# Phytochemical Screening and Anthelmintic Activity of *Entada leptostachya* (Harms) and *Rapanea rhododendroides* (L.) Mez against *Haemonchus contortus*

**Moses Ombasa Omingo**

**A Thesis submitted in partial fulfilment for the Degree of Master of Science in Medicinal Phytochemistry in the Jomo Kenyatta University of Agriculture and Technology**

**2013**

## DECLARATION

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

Signature…… Date…….....

**Moses Ombasa Omingo**

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

1. Signature……..... Date…….....

   **Prof. Patrick G. Kareru**

   **JKUAT, Kenya**

2. Signature……..... Date…….....

   **Prof. James Mbaria**

   **UON, Kenya**

3. Signature……..... Date…….....

   **Prof. Joseph M. Keriko**

   **JKUAT, Kenya**

## DEDICATION

This Thesis is dedicated first to the Almighty Father for his providence, to my beloved family members for their prayers and support. To the friends of nature who always obey the rules of nature. Continue with search for knowledge without violating her rules. Mother Nature is a merciless mother. Everything in nature is enough for all of us. Take what you need from nature but not what you want. All is here for all of us. Utilise the provisions of nature.

## ACKNOWLEDGEMENT

I am grateful to the Almighty God for giving me life and strength to overcome challenges. I wish to express my profound gratitude and thanks to my research supervisors Dr. Patrick Kareru, Department of Chemistry, Jomo Kenyatta University of Agriculture and Technology (JKUAT), The late Dr. Geoffrey Rukunga, Kenya Medical Research Institute (KEMRI), Prof. James Mbaria, Department of Public Health Pharmacology and Toxicology University of Nairobi and Prof. Joseph Keriko, Department of Chemistry, Jomo Kenyatta university of Agriculture and Technology (JKUAT) for their interest, guidance, supervision and support in the course of this research.

I am grateful to Mr. Richard O. Otieno of Parasitology and pathology laboratory, Faculty of Veterinary Medicine, University of Nairobi and Mr. Daniel Gachanja of Department of Zoology, JKUAT for their assistance in identification of the worm *Haemonchus contortus* and guidance in carrying out anthelmintic activities. Thanks also to Mr. Joseph Nderitu (University of Nairobi) for his assistance in brine shrimps bioassay and Mr. Xavier Kizito of ICIPE for running the GC-MS of the sample. My sincere thanks go to Research Production and Extension (RPE) division, JKUAT for funding this project. Sincere thanks to Dr. Bethwell Onyango Owuor, Department of Natural sciences, Catholic University of Eastern Africa (CUEA) for support and encouragements be persistently showered me with, especially when the tunnel used to appear dark. Otherwise, writing of this paper, from this research work for *publications, may not have been that easy.

I appreciate the cooperation of my fellow post-graduate students: Peter Ogoti, James Mogwambo, Dominic Menge, and Martin Magu. Thanks also to Denis Shileka, John Kamau, Josphat Muthanga, Elizabeth Waithera and Wilson Kung'u who assisted me in lab work: Vincent Rioba for his contribution, morally and materially especially for the laptop which enabled me successful complete writing of the thesis.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF PLATES

## LIST OF SCHEMES

## LIST OF APPENDICES

## LIST OF ACRONYMS

| | |
|---|---|
| **ANOVA** | Analysis of Variance |
| **BST** | Brine Shrimp Lethality Test |
| **CAE** | Crude Aqueous Extract |
| **CC** | Column Chromatography |
| **CEE** | Crude Ethanolic Extract |
| **CT** | Crude Tannins |
| **CUEA** | Catholic University of Eastern Africa |
| **DMSO** | Dimethyl sulphoxide |
| **GABA** | Gammaaminobutyric acid |
| **GI** | Gastrointestinal |
| **GIN** | Gastrointestinal Nematode |
| **IR** | Infra-Red |
| **JKUAT** | Jomo Kenyatta University of Agriculture and Technology |
| **$LD_{50}$** | Dose required that kills 50 % Brine Shrimps |
| **MI** | Motility inhibition |

| | |
|---|---|
| **MS** | Mass Spectrometry |
| **NMR** | Nuclear Magnetic Resonance |
| **PBS** | Phosphate buffer solution |
| **PS** | Physiological Solutions |
| **PT** | Post-treatment |
| **RPE** | Research Production and Extension |
| **SAS** | Statistical Analysis System |
| **S.E** | Standard Error |
| **SPSS** | Statistical package for social scientist |
| **TLC** | Thin Layer Chromatography |
| **PTLC** | Preparative Thin Layer Chromatography |
| **UoN** | University of Nairobi |
| **USA** | United States of America |
| **UV/VIS** | Ultra Violet/Visible |
| **WHO** | World Health Organization |

## ABSTRACT

*Haemonchus contortus* is parasitic helminths of the sheep capable of causing acute and high mortality. Control of gastrointestinal helminths infection in livestock relies mainly on the use of synthetic anthelmintics. However, these broad spectrum drugs are expensive, inaccessible or inadequately available to farmers in remote poor settings. The problem with synthetic anthelmintics is further compounded by development of anthelmintic resistance. The present study investigated the phytochemistry and anthelmintic activity of aqueous and solvent extracts (hexane, acetone and methanol) of root bark of *Entada leptostachya* (Harms) and stem bark of *Rapanea rhododendroides* (L.) Mez. To achieve the objectives, brine shrimp lethality test using brine shrimp (*Artemia salina*) larvae was used to determine the toxicity of the aqueous and organic extracts with modifications. The worm motility inhibition assay was utilized in order to investigate the direct effects of the plant extracts on the survival of adult worms of *H. contortus*. Four concentrations (12.5, 25, 50 and100 mg/ml) of each extract were studied in the bioassay. The *Extracts* of *E. leptostachya* exhibited higher toxicity and anthelmintic activity compared to those of *R. rhododendroides*. Methanolic extracts for both *E. leptostachya* and *R. rhododendroides* were more potent against *H. contortus* adult worms exhibiting 70 and 50 % mortality respectively. Hexane extracts of both *E. leptostachya* (20 %) and *R. rhododendroides* (10 %) were the least potent.

Methanol and aqueous extracts of *E. leptostachya* showed brine shrimp lethality of 1.1 and 2.7 g/ml, respectively. *E. leptostachya* and *R. rhododendroides* methanolic extracts have significant anthelmintic activity (70 and 50 %) and have potential for development of new anthelmintic drugs. Saponins, tannins and flavonoids were some of the phytochemicals identified in the extracts. Oleanolic acid was isolated from *E. leptostachya* methanol extract using chromatographic techniques and characterized using spectroscopic techniques (IR, UV and MS). The data revealed the dose-dependent anthelmintic activity in the *in vitro* studies. There were notable anthelmintic activities by all extracts. Methanol extract of *Entada leptostachya* showed the highest anthelmintic activity and therefore it can be used as a lead compound to prepare anthelmintic drugs.

# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Background

Gastrointestinal (GI) nematodes are a major threat and a primary constraint to sheep productivity and endanger animal welfare worldwide. The situation is particularly severe in developing countries (Dhar *et al.*, 1982). *Haemonchus contortus* is a highly pathogenic helminthes parasite of the small ruminants which is capable of causing acute and high mortality in all age groups (Githiori, 2004). It is a big threat to the overall industry of livestock (Saddiqi *et al*., 2010). Though there are many species of gastrointestinal nematodes, *H. contortus* is the dominant one which comprises 73 % of the recovered parasites in sheep in semi-arid areas (Gatongi *et al*., 2003). Haemonchosis has been identified as one of the top ten constraints to sheep and goat rearing in East Africa (Perry *et al.*, 2002).

Control of gastrointestinal helminthes infection in the livestock relies mainly on the use of synthetic anthelmintics in combination with farm management (Waller, 1998; Behnke, *et al*., 2008). However, the broad spectrum drugs are expensive, unaffordable, inaccessible or inadequately available to the poor farmers of developing countries (Hammond *et al*., 1997; Marley *et al.*, 2003). The problem has been compounded by development of anthelmintic resistance due to long term and continuous drug application which have become a threat to continuing livestock production throughout the tropics and sub-tropics (Waller, 1998).

Resistance has affected all major broad spectrum anthelmintic types: Morantel, Trichlophon, levamisole, benzimidazoles and the other nicotinic agonists, in addition to the avermectins and milbemycins (Van Wyk, 1997). Therefore, there is need to prospect for anthelmintic compounds from plant materials.

The World Health Organization (WHO) estimates that throughout the world more than 70 % of the drugs used for a therapeutic goal are from natural origin. Moreover, it has been estimated that more than 30 % of the recently introduced drugs are directly or indirectly issued from natural origins (Wilcox *et al.*, 2001).

**1.2 Phytochemistry of Secondary Metabolites**

Phytochemistry is a study of phytochemicals derived from plants and commonly referred to as natural products. These compounds are mainly organic compounds that are unique to one organism or common to a small number of closely related organisms (Macfoy**,** 1992). Natural products are generally either of prebiotic origin or originate from microbes, plants or animal sources (Nakanishi, 1999). In most instances secondary metabolites appear to be non-essential to the organisms producing them but have a great effect on the physiological functioning of other organisms including man (Macfoy, 1992). However, most defensive chemicals in plants are considered secondary metabolites since a plant will grow perfectly well without them. In the contrast, secondary metabolites are synthesized by plant, allegedly because they play certain biological/or ecological roles toward combating other plants, animals, insects and man (Mann, 1987).

Many studies on natural products have shown interesting biological and pharmacological activities as they are used as chemotherapeutic agents for centuries to treat variety of diseases or serve as the starting point in the development of modern medicines (Verpoorte, 1998; 2000). Natural products from plant have traditionally played important role in treatment of human diseases. Today, about 80 % of the world population residing in third world countries still rely almost entirely on plant product for their primary health care. The remaining 20 % of individuals living in the first world use more than 25 % of the pharmaceuticals which have been directly derived from plant products (Farnsworth, 1984; Cox, 1994).

Natural products derived drugs are extracted by a variety of solvents. The isolated drugs are separated and purified by chromatographic and characterized by various spectroscopic techniques-Columns Chromatography (CC), Thin Layer Chromatography (TLC), Fourier Transform-Infrared (FTIR), and Ultraviolet/Visible (UV/Vis), Nuclear Magnetic Resonance (NMR) Mass Spectroscopy (MS).

### 1.3 Toxicity of Secondary Metabolites

Natural products used in traditional medicine are considered non-toxic because of their long usage in treatment of animals. However, potential hazards associated with long term effects of the plant can occur. Therefore, it is necessary to evaluate the possible toxicity of natural compounds using *in vitro* assay against invertebrates like brine shrimp larvae (Fennel *et al.*, 2004). According to McLaughlin *et al.* (1998), today's work in natural product chemistry must incorporate bioassays.

Extracts must be screened for biological activity, the active extracts selected, fractionation directed with bioassay and the bioactive compounds identified and then exploited. *In vitro* assays present several advantages. They are usually simple to perform, of relative low cost, are reproducible, require a limited amount of biological material and do not need or require the slaughtering of animals (Gordon *et al*., 2007).

This study was designed to evaluate the *in vitro* anthelmintic activity of crude aqueous and organic solvent extracts of the parts of *E. leptostachya* and *R. rhododendroides* when compared to a reference drug albendazole against the GI nematodes.

### 1.4 Problem Statement

Gastrointestinal helminths continue to be a major animal health problem despite the use of anthelmintics and development of new anthelmintic drugs. Available drugs are very expensive and ineffective for the treatment of helminths. Plants with potential anthelmintic properties are not scientifically prove and validated. Since phytochemical compounds that make these plants useful in the treatment of helminths have not been identified, isolated and characterized.

### 1.5 Hypothesis

Extracts from *E. leptostachya* and *R. rhododendroides* plants used as herbal medicine in Kenya have no anthelmintic activity.

### 1.6 General objective

To investigate the phytochemical profile and anthelmintic activity of *Entada leptostachya* and *Rapanea rhododendroides* traditionally used as anthelmintic in small ruminant in Mbeere - Embu county of Kenya.

#### 1.6.1 Specific Objectives

i. To determine the phytochemical compounds in *E. leptostachya* and *R. rhododendroides*
ii. To determine the toxicity of the extracts of *E. leptostachya* and *R. rhododendroides* using brine shrimp lethality test.
iii. To determine *in vitro* anthelmintic activity of aqueous and solvent extracts of *E. leptostachya* and *R. rhododendroides* against *H. contortus.*
iv. To Isolate and characterize the compounds from the most active fraction by spectroscopic techniques.

### 1.7 Justification

In Kenya, Economic loss to the agricultural sector due to *H. contortus,* gastrointestinal parasite of small animals is estimated at over US$ 26 million per year (Githiori, 2004). Conventional synthetic anthelmintic drugs used have been reported to have side effects and some are known to be toxic. Medicinal plants with chemical compounds that can kill the GIN or reduce egg production and have no or least toxicity to the host and are considered as potential candidate for development of new anthelmintic drugs.

Methanol extracts of plants such as *Terminalia Arjuna* has been reported to have significant anthelmintic activity value $LC_{50}$ 645.65 and 467.74 µg/ml against *H. contortus* (Bachaya *et al*., 2009). In addition, ethanol and aqueous extracts of *Artemisia absinthium* have anthelmintic activity against *H. contortus* adult worm with a mean percentage mortality of 90 and 70 %, respectively. These mean percentage worm motility inhibition provide scientific proof for wide application in traditional medicine systems. Therefore, there is a strong need to come up with alternative anthelmintics for the treatment of GIN from the two selected Kenyan medicinal plants.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 Helminthes

The parasitic helminthes of grazing animals belong to the phyla Platyhelminthes and Nemathelminthes (Soulsby, 1982). Platyhelminthes are also known as flatworms and consist of two classes of parasites, the Trematoda (flukes) and the Cestoda (tapeworms).These parasites are flattened and dorsoventrally and hermaphroditic. The *monieza* spp is the most common and abundant Cestode parasites in grazing small ruminants (Soulsby, 1982). The most common and important trematode in livestock in Kenya *is Fasciola gigantica* which is endemic in marshy areas with poor drainage and high rainfall (Wamae, *et al.,* 1990) Class nematode (roundworms) belong to the Nemathelminthes. Some of the super families of veterinary importance in this phylum include *Ascaridoidea, Trichostrongyloidea* and *Strongyloidea* (Anderson, 1992).

Helminthiasis is a complex of conditions caused by parasites of the classes Nematoda, Cestoda and Trematoda in livestock (Githiori, 2004). In Kenya, GI nematodes especially *Haemonchus contortus* is prevalent because the animals remain exposed to these nematodes found in the pasture they continuously graze all year long (Dinnik and Dinnik, 1958). Infections caused by *H. contortus* were reported to affect all adult sheep and lambs in a flock, resulting in numerous deaths within six week s after the onset of clinical signs (Allonby and Urquhart, 1975).

