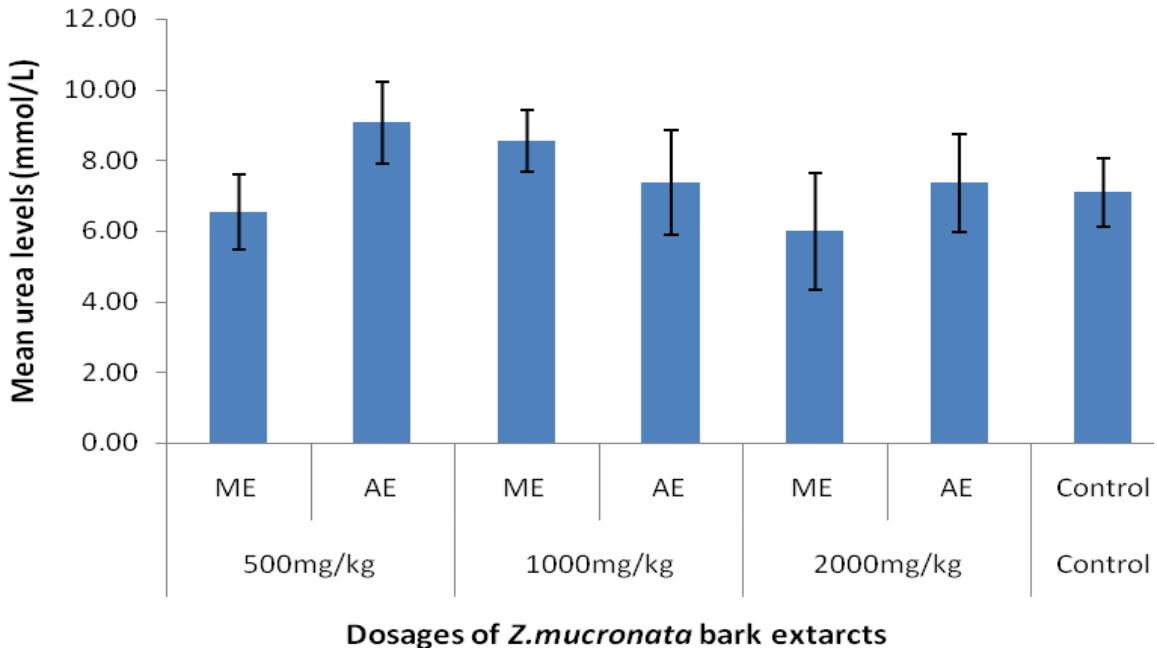

Figure 4.9: Blood Urea Nitrogen (BUN) of mice (mean ± SD) treated with dosage of (500, 1000 and 2000 mg/kg) methanolic extracts (ME) and aqueous extracts (AE) of *Ziziphus mucronata* barks compared to the control (n=5)

CHAPTER FIVE

5. 0- DISCUSSION

The present study carried out on the barks of *Z. mucronata* showed that there were no significant differences in the percentage yield when either Methanol or water was used as solvent. Since methanol is expensive and not widely available in the field setups, the use of water for extraction of *Z. mucronata* barks is recommended. The phytochemical screening in the present study showed the presence of various phytochemical constituents such as flavonoids, saponins, steroids, tannins and glycosides. These diversities in the phytochemical contents of a plant part are due to number of environmental factors as previously mentioned (Kokate *et al.,* 2004). These secondary metabolites (phytochemicals) of medicinal plants may account for their medicinal value. For instance, tannins extracted from plants have been shown to have anthelmintic, antidiarrhoeal and antimicrobial activities (Sutar *et al.,* 2010; Kumar *et al.,* 2010; Cowan, 1999), while saponins have anthelmintic, antidiarrhoeal and anticancer properties (Mali *et al.,* 2007; Roy, 2010; Prashant *et al.,* 2011). Glycosides and steroids produced by the plant extracts are known for their antidiarrhoeal action (Prashant *et al.,* 2011; Wang *et al.,* 2010), whereas flavonoids in plants have been shown to have antidiarrhoeal and antimicrobial activities (Cowan, 1999; Vidyadhar *et al.,* 2010). The preliminary phytochemical screening tests are important in finding chemical constituents in the plant material which can be used in subsequent drug discovery and development. Further, these tests facilitate their quantitative estimation and separation of pharmacologically active chemical compounds.