Direct and indirect losses caused by GIN are estimated to be very high and therefore, control of these parasites is necessary.

## 2.2 Control strategies

The main methods for control of GI nematode parasite are prophylactic treatment with synthetic anthelmintics in combination with grazing management. However, alternative strategies have also been examined for the sustainable control of GI nematode parasites (Githiori, 2004).

### 2.2.1 Conventional Anthelmintics Drugs

Anthelmintics are drugs that act against worms and they can be used prophylactically or therapeutically (Coles *et al.*, 1992). Anthelmintics that are effective against many species or genera are termed "broad spectrum" while those against a few species are termed "narrow spectrum" anthelmintics. Most of these drugs are commercially available and considerably vary in their effectiveness and toxicity (Hamilton, 2001). The common anthelmintic drugs used in Kenya are: mebendazole **(1),** levamisole **(2)** and albendazole **(3).**

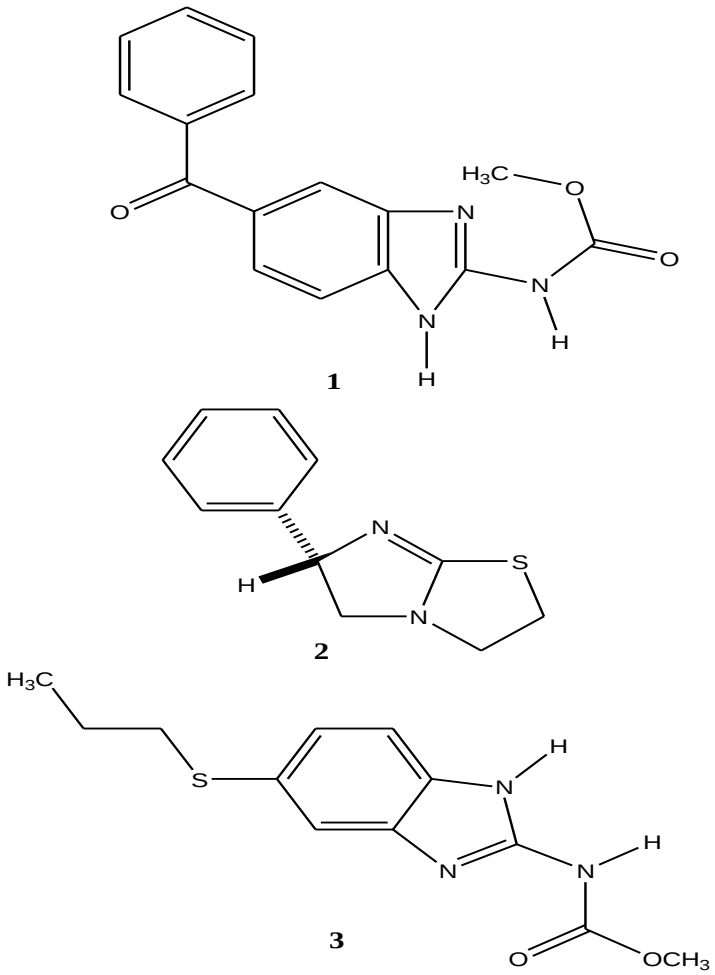



**Figure 2.1**: Structures of mebendazole **(1),** levamisole **(2)**, and albendazole **(3)**

## 2.3 Classification of Anthelmintics

The broad spectrum anthelmintics remove parasites in different stages of development within the host species. Currently, there are three major classes of synthetic anthelmintics used for control of GI nematode parasites of small ruminants (Githiori, 2004): benzimidazoles (including albendazole, fenbendazole), cholinergic agonists (including levamisole/morantel), and the macrocyclic lactones or avermectins and milbemycins (including avermectins, moxidectin) (Githiori, 2004).

### 2.3.1 The Benzimidazoles/ Probenzimidazoles (BZs) Group

The mode of action of BZs is by interference with polymerization of microtubules (Harder, 2002). These drugs bind to the protein tubulin of the parasite therefore, causing death by starvation (Roos, 1997).They have a selective inhibitory action being 250 – 400 times more effective in producing this effect in helminths than in mammalian tissue. The effect takes time to develop and the worm may not be expelled for several days (Rang *et al.,* 2007).

### 2.3.2 The Tetrahydropyrimidines/ Imidazothiazoles group (Levamisole/ Pyrantel-Morantel)

These drugs affect acetylcholine neuro-transmission by interfering with nicotinic acetylcholine receptors on the muscles (Roos, 1997; Harder, 2002). They cause contracting of the muscle followed by paralysis of the worm. The paralysed worms are then expelled in the faeces. These drugs are given orally and do not kill the ova. The unwanted effects include gastrointestinal disturbances, dizziness and skin eruptions (Rang *et al.,* 2007).

### 2.3.3 The Macrocyclic Lactones (MLs) or Avermectins/Milbemycins Group

The MLs are thought to interact with chloride channels on helminths gamma-aminobutyric acid (GABA) receptor complexes, and also inhibit pharyngeal pumping (and hence feeding), motility and fecundity in susceptible nematodes resulting in paralysis and ultimately elimination from the host (Harder, 2002; Yates *et al.,* 2003). There are other anthelmintics referred to as narrow spectrum

compounds, which have activity against fewer species of parasites and/or lack high levels of efficacy against all stages of the parasites (Bowman *et al.,* 2003). Examples of these anthelmintics include naphthalophos, salicylanilides and substituted phenols (closantel, oxyclozanide and nitroxynil) and triclabendazole (Githiori, 2004).

### 2.4 Anthelmintic Resistance

Anthelmintic resistance is defined as a decrease in the efficacy of an anthelmintic against a population of parasites that is generally susceptible to that drug (Sangster and Gill, 1999). Misuse and poor formulations of these products have led to the development of anthelmintic resistance (Lans and Brown, 1998). New approaches to nematodes parasite control in small ruminants are needed to counteract the problem of anthelmintics resistance (Waller, 1999). Furthermore, lack of veterinary services in remote pastoralist areas has worsened the problem (Lans *et al.,* 2007). Nematodes that are resistant to other, narrow-spectrum anthelmintic, such as closantel, have been reported (Sangster and Gill, 1999).

The problem of anthelmintic resistance in small ruminants has been reported in South Africa, Australia, New Zealand, Malaysia, Spain, France, Denmark UK, Brazil, and the United States (Young *et al*. 1997; Mortensen *et al*. 2003). The common gastrointestinal nematodes of small ruminants include; *Haemonchus contortus, Telodorsagia circumcinta, Trichostrongylus axei, Nematodirus spp*, and *Cooperia spp* (Radostits *et al.,* 2000). The proportions of each of these nematodes in small ruminant populations vary according to geographical location.

*Haemonchus contortus* and *T. circumcincta* represent most of the parasite burdens seen in small ruminants with *H. contortus* being present in highest numbers. Anthelmintic resistance is present in all of these parasites, but the prevalence is highest for *H. contortus* making it the most economically important gastrointestinal nematodes of sheep and goats (Uhlinger, 1992).

### 2.4.1 Conventional Anthelmintics Drug Resistance

Conventional methods used to control helminthiasis in animals include anthelmintics drenching, pasture spelling, crop rotation and integrated grazing systems using stock with low internal parasite susceptivity. Currently, the least complex of control options preferred for internal parasite management is anthelmintics. Uses of synthetic anthelmintic drugs are toxic, expensive and have side effects to both the animal and the drug administrator. Long use and poor administration leads to drug resistance. Anthelmintic resistance has been documented across the World, including Africa (Maingi, 1991) and North America (DeVaney *et al*., 1992; Fleming *et al*., 2006). Development of parasitic resistance to commercially available drugs, for example in the USA has become a serious problem due to misuse and properly adaptation of the parasite to commercially available drugs (Mascie-Taylor and Karim, 2003). Besides, the drugs are unaffordable, not available or in inadequate supply under local conditions (Hammond *et al.,* 1997). However, increasing prevalence of anthelmintic resistant strains of gastrointestinal nematodes and high cost of conventional anthelmintics have created an interest in studying medicinal plants as an alternative source of anthelmintics. This trend is especially relevant to organic small ruminant

production (Green and Kremen, 2003) although the effectiveness of herbal dewormer remains unproven. There is need for Ethno-veterinary alternatives (based on medicinal plants) for small-scale livestock farmers who cannot use allopathic drugs or for those larger conventional farmers whose economic circumstances prevent the use of veterinary services for health problems in their livestock (Lans *et al.,* 2007).

### 2.4.2 Anthelmintics Activity of Plant Extract

Studies from various parts of the world have shown that certain plant species effectively reduce the degree of parasite infestation in ruminants (sheep) and are promising alternatives to conventional anthelmintics (Githiori *et al.,* 2006; Max *et al.*, 2007; Jan *et al*., 2008; Goswami *et al*., 2011). Developing countries can derive economic benefits by using their natural resources of medicinal plants by using traditional knowledge, which has often played a major role in the development of modern science (Choudhary and Atta-Ur-Rahman, 2005). Knowledge gained from research in natural product will significantly benefit drug discovery (Gad, 2005). Traditional knowledge on herbal medicine maybe utilized as the possible alternatives to synthetic anthelmintics. Considerable research has shown that some plants not only affect the nutrition of animals, but also have antiparasitic effects (Waghorn and McNabb, 2003; Adam *et al*., 2012). Therefore, indigenous knowledge on herbal medicines can be used as the possible alternative to synthetic anthelmintics.

Study of *Nauclea latifola* was shown to prevent *Strongyloide* nematode eggs from hatching and was observed to have comparable anthelmintics efficacy with commercial anthelmintics drugs, levamisole and albendazole at a concentration of 100 mg/ml (Onyeyili *et al*., 2001). Githiori (2004) reported ethno-medicinal uses of a number of plants with purported anthelmintics activity used by pastoralists and small scale holders in Kenya. The plants documented are *Aframomum sanguineum, Albizia anthelmintica, Ananas comosus, Annona squamosa, Azadirachta indica, Dodonaea angustifolia, Hagenia abyssinica, Hildebrandtia sepalosa, Myrsine africana, and Olea europaea* var. *Africana*. Gefu *et al.* (2000) has documented on plants used as anthelmintics in Nigeria which include *Allium cepa, Citrus aurantifolia, Khaya senegalensis and Terminalia avicenmoides, Ananas comosus* (Baldo, 2001) and *Azadirachta indica* (Chandrawathani *et al*., 2002) have been reported to have nematocidal activity in ruminants. Wasswa and Olila (2006) have reported *in vitro* ascaricidal activity of selected medicinal plants endemic to Uganda. Iqbal *et al*. (2004) found alcoholic extracts of *Artemisia brevifolia* at 25 mg/ml more effective in causing mortality of *H. contortus* as compared to the aqueous extracts. Similarly, the hydro-alcoholic extract of *Coriandrum sativum* and *Hedera helix* also showed better anthelmintic activity against *H. contortus* as compared to aqueous extracts (Eguale *et al*., 2007).

Tariq *et al*. (2009) reported *In vitro* studies, revealed significant anthelmintic effects of aqueous extracts and ethanol extracts on live *H. contortus* worms ($p = 0.05$) as evident from their paralysis and/or death at 8 hr post exposure.

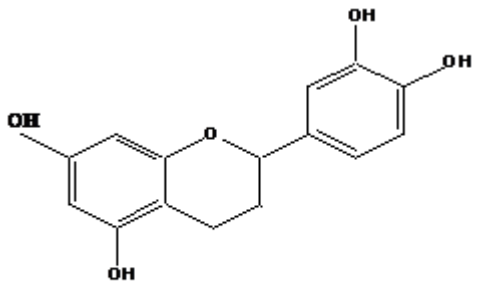
Aqueous extracts of *Achilea millifolium* resulted in a mean worm mortality of 94.44 %, while ethanol extracts resulted in mean worm mortality of 88.88 %. The extracts of several plants growing in Kenya have been reported with high anthelmintic activity both *in vitro* and *in vivo.* In most cases the method of accessing the efficacy of the drug is to determine the effect on worm eggs counts before and after treatment (Jorgen and Brian, 1994). Recently, Kakari *et al*. (2013) described the *in vitro* anthelmintic activity of crude methanol extract, n-hexane, ethyl acetate, chloroform extract and aqueous fractions of *Ferula costata* (Kor) against *H. contortus.* Crude methanol extract showed the best activity (33.77 mg/ml) followed by hexane (39.77 mg/ml, ethyl acetate (42.76 mg/ml, chloroform (67.32 mg/ml) and aqueous (539 mg/ml while $LC_{99}$ of positive control (Levamisole) was 1.257 mg/ml.

One of the most successful strategies of the extraction of medicinal compounds from higher plants include the pharmacological screening of plant extracts followed by bioassay guided fractionation of the active plants and leading to the isolation of the pure constituents (Vlientich *et al.,* 1997). However, herbal medicine can be poisonous if taken in large quantities and more so if administered by inexperienced practitioners, hence, a correct dosage is of outmost importance (Kareru *et al.*, 2007a). There is also need to carry out the phytochemical and bioactivity studies of compounds from *E. leptostachya* and *R. rhododendroides* used as anthelmintic (dewormer).

## 2.5 Secondary Metabolites with Reported Anthelmintic Activity

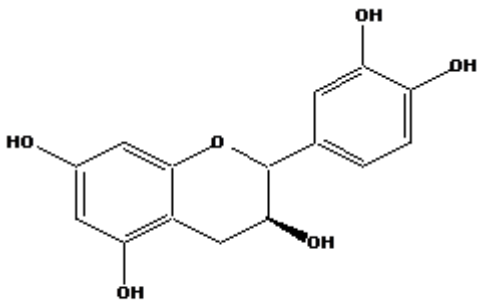
### 2.5.1 Condensed Tannins

Condensed tannins are polymeric flavonoids (polyphenols) often referred to as proanthocyanidins. The most widely studied condensed tannins are based on the flavan-3-ols namely epicatechin **(4)** (Fig. 2.2) and catechin **(5**) (Fig. 2.3)




4

**Figure 2.2:** Structure of epicatechin **(4)**




5

**Figure 2.3:** Structure of catechin **(5**)

Simple polyphenols polymerize to form linear procyanidins which are formed by linkage of monomers like (-) epicatechin and (+) catechin for example sorghum polycyanidin **(6).**

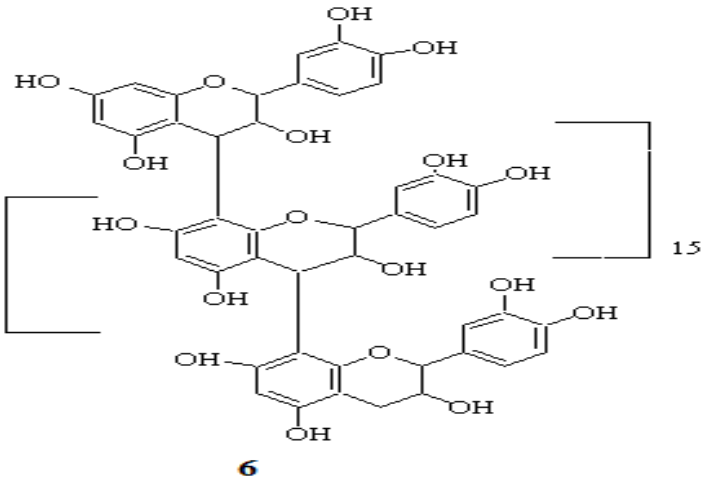



**Figure 2.4:** Structure of polycyanidin **(6)**

High concentration of the condensed tannins is reported to have anthelmintic effects against nematodes parasites (Khan and Diaz-Hermandex, 2000). Cassava foliage is relatively rich in condensed tannins (Wanapat *et al*., 1997). High content of condensed tannins in cassava, exhibit protection against nematode parasites in goats (Mekarn, 2003).

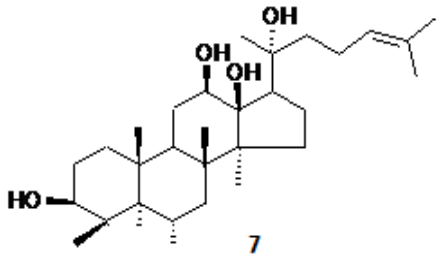
Anthelmintic activity of condensed tannins against nematodes parasites in goats has also been demonstrated (Granum *et al.,* 2003; Butter *et al*., 2000; Kabasa *et al.,* 2000; Molan *et al.*, 2002; Nielzen *et al*., 1996).

In traditional medicine which is practiced in many parts of the world, the use of plants with anthelmintic properties has been considered as an alternative to synthetic anthelmintic drugs (Niezen *et al*., 1998).

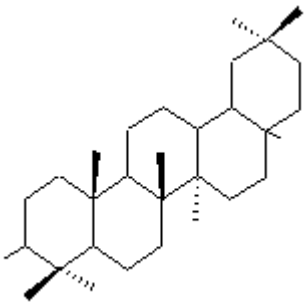
Grazing forage high in tannins or adding purified condensed tannins (CT) to the diet has been shown to reduce parasite eggs in sheep and goat faeces in a number of studies (Niezen *et al*., 1998; Athanasiadou, 2000; Min and Hart, 2003; Paolin, 2003).

### 2.5.2 Saponins

Saponins occur naturally as triterpenoids and steroid saponins and are mainly distributed in higher plants. Steroidal saponins are commonly found in Lilaceae, Dioscoreaceae and Scrophuhrhceae while triterpenoids saponins exist in Araliaceae, Leguminosae, Polygalaceae and Cucurbitaceae (Wu and Yao, 2003). Saponins consist of a glycone part which is a sugar moiety (oligosaccharides). The aglycone part is referred to as a sapogenin which is either steroid **(7)** or triterpenoids **(8)**. The sugar moiety may be attached to the sapogenin by an ester or ether linkage at one or two glycosylation sites. The oligosaccharide is mainly attached to $C_3$ position giving rise to a monodesmosidic while an additional oligosaccharide at $C_{26}$ or $C_{28}$ forms bidesmosidic saponin. Attachment of glycone to a third site (tridesmodic) in a sapogenin is rare (Natori *et al*., 1981).




7



**8**

**Figure 2.5:** Structures of steroid **(7)** and triterpenoids **(8)**

Saponins containing plants have been used for medicinal purpose among the oriental drugs where many herbal drugs contain saponins as their principal constituents (Osamu, 1990). In addition, saponins are precursors of important therapeutically drugs such as cortisone and contraceptive oestrogen. Pharmaceutical activities associated with saponins include cytotoxity, anti-tumor, anti-mutagenic, anti-inflamatory, anti-viral and cardiac activities in addition to anthelmintic activity (Watt and Brayer, 1962).

Monodesmosidic saponins destabilize membranes and increase cell permeability by combining with membrane-associated sterols [Price *et al*., 1987; Gee and Johnson, (1988)]. Mc-Allister *et al*. (2001) reported anthelmintics effect of *Combretum molle* due to the presence of saponins. The leaves of *Combretum molle* contain steroidal acids and saponins.

Other plant extracts with anthelmintic activity *in vitro* are *Peltophorum africanum* and *Leucaena leucocephala* (Bizimenyenra *et al*., 2006; Ademola and Idowu, 2006). Saponins were first reported to kill worms as early as 1962 by Watt and Brayer, using an extract from *Albizia anthelmintica*. Some medicinal plants containing saponins (in the *Entada* genera, Fabaceae family) have been associated with anthelmintic properties. Manandhar (1995) documented *Entada scandens* as one of the anthelmintic plants among the Nepal medicinal folklore. Baoura *et al*. (1976) reported *Entada africana* as a plant used as a vermifuge. Rotenone (Gaudin and Vacherat, 1938) and alkaloids (Baoua *et al*., 1976) are among the compounds found to be present in *Entada africana*. A triterpene saponin (Okada *et al.*, 1988), lupeol triterpene, beta-sitosterol, prosapogenin, a triterpene glycoside called Entanine (Hariharan and Rajagopalan, 1976); Entadamide B (Ikegami *et al*., 1989), are among the reported saponins related compounds isolated from *Entada scandiens.* Saponins make the highest proportion in the latter plants and they are probably associated with its observed anthelmintic activity (Manandhar, 1995).

### 2.5.3 Alkaloids

Alkaloids are plant secondary metabolites found in about 5,500 known plant species. They contain nitrogen found in the chemical ring structure, alkali-like chemical property and pharmacological activity hence their use in medicine. Alkaloids have a bitter taste with quinine being one of the bitterest substances known (Trease and Evans, 1989)

The Chinese herbal medicines which contain alkaloids have been used to treat diseases like asthma, chronic bronchitis, and flu symptoms. Some studies have shown alkaloids to be responsible for anthelmintic properties. Recently, Sorajin *et al.* (2011) and Patel *et al.* (2011) reported anthelmintics effects of *Saraca indica* and *Lantana camara*, respectively which were due to the presence of alkaloids.

**Table 2.1:** Ethno medical uses of some *Entada* genera

| **Species Name** | **Ethnomedical use(s)** | **Compounds Present** |
|---|---|---|
| Entada abyssinica (A.Rich) | Epilepsy (Mathias, 1982) Sleeping sickness(Freiburghaus et al., 1996 ) Cancer (Kamuhabwa et al., 2000) | Flavonol glycoside, Phytosterol glycoside (Debella et al., 2000) |
| Entada africana (Guill and Perr) | Abortifacient (Watt and Brayer, 1962) Coughs, Bronchitis, Sores (Le Grand, 1989) Vermifuge (Baoua et al.,1976), Fish poison (Gaudin and Vacherat, 1938) Liver disease (Sanogo et al.,1998), Diarrhea, Eye inflammation (Le Grand, 1989) | Rotenone (Gaudin and Vacherat,1938) Alkaloids (Hussain and Deeni, 1991). |
| Entada gigas (L.Fawc and Rendle) | Aches and pains (Coe and Anderson, 1996 a) Skin rashes and sores (Coe and Anderson, 1996 b) Rheumatism (Kamdem et al., 1986) | Archidic acid, Behenic acid, Linoleic acid, Linolenic acid, Oleic acid, Palmitic acid, Stearic acid, (Derbesy and Busson, 1968) |
| Entada glandulosa (Gagnep) | Cancer (Wasuwat, 1967) | No information |
| Entada monostachya DC | Wounds or Bruises (Martinez, 1984) | No information |

| | | |
|---|---|---|
| *Entada phaseoloides* L.Merr | Stomach ache, Convulsions (Croft and Tutpulotu,1980) | Entadamine A, Entamide-C,Beta-Entadamine A,D-glucoside, Gentisic acid (Ikegami *et al.*, 1989) |
| *Entada scandens* Benth | Diabetes (Murakami *et al.*, 1993) Anthelmintic (Manandhar, 1995) Fish poison, Soap (Duke, 1975) Contraceptive Oral thrush in children (Holdsworth, 1990) Stomach pains (Bourdy and Walter, 1992) Sterility (De Laszlo and Henshaw, 1954) Piles (Girach *et al.*, 1994) Snake bite (Nagaraju and Rao, 1990) Cancer (Wasuwat, 1967) | Archidic acid, Behenic acid, (Sengupta and Basu, 1978), Entada I Triterpene saponin(Okada *et al.*, 1988) Lupeol triterpene, Beta-sitosterol (Hariharan,1975) Prosapogenin A triterpene, (Hariharan *et al.*, 1975), Triterpenoid glycoside (Hariharan and Rajagopalan, 1976) Entadamide B sulfur compound (Ikegami *et al.*, 1987) Phaseoloidin Benzenoid (Barua *et al.*, 1988) |
| *Entada spiralis* Ridl | Chest pains (Goh *et al*., 1984) | No information |
| *Entada stuhlmannii* Taub (Harm) | Aphrodisiac(Kokwaro, 1976 ) | No information |

| | | |
|---|---|---|
| *Entada sudanica* Schweinf | Fish Poison (Gaudin and Vacherat, 1938) | Rotenone(Gaudin and Vacherat,1938) |
| *Entada polystachya* L (DC) | No information | Phenyl Alanine, Cystine, Quinolizidine Alkaloid, Methionine proteid, (Giral *et al*., 1978) |
| *Entada mannii* Oliv.Tisser | No information | Behenic acid, Lauric acid, Linoleic acid, Linolenic acid, Oleic acid Palmitc acid, Stearic acid, (Derbesy and Busson, 1968) |
| *Entada leptostachya* (Harm) | Concoction of bark used as anti-venin in case of snake bite (Owuor and Kisangau, 2006) | No information |

*__Source:__ Kareru (2008)

## 2.6 Ethno-Botanical Information of Plants with Anthelmintic Activity

### 2.6.1 *Entada leptostachya* (Harms) (Mimosaceae)

*Entada leptostachya* belongs to the Mimosaceae family, *Entada* genus and species *E. leptostachya* (Plate 2.1 and 2.2)**.** This plant is a climbing shrub or a small tree 3 - 10 m with a grey smooth bark. The leaves are arranged almost alternate and the flowers are yellow (or white) and bisexual. The fruits are brown straight or curved abit. The plant is distributed in most part of Kenya, Tanzania, Somalia and Ethiopia (Kareru, *et al*., 2007b). It is locally known as “Hundad” among the Boran, “Mwaitha” (Kamba), “Kobagor” (Somali), "Mgambari” (Swahili) and “Ldalampo” among the Samburu (Beentje, 1994). The bark decoction is used by the Kamba people to treat snake bites (Owuor and Kisangau, 2006). The Embu and the Mbeere communities in Eastern Province, Kenya use the bark decoction to treat worms in animals. Additional information on some of the *Entada* genera is summarized in Table 2.1.

**Plate 2.1:** *Entada leptostachya* aerial parts

**Plate 2.2:** *Entada leptostachya* plant showing fruit pods (dry season)

### 2.6.2 *Rapanea rhododendroides* (L.) Mez (Myrsinaceae)

Synonyms: *R. pulchra* (Gilg and Schellemb.) (L.) Mez. *Rapanea melanophloeos* (L.) Mez. It is an evergreen tree growing to 20 m and commonly found in upland forests on edge of moorlands. Its leaves cluster at the branch ends which are elliptic to obovate. Found from the Coast to the Western part of the country and most of tropic Africa to South Africa. More common in high land forest to altitude as high as 3800 m (Maundu and Tengnas, 2005). Flower clusters below leaves on old wood from scaly knobs. The plant is known as "Ol-engabbura" among the Maasai, "Muthitha" among the Ameru and "Mugaita" by the Agikuyu communities. The bark, roots and fruits are reputed to have anthelmintic properties (Beentje, 1994). The fruits were reported to be used as anthelmintics in livestock and in humans, to be used primarily against cestodes but also as general anthelmintics (Kokwaro, 1993).

# CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1 Cleaning Procedures

The glassware used in this study were soaked in chromic acid overnight then cleaned thoroughly with hot water and detergent, rinsed with distilled water followed by acetone and dried at 150 ℃ in an electric oven for one hour. All the general purpose solvents were freshly distilled prior to use. Silica gel and Thin Layer Chromatography plates were from Aldrich Chemical Company Ltd., England and Merck, Germany.

A synthetic anthelmintics drug (1.5 % albendazole) was purchased from an Agro veterinary store at Juja and was used as a positive control in anthelmintic study. The solvents (hexane, acetone and methanol,) used were general purpose reagents and were obtained from Kobian (K) Ltd, Nairobi-Kenya and were distilled before use.