The current study evaluated the activity of bark extracts of *Z. mucronata* against different stages of *H. contortus*. The egg hatch assay was initially developed for the diagnosis of benzimidazole resistant helminths but has, however, been used for screening of plants

compounds for their anthelmintic activity (Min and Hart, 2003; Iqbal *et al.,* 2005; Hamad *et al.,* 2013). In this study, methanolic extract showed a higher activity with IC_{50} value of 3.9 mg/ml as compared with aqueous extract with IC_{50} value of 14.7 mg/ml for egg hatch inhibition. This could be attributed to the difference in proportions of active components that were responsible for anthelmintic activity. A study done by Eguale *et al.,* (2007), on aqueous extracts of *Hedera helix* showed IC_{50} value of 0.12 mg/ml respectively when tested against *H. contortus* egg. Aqueous extract of *Coriandrum sativum* had an IC_{50} of 0.12 mg/ml while the hydroalcoholic extract had an IC_{50} of 0.18 mg/ml on egg hatch inhibition test (Eguale *et al.,* 2006). Thus, these plants had a relatively higher activity compared than *Z. mucronata* aqueous extract. Albendazole, in the present study, showed comparable results with that of *in vitro* anthelmintic activities of four Ethiopian medicinal plants against *H. contortus* which had IC_{50} value of 0.04 mg/ml (Eguale *et al.,* 2006), a value which is close to that reported in the current study. This shows the parasite is susceptible to the drug.

Similar to egg inhibition, the highest EC_{50} value in causing larval mortality was by methanolic extract (EC_{50} = 2.6 mg/ml) and least was aqueous extract (EC= 7.5 mg/ml). Comparable results are reported by Al-Shaibani *et al.,* (2009) on aqueous extract of *Fumaria parviflora* against *H. contortus* with EC_{50} value of 10.23 mg/ml. Findings of this study showed that increasing the concentration of the plant extracts increased the activity. Same observation have also been made by Lullman *et al.,* (1993) who demonstrated that the receptors get saturated with increasing concentration of active ingredient. It is likely that at higher concentration all binding receptors on the larvae were occupied thus leading to hyper-polarisation of membranes limiting excitation and impulse transmission causing flaccid paralysis of larvae muscles (Wasswa and Olila, 2006). The present study showed that larval development inhibition by the extracts showed a better activity in contrast with egg hatch inhibition. The possible explanation could be due to the difference in structure of the egg

shell and cuticle of larvae of *H. contortus* through which absorption of chemicals take place (Eguale *et al.,* 2006). Two mechanisms of nematode uptake of drugs have been reported: either by transcuticular diffusion or by diffusion through intestinal cell (Eguale et al., 2007).

The paralysis and mortality of adult worms observed from the present study can be attributed to combination of active compounds resulting in a synergistic effect on the parasite (Rates, 2001). Adult mortality assay is the most convenient test developed to detect anthelmintic resistance in nematodes (Craven *et al.,* 1999). In the present study, the concentrations required to cause mortality of 50% (EC_{50}) adult *H. contortus* after 24 hours post exposure to aqueous extract, methanolic extract and Albendazole were 5.0, 2.4 and 0.04 mg/ml, respectively. The higher larval and adult worm activity of both the extracts and Albendazole compared to activity on eggs could be explained by the fact that the larvae and adult worms are the feeding stages of the parasite and therefore the effect of the drugs could be through ingestion and penetration (or effect on) through the cuticle (Ferhat *et al.,* 2011). Helminth eggs also have a thick shell which inhibits drug penetration (Martin and Lejambre, 1979).

The safety study was carried out by performing oral acute toxicity test of extracted barks of *Z. mucronata* in Swiss albino mice. In the present study, single dose of oral administration of both aqueous and methanolic extracts to mice at different dosages 500, 1000 and 2000 mg/kg body weight did not cause any mortality and clinical signs. Therefore, the LD_{50} of both aqueous and methanolic extracts of *Z. mucronata* may be considered to be greater than 2000 mg/kg, giving room for further efficacy studies using higher doses. According to the Globally Harmonized System (GHS) of Classification and Labelling of Chemicals, the substances having an LD_{50} value greater than 2000 mg/kg are considered as relatively safe (Anonymous, 2005). The mice were bought at an age after weaning which meant that they continued their growth. Body weight changes are an indicator of adverse side effects of drugs (Feres *et al.,*

2006). Therefore, the absence of significant differences in mean body weight of experimental mice compared to the normal control group provides support for the safety of both aqueous and methanolic extracts of *Z. mucronata*.