### 3.2 Plant Material

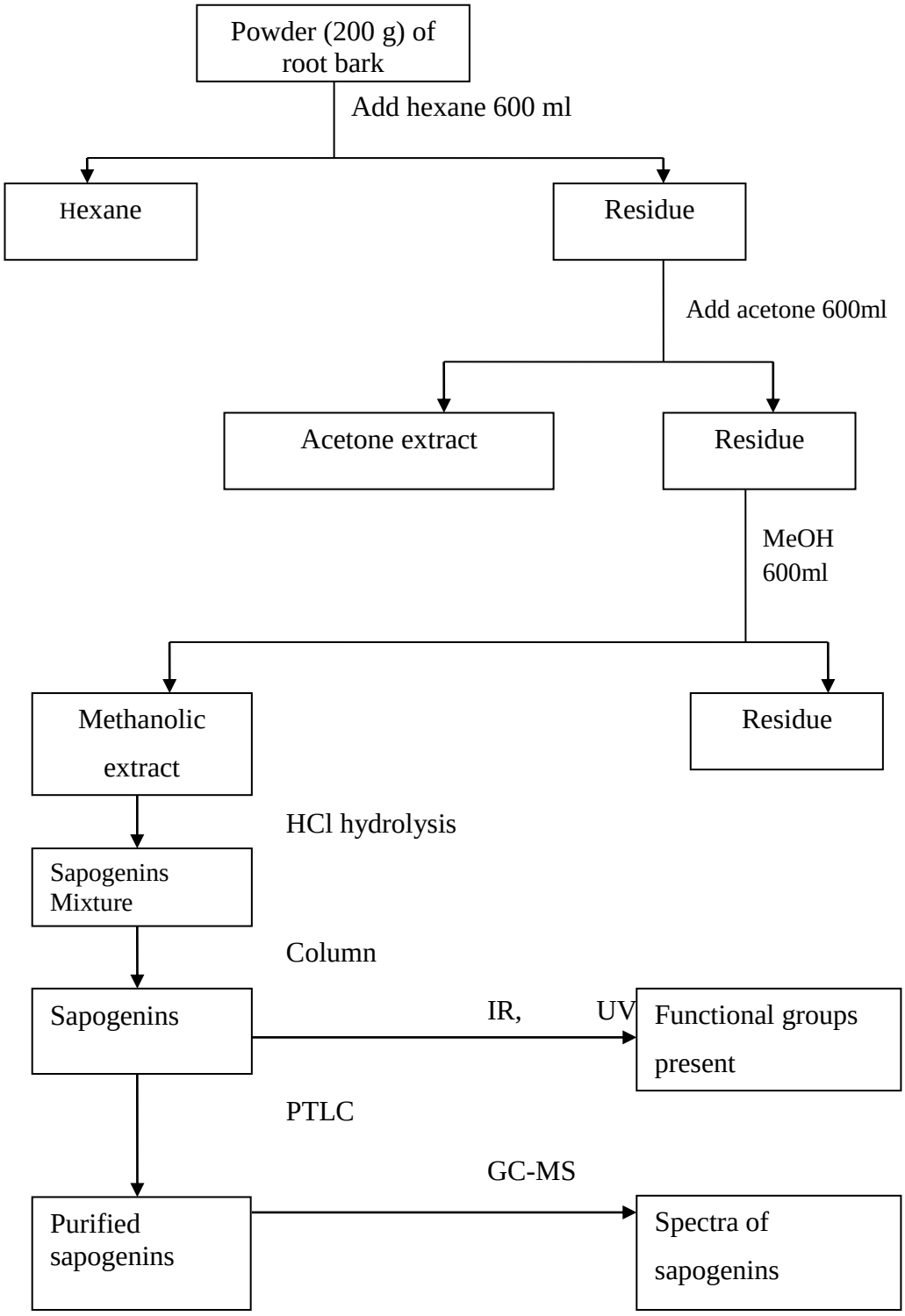
The root bark of *E. leptostachya* and stem bark of *R. rhododendroides* were collected from Mbeere in Embu County. The plant materials were identified and authenticated by a plant taxonomist and reference specimens were deposited at Jomo Kenyatta University of Agriculture and Technology Herbarium in the Department of Botany. The voucher specimen of *E. leptostachya* and *R. rhododendroides* were recorded as OMO/01 and OMO/02, respectively.

The plant materials were air dried in a well-ventilated room under normal room temperature, then ground into powder form using a laboratory mill and stored in a dry closed container for further use.

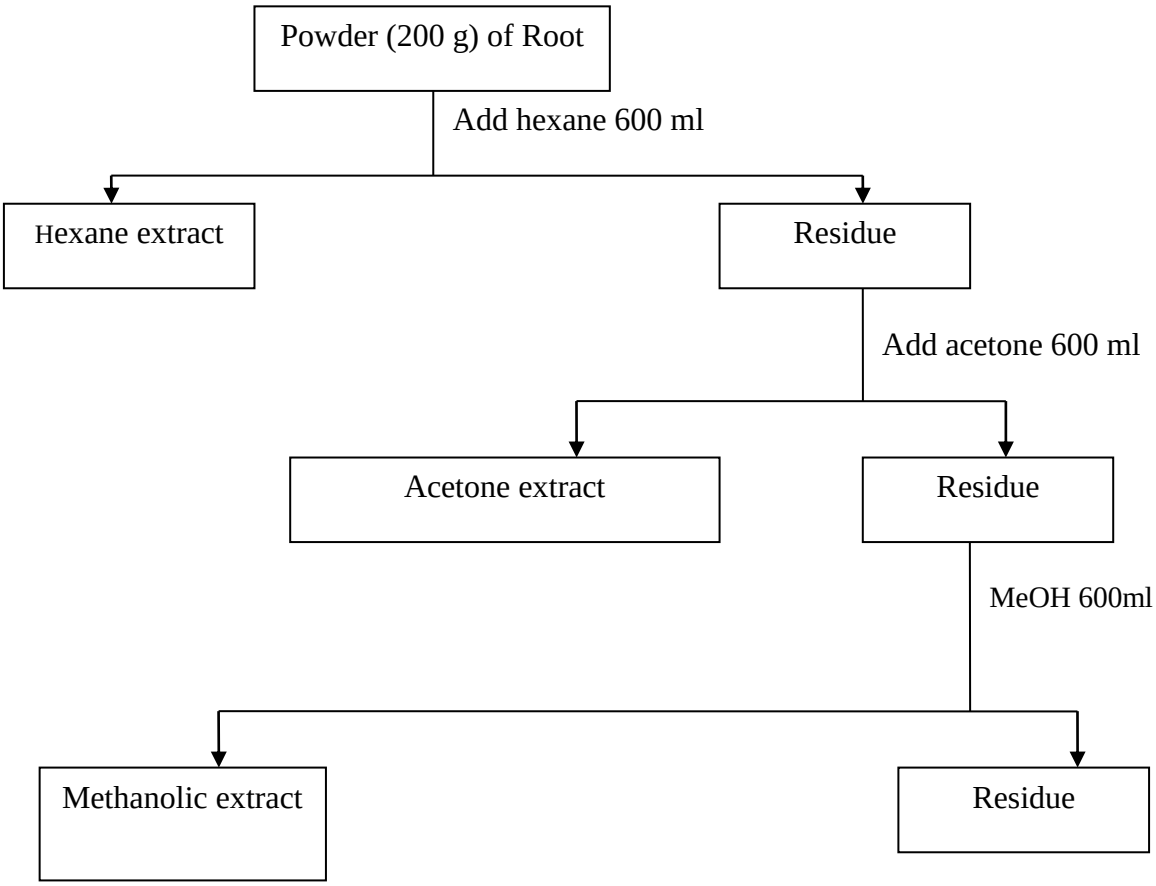
### 3.3 Extraction of Plant Materials Using Polar and Non - Polar Solvents

Aqueous extracts were prepared by adding 100 g of pulverized plant material in 500 ml of hot distilled water in a glass percolator. It was allowed to macerate for 24 hr at room temperature and the brew was filtered using Whiteman No 1 filter papers. The process of percolation was repeated three times (× 500 ml). The combined filtrates was then concentrated in a Rotary Evaporator to ensure complete evaporation of the solvent. The final crude aqueous extract was transferred to a vial, labelled for easy identification and kept in a clean air tight bottle and was stored at 4 $^{o}$C until used (Tariq *et al.*, 2009).

Solvent extracts were prepared by placing about 200 g of the air-dried pulverized powder sample and sequentially extracted in a soxhlet apparatus for 6 hr using distilled solvent of increasing polarity. The solvents used were hexane, acetone and methanol. The crude extracts were concentrated in vacuum at 50 ℃ using a Buchi Rotary Evaporator coupled with a thermal regulator. The weight of each dry extract was taken and percentage yield determined and recorded for each extract. Each extract was put in a clean labelled bottle for easier identification and stored in a freezer at 4 $^{o}$C until used. Scheme 3.1 summarizes the extraction and bio-activities carried out.




**Scheme 1:** Flow diagram for the solvent extraction of the powdered root bark of *Entada leptostachya*




**Scheme 2:** Flow diagram for the solvent extraction of the powdered root bark of *Rapanea rhododendroides*

### 3.4 Brine Shrimp Lethality Test

Brine shrimp (*Artemia salina*) larvae were used to determine the toxicity of the aqueous and organic extracts of *E. leptostachya* and *R. rhododendroides* with some modifications. The brine shrimp (*Artemia salina*) toxicity test is used as a basic indicator of toxicity (McLaughlin *et al.*, 1991; Meyer *et al*., 1982). Brine shrimp are also used since they respond similarly to the mammalian systems.

For instance, the DNA - dependant RNA polymerases of *A. salina* have been shown to be similar to the mammalian type (Birndorf *et al*., 1975; Solis *et al*., 1993). Brine shrimp (*Artemia salina*) eggs (Interpet Ltd. Dorking, England) were hatched in artificial sea water which was prepared by dissolving 40 g of sea salt (Sigma Chemical Co., UK) in a litre of distilled water as described by Rabel *et al*. (1994).

After 48 hr incubation in a warm room (22 – 29 ℃), nauplii were collected with a Pasteur pipette after attracting the organisms to one side of the vessel with a source of light. Nauplii were separated from the eggs by pipetting them 2 – 3 times into small beakers containing seawater. The aqueous or organic plant extracts (10 mg) were dissolved in 1 ml of dimethyl sulphoxide (DMSO) for organic extracts and 1 ml distilled water for aqueous extracts and this was called solution A. Solution B was prepared by adding 0.5 ml solution A in 9.5 ml of either water for aqueous or DMSO for organic extracts. The following volumes of solution B were added to the vial (20 µl, 200 µl and 2 ml) giving the following concentrations of plant extract used: 10 µg/ml, 100µg/ml and 1000 µg/ml.

A set of controls were prepared using DMSO for the organic extracts and distilled water for the aqueous extracts. The discs impregnated with the above extracts were dried at room temperature (22 – 27 ℃) to evaporate the DMSO, leaving only the test compound on the disc. The discs for water extracts were used while still wet. Five millilitres of sea water was added to each of the vials and 10 live one-day old brine shrimp larvae were added to each vial containing sea water and filter paper discs.

A drop of yeast suspension was supplied to each vial (3 mg in 5 ml sea water) to serve as source of food for the shrimp. The vials were incubated at 27 ºC for 24 hr. After the incubation period, live shrimp were counted. The percentage lethality was calculated from the mean survival larvae of extracts treated tubes and control. The Lethality Dose Fifty ($LD_{50}$) values were obtained by probits method by plotting the mean (%) dead brine shrimp against extract concentration (on log scale) for each concentration (McLaughlin *et al*., 1991).

### 3.5 *In vitro* Anthelmintic Activity of *E. leptostachya* and *R. rhododendroides*

Mature *H. contortus* worms were collected from the abomasums of freshly slaughtered sheep at Ruiru abattoir. The worms were collected by the meat inspector at the abattoir and later identified by a parasitologist at Zoology department, JKUAT. The worms were washed and finally suspended in phosphate buffered saline (PBS) and transported to the laboratory in air tight can where they were left for 2 hr to acclimatize before use. The dry plant extract samples were retrieved from 4 ℃ and a stock solutions was prepared by dissolving 10 mg in 1 ml of DMSO for organic extract and 1 ml distilled water for aqueous extract and this was called solution A. Solution B was prepared by adding 0.5 ml solution A in 9.5 ml of either water for aqueous or DMSO for organic extracts. Forty micro litres (40 µl) of solution B was added to filter discs to give concentrations of plant extracts of 20 µl/ml. The discs impregnated with the above extracts were dried at room temperature to evaporate the DMSO, leaving only the test compound on the disc; the discs for water extracts were not dried. Five sterile Petri dishes were labelled A, B, C, D and E.

Ten (10) adult *H. contortus* worms were placed in each sterile Petri dish containing 10 ml of Physiological solution (PS). Dry filter disc containing aqueous extract was added and agitated. The procedure was repeated for all the other plants extracts. Albendazole was used as the reference drug (positive control) while distilled water was used as a negative control. Three replicates were performed for each treatment. Inhibition of worm motility and mortality were the rationale for anthelmintic activity. After periods of 12, 24 and 36 hr, the extracts and albendazole were washed away and the parasites were suspended in PBS for 30 min. for possible recovery of the parasite motility. The number of motile (alive) and immotile (dead) worms were counted using a hand lens, and recorded for each concentration. Death or paralysis of worms was ascertained by absence of motility for an observation period of 5 - 6 sec. (Rabel *et al.*, 1994).

### 3.6 Phytochemical Analysis of Medicinal Plants

Chemical tests were carried out on all extracts to qualitatively determine the phytochemical constituents of *E. leptostachya* and *R. rhododendroides* as described by (Harborne, 1998).

#### 3.6.1 Test for Saponins

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. Ten (10) ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously (for 2 min) for appearance of stable foam that persists for 15 min. The foam was mixed with 2 drops of olive oil and shaken, then observed for the appearance of emulsion (Harborne, 1998).

### 3.6.2 Test for Tannins

About 0.5 g of the dried samples were mixed with 2 ml of distilled water in a test tube and boiled and then filtered. A few drops of 0.1 % of Ferric chloride was added and observed for brownish green or dark blue coloration (Harborne, 1998).

### 3.6.3 Test for Reducing Compounds

Two(2) ml of the extract were mixed in a test tube with 2 ml mixture of equal volumes of Fehling's solution A and B were added and boiled in a water bath for 2 min. The test tube was checked for a brick red precipitate (Harborne, 1998).

### 3.6.4 Test for Alkaloids

About 0.2 ml of the extract were stirred and placed in 5 ml of 1 % hydrochloric acid in a steam bath and filtered. One (1) ml of the filtrate was treated with 3 drops of Dragendorff's reagent. A yellow precipitation with this reagent was considered as evidence for the presence of alkaloids (Harborne, 1998).

### 3.6.5 Test for Steroids and Triterpenoids

Ten (10) ml of aqueous extract were placed in a 50 ml beaker and evaporated to dryness. The residue was dissolved in 0.5 ml of acetic anhydride and 0.5ml of chloroform. The solution was then transferred to a dry test tube and 2 ml of concentrated sulphuric acid was added along the sides of the test tube. Brownish red or violet ring at the zone of contact with the supernatant and green or violet coloration denoted the presence of steroids and triterpenoids (Harborne, 1998).

### 3.6.6 Extraction of Crude Saponins

Crude saponins were extracted with methanol according to Patnaik *et al.* (2007) with slight modifications. Five grams (5 g) of methanol extract of *E. leptostachya* was dissolved in some methanol until it all dissolved. Water was added to the mixture followed with butanol. The water soluble layer was discarded since it contained mainly tannins. The butanol layer was concentrated *in vacuo* at 50 ℃ using a Buchi Rotary Evaporator coupled with a thermal regulator. Ethyl acetate was added to the mixture and the ethyl acetate layer decanted. The precipitate composed of crude saponins.

## 3.7 Characterization of *E. leptostachya* Methanol Extract

### 3.7.1 Separation of the Crude Methanol Extracts of *E. leptostachya* by Chromatographic Method

The most active extract was subjected to the standard chromatographic methods namely column chromatography (CC), thin layer chromatography (TLC) and preparative thin layer chromatography (PTLC) for separation while spectroscopic techniques were used for characterization.

### 3.7.2 Separation of the Extracts by Thin Layer Chromatography

Thin Layer Chromatography (TLC) was carried out on Aluminium pre-coated sheets with Silica Gel 60 $F_{254}$, (Merck, German) (0.25 mm layer thickness). The TLC plates were used in the present study as a scouting technique in the isolation and purification process.

The separated compounds on the chromatograms were examined under UV light at 254 nm and 366 nm using a hand lamp (model UV GL-58 mineral lamp light of 254 nm and 366 nm). Visualization was done by spraying with vanillin reagent for the identification of terpenoid spots which are UV inactive. The TLC plates were left to dry at room temperature before they were heated at 110 $^{o}$C for 10 min. in an oven. Preparative TLC was done using normal phase gel ($F_{254}$ Merck) pre-coated on Aluminium plates (20 x 20 cm, thickness of 0.25 mm).

### 3.7.3 Column Chromatography (CC)

Column Chromatography was performed using a glass column (1.5 - 5.5 cm in diameter 12 - 55 cm in length) on silica gel 60 (0.40 – 0.0630, 230 – 400 mesh, Merck) and eluted with slow gradient of different solvent systems. The slurry method was used when packing the column. The plant extract was dissolved in minimum possible solvent, mixed with equal amount of silica gel, and ground into fine powder and the solvent removed. The dry powder was introduced at the top of the column and covered with a small amount of silica and cotton wool to minimize disturbances during the addition of the eluting solvent. Fractions which showed similar TLC profiles were combined and concentrated to give pure or semi-purified portions (Hayashi *et al*., 1990).

## 3.8 Fractionation and Isolation of Crude Saponins of *E. leptostachya*

The crude saponins from methanol extracts of *E. leptostachya* 20 g was loaded on Sephadex column (Sephadex ® LH-20 Pharmacia) 4 cm x 30 cm and eluted with DCM: MeOH in the ratio of 1:1 with a flow rate of a drop per min.

Eighty eight fractions (20 ml each) were collected and concentrated in vacuum at 50 ℃ using a Buchi Rotary Evaporator coupled with a thermal regulator. The fraction were monitored using TLC plate using a solvent system (Butanol: Acetic: Water - 4:1:5). Fractions with the same TLC profile were pooled together and seven combined fractions; A, B, C, D, E, F and G were obtained. The fractions were tested for presence of saponins and fraction F was subjected to acid hydrolysis to separate the aglycone moiety from the sugars.

### 3.8.2 Hydrolysis of Crude Saponins Mixture

Fifteen (15 g) of fraction 57 - 80 crude saponins of *E. leptostachya* was refluxed in methanolic hydrochloric acid mixture for 6 hr. The methanolic hydrochloric acid was prepared by adding together 225 ml of methanol, 25 ml of water and 35 ml of concentrated hydrochloric acid. After the 6 hr, the reaction mixture was concentrated in a rotary evaporator to about 3 ml and 100 ml of distilled water added. The mixture was filtered and the resulting residue contained the sapogenin mixture which was washed with warm distilled water several times for the purpose of removing sugars and monitored with Benedict solution until there were no reducing sugars. The filtrate was mixed with acetonitrile and stored at 4 ℃ for later analysis.

### 3.8.3 Thin Layer Chromatography (TLC) of the Sapogenin Mixture

The sapogenin mixture from hydrolysis of crude saponins of methanol extract of *E. leptostachya* was dissolved in methanol and spotted on silica plate (silica gel 60 $F_{254}$, Merck, Germany).The chromatogram were developed in a 10 cm x 5 cm x 10 cm tank and eluted with several solvent systems. The best solvent system was found to be ethyl acetate - methanol (4:1) and was used as a guide for column chromatography of the sapogenin. Vanillin reagent was used to identify the presence of terpenoid which are UV inactive.

### 3.8.4 Column Chromatography of the Sapogenin Mixture of *E. leptostachya*

A column 4.0 cm in diameter and 50 cm long was packed with 150 g of silica gel 60 (70 – 230 mesh, Merck). Two (2 g) of sapogenin was thoroughly mixed with equal amount of silica gel 60 (70 – 230 mesh, Merck). The mixture was loaded onto the column and then eluted with solvent system ethyl acetate - methanol (4:1). The fractions were subjected to TLC and monitored with UV lamp at the two wavelengths of 254 and 366 nm.

### 3.8.5 Preparative TLC of Sapogenin

Preparative TLC was carried out on Aluminium sheets precoated with silica gel 60 $F_{254}$, (Merck, German) with a 0.25 mm layer thickness. A glass development tank (20 x 20 x 5) was used. Ethyl acetate: methanol (4:1) was used as the mobile solvent. 5 g of the hydrolyzed crude saponins were dissolved in methanol (10 ml). The resulting mixture was spread on the base of the silica gel plate.

After the solvent had covered above 70 % the silica gel plate was removed from the development tank and the solvent front marked. The plate was left to dry at room temperature before they were covered using glass but exposing the edges of the plate which were then sprayed using vanillin-sulphuric acid and left to dry before heated at 110 $^{o}$C for 10 min. The positions of the bands were marked and each band scrubbed and dissolved in hexane and then filtered into clean vials which were covered with perforated aluminium foil to allow slow evaporation of the solvent.

### 3.8.6 Thin Layer Chromatography (TLC) of Separated Sapogenin

The sapogenin from each band was spotted on aluminium sheets pre-coated with silica gel 60 $F_{254}$, (Merck, Germany) with a 0.25 mm layer thickness and developed using ethyl acetate-methanol mixture (4:1) as a mobile phase. Vanillin was used to identify terpenoid which are UV inactive develop the chromatograms.

## 3.9 Spectroscopic Methods (SM)

Spectroscopic methods (SM) usually used are Fourier Transform-infrared (FTIR), Violet/ Visual mass spectroscopic and Nuclear Magnetic Resonance (NMR).

### 3.9.1 Melting Point

The melting point of the aglycone from hydrolyzed crude saponins of methanol extracts of *E. leptostachya* samples in open capillary tubes were recorded using Gallenkamp melting point apparatus and expressed in degree centigrade ($^{0}$C) in the chemistry laboratory at JKUAT.

### 3.9.2 Infrared spectra (FT-IR) of the Sapogenin from *E. leptostachya*

The Infrared (IR) spectrum of the aglycone from hydrolyzed crude saponins of methanol extracts of *E. leptostachya* were recorded on an FT-IR Shimadzu Infrared Spectrometer, 8000 series as KBr pellets in the Chemistry Laboratory at JKUAT.

### 3.9.3 Ultraviolet (UV) of the Sapogenin from *E. leptostachya*

The Infrared spectrum of the aglycone from hydrolyzed crude saponins of methanol extracts of *E. leptostachya* samples were dissolved in pure methanol (1mg in 5ml spectroscopic methanol). The Ultraviolet spectra were recorded between 200 and 4000 nm using a Shimadzu UV recording Spectrophotometer equipped with a UV detector in the Food Science Laboratory of JKUAT.

### 3.9.4 MS of the Sapogenin

MS analyses were carried out in Finigan Mat SSQ700 single quadruple instrument and Autospec time of flight (ToF) spectrometer Ei-MS and ES-MS at ICIPE.

## 3.10 Statistical Analysis of the Data

The statistical analyses were performed using the statistical package for social scientists (SPSS) – Version18.0 software for Windows. Means were evaluated by *t* - test to compare the anthelmintics activity of different concentrations and doses of each extract and control and between the extracts tested. The $LD_{50}$ were determined using the Finley's probits and p-values $< 0.05$ were considered statistically significant whereas those $> 0.05$ as not significant.

The p-values are used to determine whether a null hypothesis should be accepted. The p- value is a statistical measure which helps scientist to determine whether the hypotheses are correct.

### 3.11 Ethical Considerations

Human subjects were not involved in this study. The nematodes were obtained from ruminant faecal material. Intellectual property rights (IPR) were handled as stipulated in the KEMRI - IPR policy document. The ethno-medical information about these plants is already in the public domain in journals and in the books (Kokwaro, 2007; Githiori *et al.*, 2006; Beentje, 1994).

# CHAPTER FOUR

## 4.0 RESULTS

### 4.1 Yields of Solvent Extracts

The percentage yields of plant extracts by hexane, acetone and methanol are shown in Table 4.1.

**Table 4.1:** Percentage yields of plant extracts (*Entada leptostachya* and *Rapanea rhododendroides*) using various solvents

| Solvent | Plant | Weight (g) | % yield | Appearance |
|---|---|---|---|---|
| **Hexane** | *E.l.* | 3.3 | 1.15 | Yellow solid |
| | *R.r.* | 2.0 | 1.0 | Yellow solid |
| **Acetone** | *E.l.* | 15.0 | 7.5 | Brown solid |
| | *R.r.* | 10.0 | 5.0 | Light brown solid. |
| **Methanol** | *E.l.* | 20.0 | 10.0 | Dark brown solid |
| | *R.r.* | 15.0 | 7.5 | Brown solid |

Key: *E.l. = Entada leptostachya*. *R.r. = Rapanea rhododendroides*

The percentage (%) yield was calculated against 200 g of the plant material subjected to each extraction technique.

The extraction yields were calculated using the formula:

$$(\%)\ \text{Yield} = \frac{\text{Weight of crude extract obtained x 100}}{\text{Weight of dried plant material used}}$$

The *E. leptostachya* extracts for hexane, acetone and methanol appeared yellow, brown and dark brown respectively while the extracts for *R. rhododendroides* were yellow, light brown and brown for hexane, acetone and methanol respectively. Hexane extract of *R. rhododendroides* gave the lowest yields (1.0 %) while methanol extract of *E. leptostachya* gave the highest yield (10 %).

### 4.2 Phytochemical Analyses of the Crude Extracts

Table 4.2 summarizes the phytochemicals compounds present in the extracts of *E. leptostachya* and *R. rhododendroides*. Terpenoid and steroid were present in all extracts while saponin, tannins and flavonoids were present in all extracts except in the hexane extract. Tannins and reducing sugars were present in all extracts except in n-hexane extracts for *R. rhododendroides* and *E. leptostachya*.

**Table 4.2:** Phytochemicals in *R. rhododendroides* and *E. leptostachya* crude extracts.

| | | Phytochemicals | | | | | |
|---|---|---|---|---|---|---|---|
| **Solvent** | **Plant** | **Saponins** | **Tannins** | **Steroids** | **Flavonoids** | **Alkaloids** | **Reducing Sugars** |
| Hexane | *E.l* | - | - | + | - | - | - |
| | *R.r* | - | - | + | - | - | - |
| Acetone | *E.l* | + | + | + | + | - | + |
| | *R.r* | + | + | + | + | - | + |
| Methanol | *E.l* | + | + | + | + | - | + |
| | *R.r* | + | + | + | + | - | + |
| Aqueous | *E.l* | + | + | + | + | - | + |
| | *R.r* | + | + | + | + | - | + |

Key: + present - absent *E.l. = Entada leptostachya; R.r. = Rapanea rhododendroides*

## 4.3 Brine Shrimp Lethality Test

The two plant extracts showed varying toxicity to the brine shrimps in a dose dependent manner (Tables 4.3, 4.4, and 4.5) for *E. leptostachya* and *R. rhododendroides* respectively. *Entada leptostachya* extracts exhibited higher toxicity compared to those of *R. rhododendroides.* The methanol extract of *E. leptostachya* was the most toxic ($LD_{50}$ 1.1 µg/ml) while its hexane extract was the least toxic ($LD_{50}$, 11.0 µg/ml). For *R. rhododendroides,* the methanol extract ($LD_{50}$, 2.8 µg/ml) was the most toxic extract while the hexane extract was the least toxic ($LD_{50,}$ 20.4 µg/ml).

**Table 4.3:** Number of dead (mean values) nauplii in different concentrations of extracts of *E. leptostachya* done in triplicates

| **Plant extract** | **10ppm** | **100ppm** | **1000ppm** | **$LD_{50}$ µg/ml** |
|---|---|---|---|---|
| Aqueous | 8.0 ± 1 | 9.0 ± 1 | 10.0 ± 0 | 2.8 |
| Methanol | 9.0 ± 1 | 10.0 ± 0 | 10.0 ± 0 | 1.1 |
| Acetone | 7.0 ± 1 | 9.0 ± 1 | 10.0 ± 0 | 3.7 |
| Hexane | 5.0 ± 1 | 8.0 ± 1 | 10.0 ± 0 | 11.0 |

**Table 4.4:** Number of dead (mean) nauplii in different concentrations of extracts of *R. rhododendroides* done in triplicates

| **Plant extract** | **10ppm** | **100ppm** | **1000ppm** | **$LD_{50}$ µg/ml** |
|---|---|---|---|---|
| Aqueous | 7.0 ± 1 | 9.0 ± 0 | 10.0 ± 0 | 4.5 |
| Methanol | 8.0 ± 0 | 9.0 ± 0 | 10.0 ± 0 | 3.4 |
| Acetone | 5.0 ± 1 | 8 ± 1 | 10.0 ± 0 | 12.4 |
| Hexane | 4.0 ± 1 | 7.0 ± 0 | 10.0 ± 0 | 20.4 |

**Table 4.5:** The mean $LD_{50}$ for hexane, acetone, methanol and aqueous extracts of *E. leptostachya* and *R. rhododendroides* against *H. contortus* at 24 hr.

| | **Extracts mean $LD_{50}$ (µg/ml)** | | | |
|---|---|---|---|---|
| **Plant** | **Hexane** | **Acetone** | **Methanol** | **Aqueous** |
| *Entada leptostachya* | 11.0 | 3.7 | 1.1 | 2.8 |
| *Rapanea rhododendroides* | 20.4 | 12.4 | 3.4 | 4.5 |

## 4.4 Anthelmintic Activity

In the search for natural anthelmintics in vitro test are used as preliminary studies of plants. In these tests the extracts are directly placed in contact with adult worms to evaluate the effect on mortality of the worms.

### 4.4.1 Comparison of In Vitro Anthelmintic Activity of *E. leptostachya* and *R. rhododendroides*

In the search for natural anthelmintics in vitro test are used as preliminary studies of plants. In these tests the extracts are directly placed in contact with adult worms to evaluate the effect on mortality of the worms. All extracts of *E. leptostachya* and *R. rhododendroides* showed some degree of anthelmintic activity against *H. contortus* adult worms. The methanol extracts from both *E. leptostachya* (70 %) and *R. rhododendroides* (50 %) were more potent against *H. contortus* adult worms while, *n*-hexane extracts were the least potent [*E. leptostachya* (20 %) and *R. rhododendroides* (10 %]. Aqueous extracts of both *E. leptostachya* (60 %) and *R. rhododendroides* (40 %) showed medium potency against *H. contortus* adult worms. Acetone extracts were the second least potent, compared to other extracts. There were no significant differences ($p = 0.165$) in anthelmintic activity between acetone extracts of *E. leptostachya* and *R. rhododendroides*. However, in aqueous, *n*-hexane and methanol extracts there were significant differences ($p < 0.05$).