In the present study, ALT and BUN were used to assess liver function and kidney function of the experimental animals treated with both aqueous and methanolic extracts of *Z. mucronata*. The liver and heart release ALT and AST and an elevation in their plasma concentrations are indicators of liver and heart damage (Wasan *et al.,* 2001; Mythilypriya *et al.,* 2007). However, ALT is more specific to the liver and is thus a better parameter for detecting liver injury (Ozer *et al.,* 2008). According to Satyanarayana *et al.*, (2001), a rise of BUN provides a positive indication towards adverse effects in kidney functions. In the current study, the mean values of ALT of mice treated with methanolic extracts did not differ significantly compared to those of the normal mice values and this suggests that *Z. mucronata* barks may not be toxic to the liver. However, significantly high ALT values were observed in mice treated with aqueous extracts in concentrations above 1000mg/kg, showing that such dosages may have an effect on the hepatocytes. In contrast, all the dosages tested in the study did not significantly the BUN levels showing that the extracts might not have any effect on kidney functions.

CHAPTER SIX

6. 0- CONCLUSIONS AND RECOMMENDATIONS

6. 1- Conclusions

i. This study showed that there were no significant differences in percentage yield whether methanol or water was used as solvent. Since water is widely available and cheaper than methanol, water as currently used for extraction in field set-ups is recommended. A variety of phytochemicals including flavonoids, saponins, steroids, tannins and glycosides were found to be present in the plant extracts. Tannins and saponins have been reported in litertaure to have anthelmintic activity.

ii. The plant extracts had activity against egg, larvae and adult *H. contortus* but the activity was lower than that of Albendazole, a conventional anthelmintic drug. Hence, the plant extracts can be further developed as an alternative herbal drug in the treatment of gastrointestinal nematode infections.

iii. The oral acute toxicity results from this study showed that the barks extract of *Z. mucronata* are moderately safe in mice at dosage of 500, 1000 and 2000 mg/kg body weight. Biochemical parameters (ALT and BUN) evaluating liver and kidney toxicity did not reveal any significant change in mice treated with methanolic extracts when compared to those of control group. However, significantly high ALT values were observed in mice treated with aqueous extracts in concentrations above 1000mg/kg, showing that such dosages may have an effect on the hepatocytes.

6. 2- Recommendations

i. Quantification of the phytochemicals present in the extracted barks of *Z. mucronata* using GCMS or LCMS is recommended.

ii. Identification and isolation of the bioactive compounds responsible for anthelmintic activity of the plant extracts are needed.

iii. Higher dosage greater than 2000 mg/kg body weight and chronic toxicity evaluation of *Z. mucronata* barks extract should be done to ascertain its long term toxicity.

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APPENDICES

Appendix 1: Procedure for biochemical analysis using clinical chemistry analyser

1. Switch on the instrument and when is display “ready”
2. Use clean & check strip to standardize the machine
3. Use control precinum U for control machine at 37^{o} C special for ALT and BUN
4. Remove a test strip (ALT/BUN) from the container and replace the desiccant stopper on the container immediately
5. Unwrap the strip
6. Using Reflotron pipette apply 32µl of the serum to the center of the red application zone in the strip (ALT/BUN)
7. With the flap open, place the test strip on to the guide within 15 seconds and slide forward horizontally until it locks in to place
8. Close the flap
9. The instrument will display either ALT/BUN to confirm that it has correctly read in the test-specific magnetic code.
10. The ALT/BUN concentration was calculated automatically from the reading taken using function and conversion factors that was entered in the instrument via the magnetic strip on the underside of each test strip.
11. The ALT/BUN concentration was displayed in (u/l / mmol/l) respectively.
12. Remove the test strip from the instrument and dispose it off.

Appendix 2: Article published from this thesis

1. **Ngaradoum O,** Kagira JM, Karanja SM, Kipyegon C and Maina N. (2017). *In vitro* ovicidal and larvicidal activity of aqueous and methanolic extracts of *ziziphus mucronata* barks against *haemonchus contortus*. *European Journal Experimental Biology*, 7:1. doi: 10.21767/2248-9215.100001