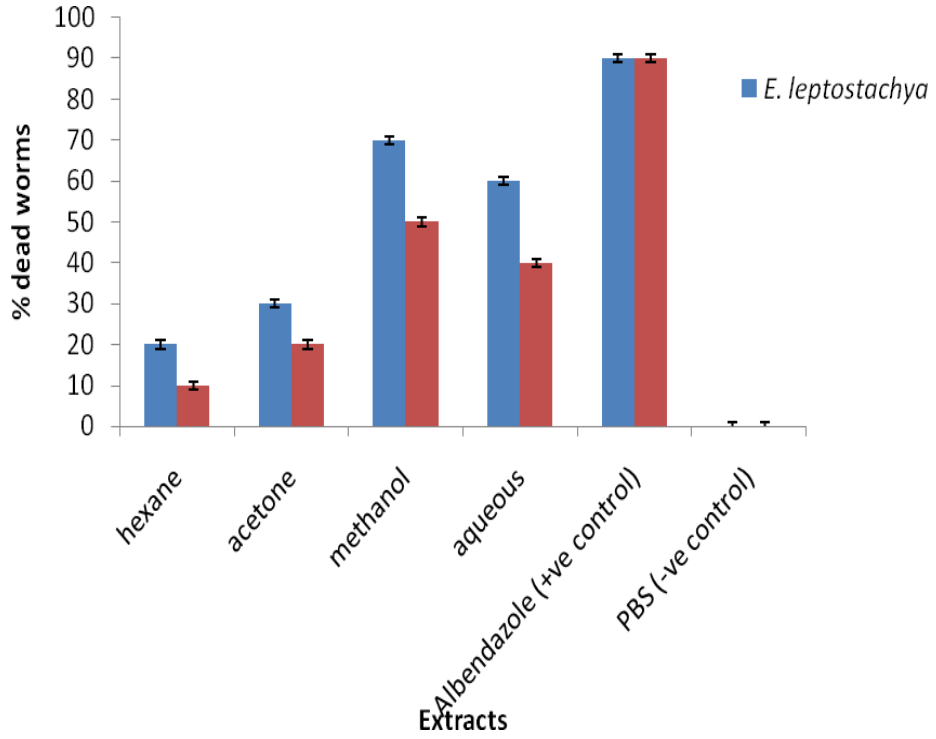



**Figure 4.1:** *In vitro* anthelmintic activity of acetone, aqueous, hexane and methanol extracts of *E. leptostachya* and *R. rhododendroides* extracts against *H. contortus* (24 hr)

### 4.4.2 Anthelmintic Activity of *R. rhododendroides* Plant extracts in 24 hr

Methanol extract of *R. rhododendroides* was the most potent of all other solvent extracts against *H. contortus* adult worms while *n*-hexane extracts were the least potent.

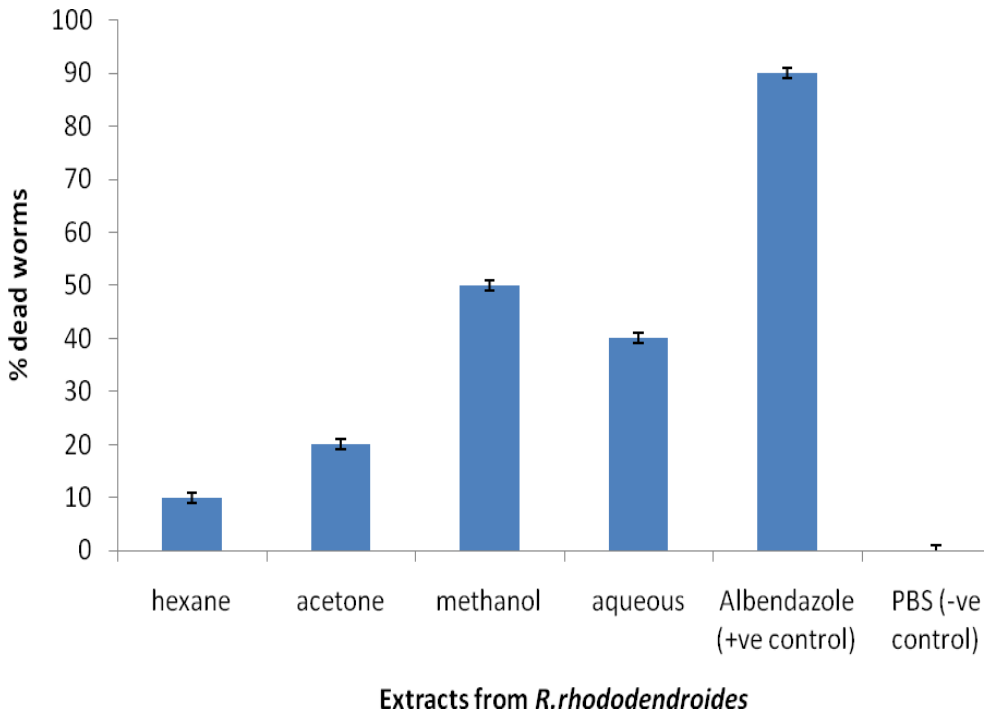



**Figure 4.2:** *In vitro* anthelmintic activity of *R. rhododendroides* extracts compared to albendazole (positive control) against *H. contortus* (24 hr).

### 4.4.3 Anthelmintic Activity of *E. leptostachya* Plant Extracts Compared to Albendazole 24 hr

Methanol extract of *E. leptostachya* was the most potent solvent extract against *H. contortus* adult worms while hexane extract was the least potent (Fig. 4.3). The methanol extract compared favourably with the positive control (albendazole). There were significant differences between methanol, aqueous and acetone extract compared to albendazole (positive control) $p < 0.05$.

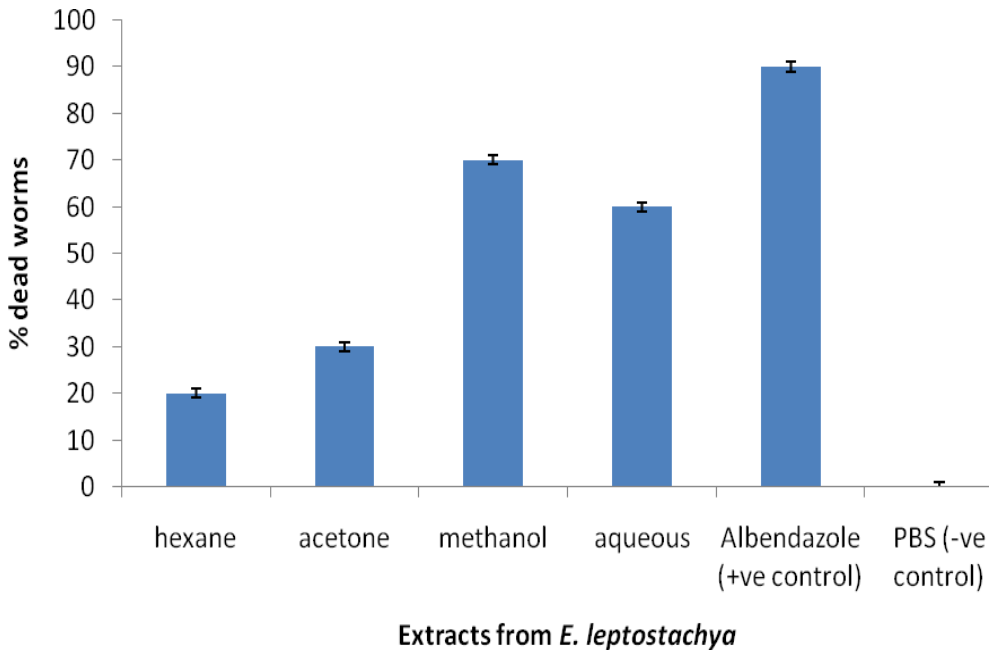



**Figure 4.3:** *In vitro* anthelmintic activity of *E. leptostachya* extracts compared to albendazole (positive control) against *H. contortus* (24 hr)

### 4.4.4 Concentration and Time Dependence of the In Vitro Anthelmintic Activities of the Aqueous and Solvent Extracts (*E. leptostachya* and *R. rhododendroides*)

The activity of the *n*-hexane extracts of *E. leptostachya* were concentration and time dependent (Fig.4.4). All concentrations of *n*-hexane extracts of *E. leptostachya* showed a below 50 % anthelmintic activity against *H. contortus* 100 mg/ml of *n*-hexane (*E. leptostachya*) extracts was the most potent at 36 hr while 12.5 mg/ml did not show any potency within 24 hr.

There were significant differences in anthelmintic activity of the extracts with increase in concentration, probably for the same reason given earlier.

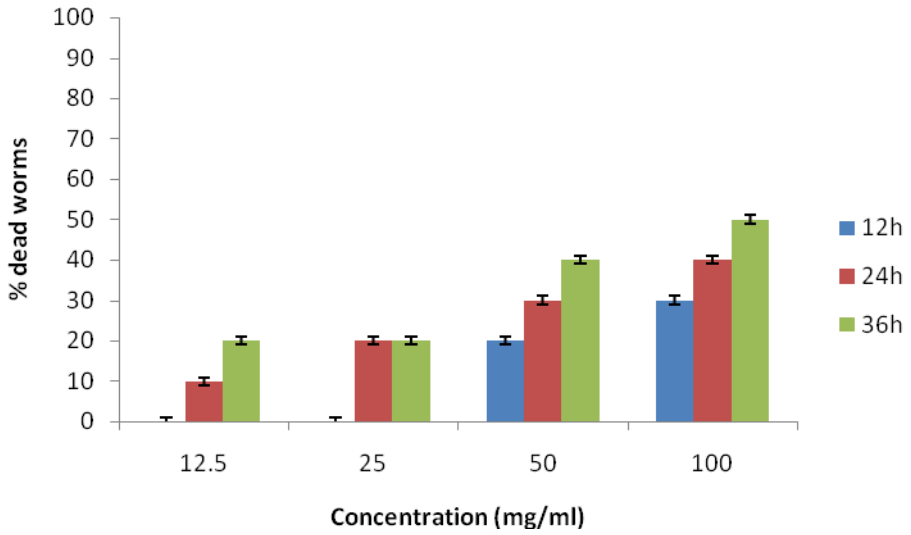



**Figure 4.4:** Anthelmintic activity of hexane extract of *E. leptostachya* against *H. contortus*

Again, the anthelmintic activity of the acetone extracts exhibited concentration and time dependence (Fig.4.5). Acetone extract of *E. leptostachya* 100 mg/ml and 50 mg/ml showed anthelmintic potencies above 50 % in 36 hr. 100 mg/ml showed the highest potency of 60 % in 36 hr. while 12.5 mg/ml did not show any potency within 24 hr. There were significant differences in anthelmintic activity of the extracts with increase in concentration.

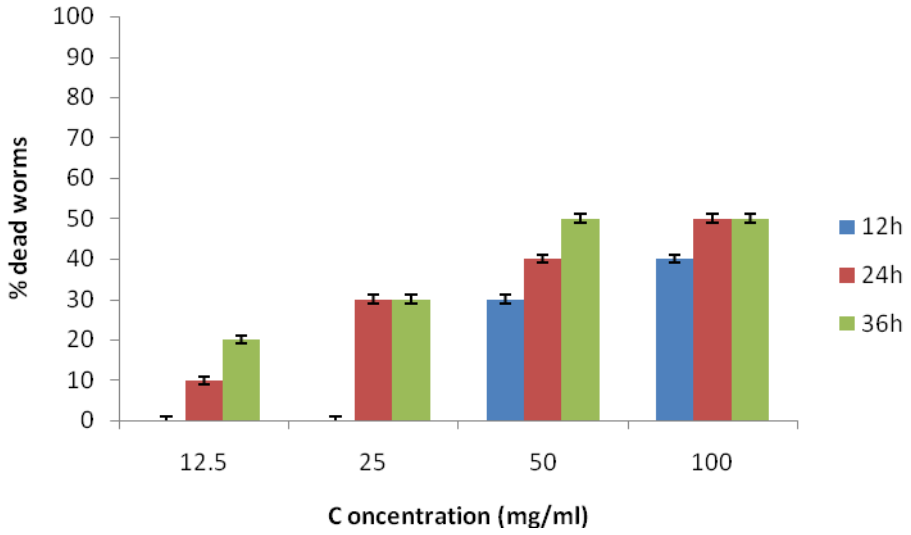



**Figure 4.5:** Anthelmintic activity of *E. leptostachya* acetone extract against *H. contortus.*

The activity of the aqueous extracts was concentration and time dependent (Fig.4.5). A dose of 100 mg/ml of aqueous extract of *E. leptostachya* showed the highest anthelmintic activity of 90 % in 12 hr while 50 mg/ml showed 90 % potency in 36 hr and 12.5 mg/ml was the least potent.

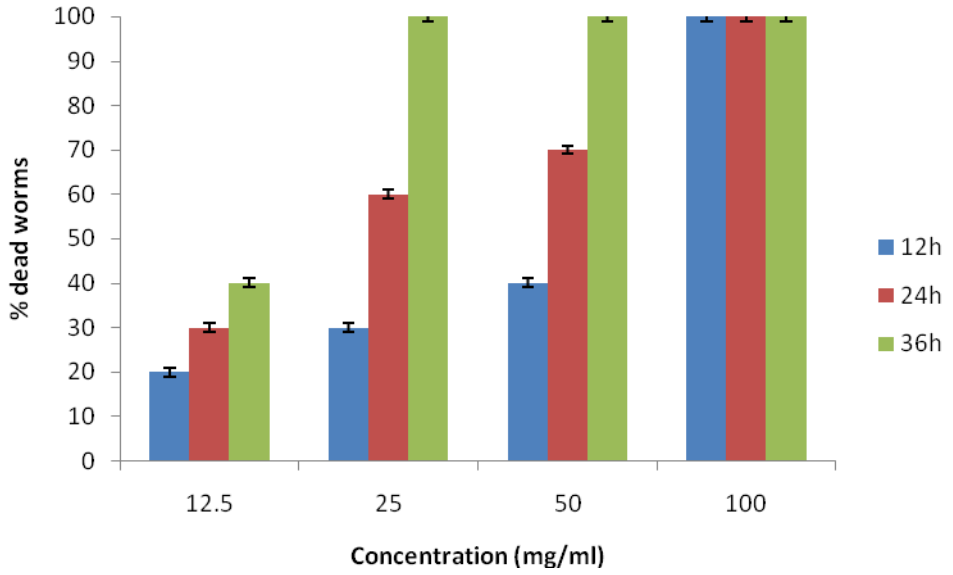



**Figure 4.6:** Anthelmintic activity of *E. leptostachya* aqueous extract against *H. contortus*

The activity of the methanol extracts were concentration and time dependent (Fig.4.7). All doses of methanolic extracts of *E. leptostachya* showed anthelmintic activities above 35 %. 25 mg/ml and 50 mg/ml showed potencies of above 90 % within 36 hr.

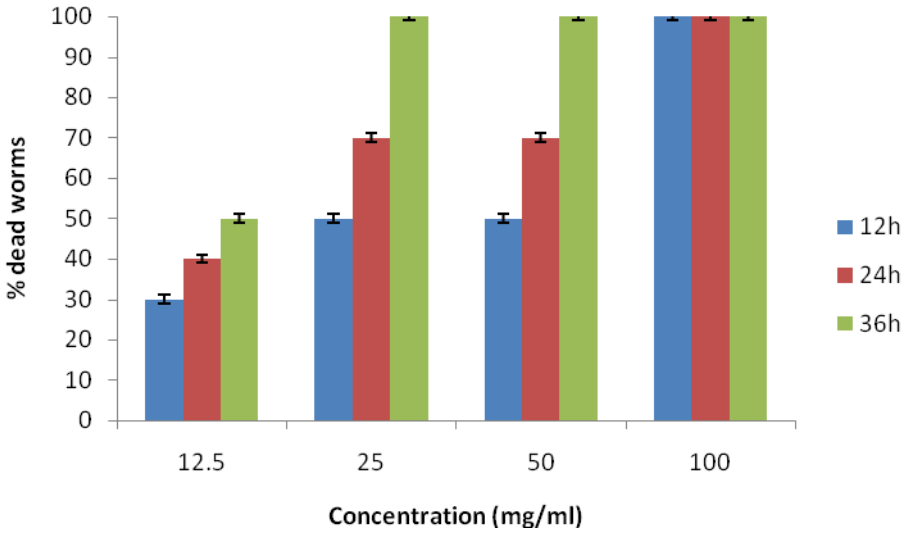



**Figure 4.7:** Anthelmintic activity of *E. leptostachya* methanol extracts against *H. contortus*

The activity of the *n*-hexane extracts were concentration and time dependent (Fig.4.8).All doses of hexane extracts of *R. rhododendroides* showed a below 35 % anthelmintic activity against *H. contortus*. 100 mg/ml and 50 mg/ml of hexane *R. rhododendroides* extracts were the most potent at 36 hr while 12.5 mg/ml did not show any potency within 36 hr. However, at 24 hr there were no differences in 100 and 50 mg/ml.

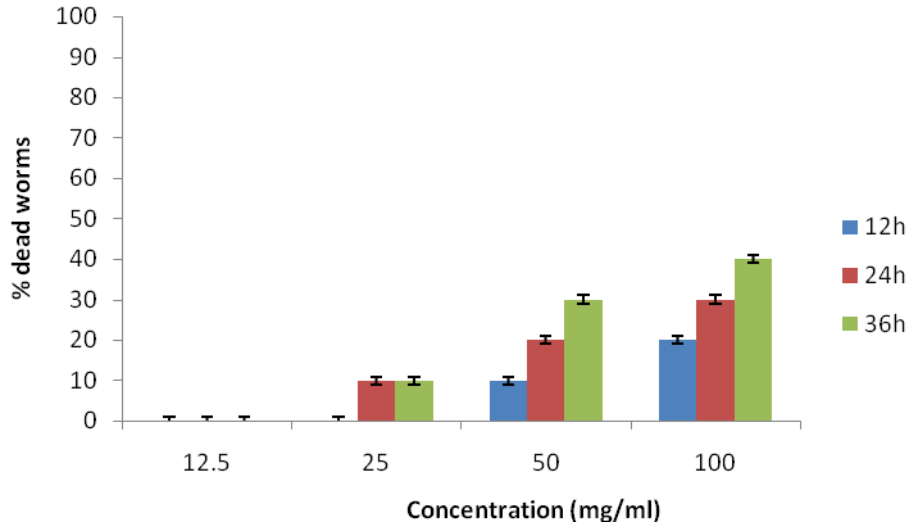



**Figure 4.8:** Anthelmintic activity of *R. rhododendroides n*-hexane extracts against *H. contortus*

The activity of the *n*-hexane extracts were concentration and time dependent (Fig.4.9). All doses of acetone extract of *R. rhododendroides* showed a below 50 % anthelmintic activity against *H. contortus* 100 mg/ml showed the highest anthelmintic (50 %) in 36 hr.12.5mg/ml showed no anthelmintic activity.

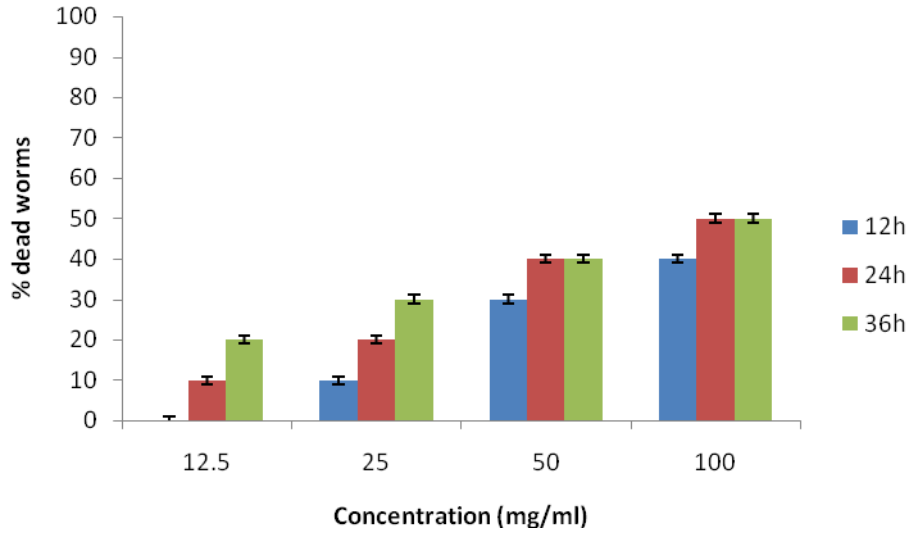



**Figure 4.9:** Anthelmintic activity of *R. rhododendroides* acetone extracts against *H. contortus*

The activities of the aqueous extracts were concentration and time dependent (Fig.4.10). All doses of *R. rhododendroides* aqueous extract showed anthelmintic activity against *H. contortus* above 30 %. 100 mg/ml dose of aqueous extracts was the most active in 36 hr while 12.5 mg/ml was the least potent in 12 hr. Aqueous extract of *Chenopodium ambrosioides*, has been shown to be effective against the adult *H. contortus* parasite.

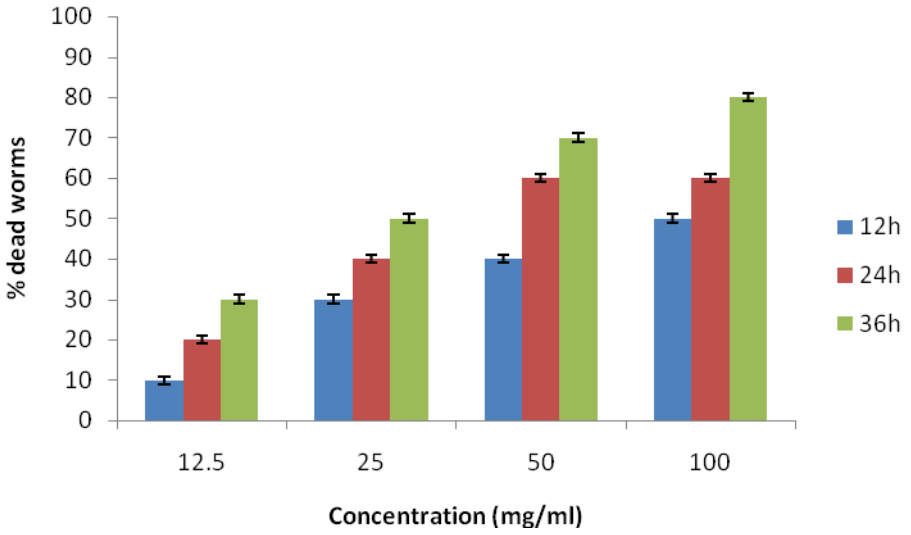



**Figure 4.10:** Anthelmintic activity of *R. rhododendroides* aqueous extract against *H. contortus*

The activity of the methanol extracts were concentration and time dependent (Fig.4.11). All doses of *R. rhododendroides* methanol extract showed anthelmintic activity against *H. contortus* above 30 %.100 mg/ml dose of aqueous extract (90 %) was the most active in 36 hr. while 12.5 mg/ml (32 %) was the least potent in 12 hr**.**

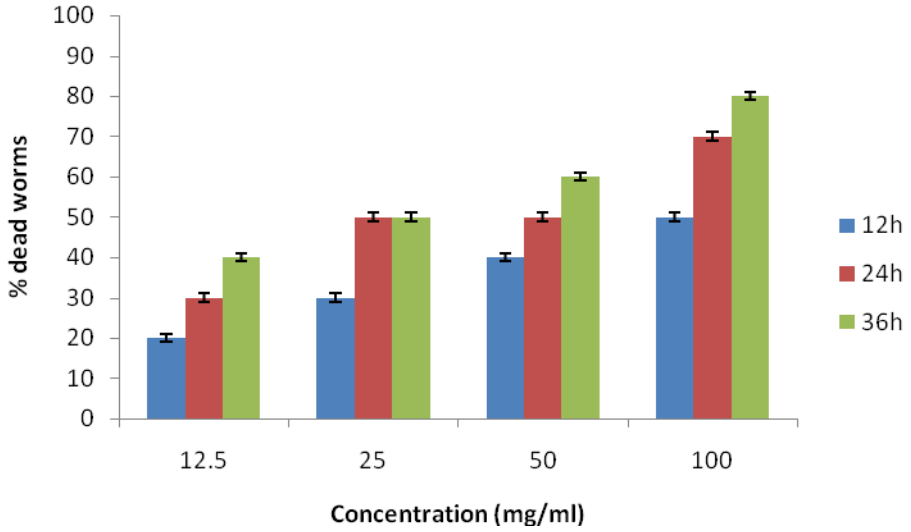



**Figure 4.11:** Anthelmintic activity of *R. rhododendroides* methanol extract against *H. contortus*

### 4.4.5 Comparisons of Anthelmintic Activities of *E. leptostachya* and *R. rhododendroides* n-Hexane Extracts

The anthelmintic activities of crude *n*-hexane extracts of *E. leptostachya* and *R. rhododendroides* were concentration and time dependent.100 mg/ml of *n*-hexane extract of *E. leptostachya* recorded the highest anthelmintic activity in 36 hr (Fig.4.12). 100 mg/ml of *E. leptostachya* was the most active concentration.12.5 mg/ml of *E. leptostachya* showed activity at 36 hr only. There was no significant difference between the two plants at 12.5 mg/ml (p = 0.42265). There is no significant difference between the two plants at 100 mg/ml (p = 0.0705).

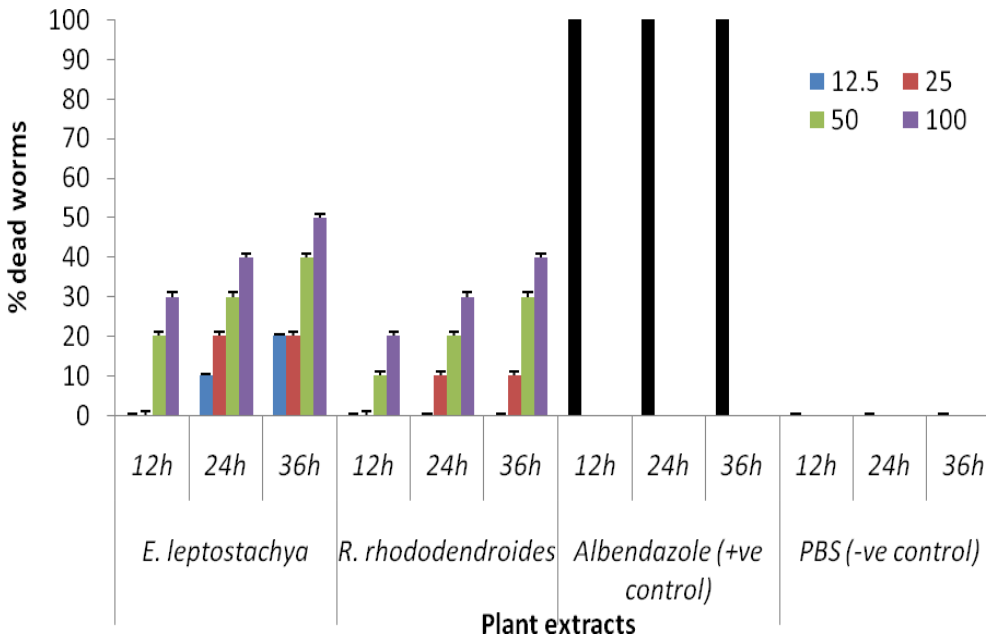



**Figure 4.12:** *In vitro* anthelmintic activity of *n*-hexane extract of *E. leptostachya* compared to *R. rhododendroides* against *H. contortus*

### 4.4.6 Comparisons of Anthelmintic Activities of *E. leptostachya* and *R. rhododendroides* Methanol Extracts

The anthelmintic activities of crude methanol extracts of *E. leptostachya* and *R. rhododendroides* were concentration and time dependent. Hundred milligrams (100 mg/ml) of *E. leptostachya* recorded the highest activity in 12 hr (Fig. 4.13) while 100 mg/ml of *R. rhododendroides* showed highest activity in 36 hr 12.5 mg/ml of *R. rhododendroides* was the least active concentration. There was no significant difference at 12.5 mg/ml concentrations between the two plants ($p = 0.42265$). There is no significant difference at 100 mg/ml concentrations between the two plants extracts ($p = 0.0705$). There is no significant difference between the two plants at 100 mg/ml ($p = 0.089$). There is no significant difference between the three times of analysis (12, 24, 36), $P = 0.0796$. The phytochemicals in the methanol extracts of *E. leptostachya* and *R. rhododendroides* (Table 4.2) may be responsible for the anthelmintic activity.

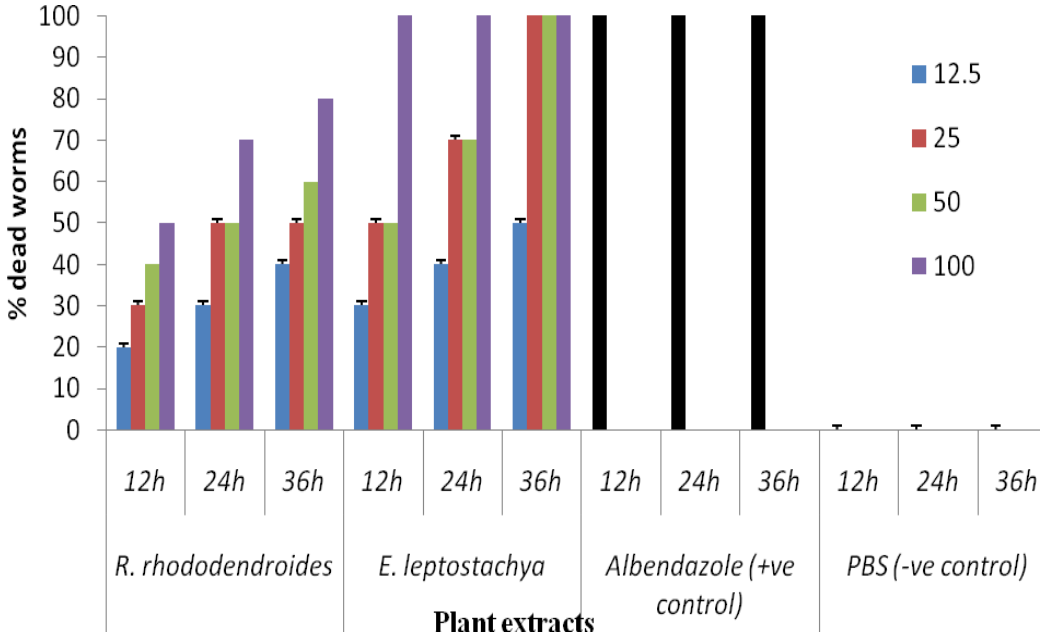



**Figure 4.13:** *In vitro* anthelmintic activity of methanol extract of *E. leptostachya* compared to *R. rhododendroides* against *H. contortus*

### 4.5 Characterization of *E. leptostachya*

#### 4.5.1 Column Separation of the Crude Saponins of *E. leptostachya*

Eighty- eight fractions (20 ml each) were collected and concentrated. The column elutes were monitored by TLC using butanol: acetic acid: water (4:1:5) as the mobile phase. Fractions with the same $R_f$ values were pooled together to give seven combined fractions.

### 4.5.2 TLC of the Aglycone (Sapogenin) Mixture of Hydrolyzed Crude Saponins

Seven spots developed which were purple in colour (with $R_f$ values; 0.10, 0.18, 0.20, 0.53, 0.67, 0.80) were obtained after spraying with vanillin reagent and heating in the electric oven at 110 ℃ for 10 min.

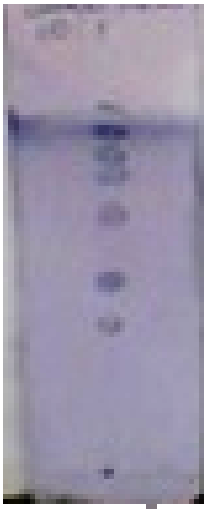


**Figure 4.14:** TLC of the Aglycone Mixture of Hydrolyzed Crude Saponins

### 4.5.3 PTLC of the Aglycone (Sapogenin) Mixture of Hydrolyzed Crude Saponins

PTLC of the aglycone mixture of hydrolyzed crude saponins of methanol extract of *E. leptostachya* gave five bands which were scrubbed and each dissolved in hexane. The filtrates of common band were put in vial and left in the fume cupboard for the solvent to evaporate slowly.

### 4.5.4 TLC of Separated Sapogenin

Separated sapogenin of bands 3 gave a single spot ($R_f$ = 0.67) when it was spotted on a TLC plate **(**Fig. 4.15)

**Figure 4.15:** TLC of the Aglycone Mixture of Hydrolyzed Crude Saponins

### 4.5.5 The IR (KBr) Spectrum of the Purified Sapogenin (Aglycone) from Hydrolyzed Methanol Extracts (*E. leptostachya*)

The IR (KBr disc) spectrum (Appendix 6 ) of band 3 show absorption band at 3444 and 1628$cm^{-1}$ indicating the presence of OH and C=C groups respectively.

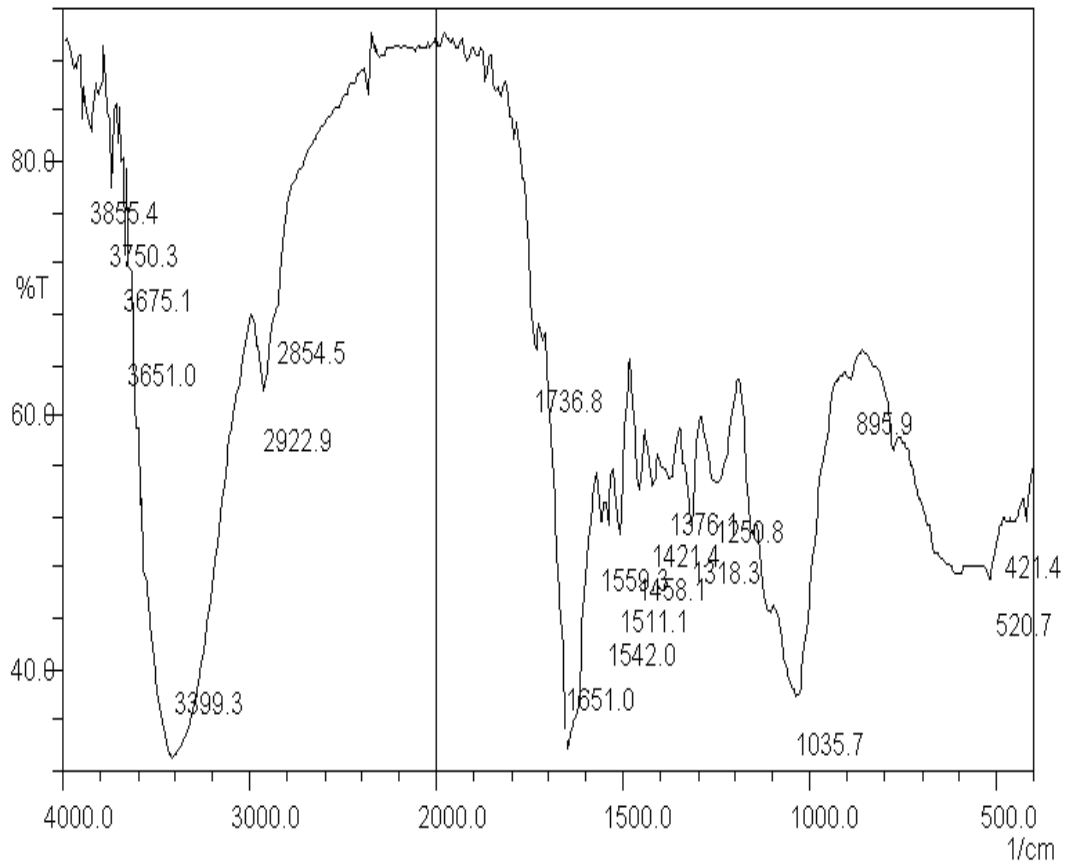



**Figure 4.16:** The IR (KBr) Spectrum of the Purified Sapogenin (Aglycone) from Hydrolyzed Methanol Extracts (*E. leptostachya*).

### 4.5.6 The UV of the Sapogenin (Aglycone) form Hydrolyzed Methanol of *E. leptostachya*

The UV spectrum of the sapogenin suggests a double bond peak at 225.0 nm and 275.0 nm

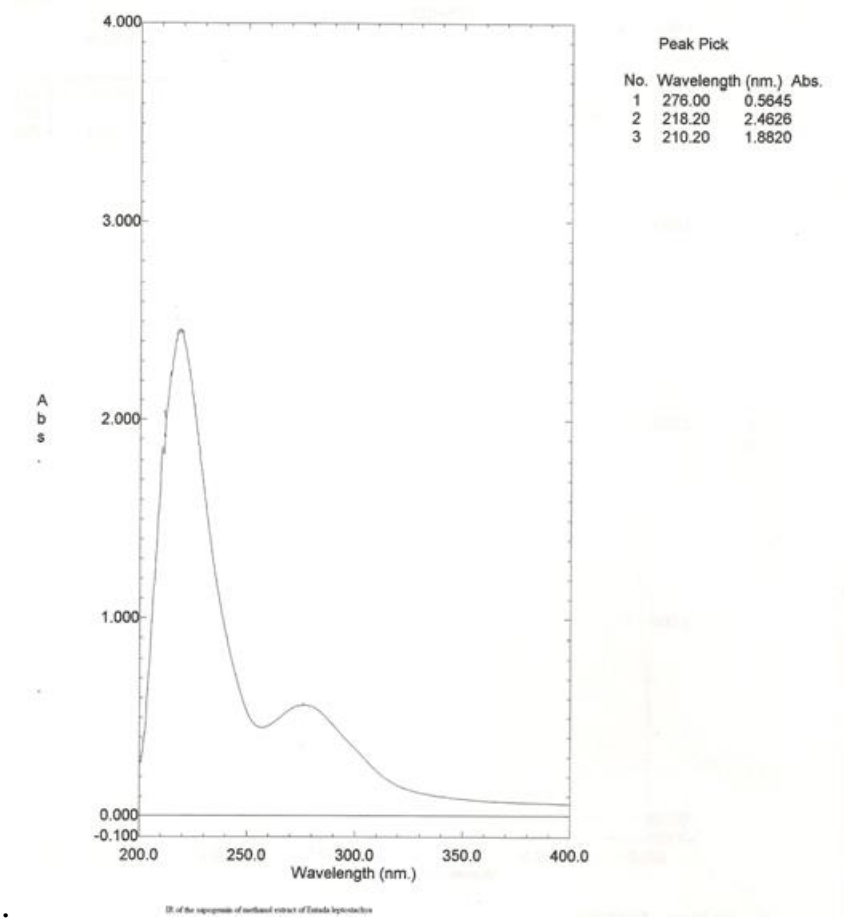
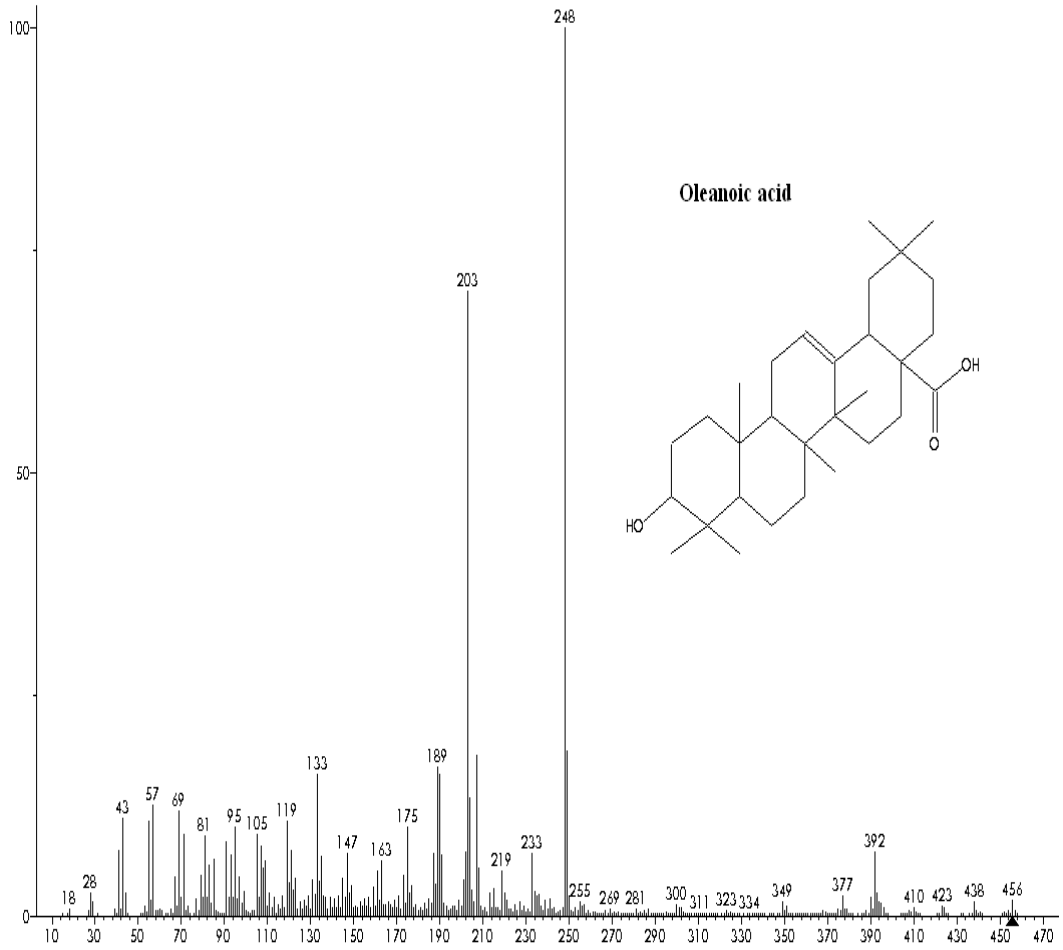



**Figure 4.17:** The UV of the Sapogenin (Aglycone) form Hydrolyzed Methanol of *E. leptostachya*

### 4.5.7 MS of the Sapogenin (Aglycone) form Hydrolyzed Methanol of *E. leptostachya*

The mass spectrum (Scheme 4.1) gave the highest peak at m/z 456 corresponding to the molecular ion [$M^+$] ion at m/z 457. A peak m/z 248 was due to loss of $[C_{16}H_{26}O_2]^+$ supported the presence of a carboxylic acid group. The peak at m/z 203 was due to the loss of $[C_{15}H_{23}]^+$. The peak at m/z 192 was due to $[C_{14}H_{25}]^+$. Scheme 4.2 shows the fragmentation pattern of the sapogenin (aglycone).




**Scheme 3:** GC-MS spectrum for aglycone form hydrolyzed methanol of *E. leptostachya*

# CHAPTER FIVE

## 5.0 DISCUSSION

### 5.1 Yields of Solvent Extracts

A higher percentage yield of methanol extract could be explained by the fact that methanol is more polar hence it was able to extract more compounds from a plant material than acetone and hexane. The results of this study are in accordance with the findings of Cowan *et. al.* (1999) and Alawan *et al* (2003), who showed that, isolation of botanical compounds from plant material, largely depend on the solvent and method of extraction. They also showed that organic solvents extracted more bioactive constituents compared to water. These, maybe the reason as to why methanol extract, showed a high anthelmintic activity (70 %) than aqueous extracts (60 %) of *Entada leptostachya*.

### 5.2 Phytochemical Analyses of the Crude Extracts

Medicinal plants are a source of extremely large number of chemical compounds known as phytochemicals. These phytochemicals are known to exhibit various pharmacological activities such as, anti-microbial, anti-protozoal, anthelmintic (Wasswa and Olila, 2006), cytotoxicity (Alluri *et al*., 2005). Several classes of secondary plant metabolites are responsible for anthelmintic activities. Phytochemicals have potential roles in health care and as lead compound for new drug development.

The phytochemicals present in the extracts were associated with the anthelmintic activities. Saponins (Watt and Brayer, 1962; Bishnu and Zeev, 2005) and Tannins (Khan and Diaz-Hermandex, 2000; Kabasa *et al*., 2000; Granum *et al.,* 2003) have been associated with anthelmintic activity. Other phytochemicals reported to have an anthelmintic activity include essential oils, flavonoids and terpenoid (Pessoa *et al*., 2002; Lahlou, 2002).

### 5.3 Brine Shrimp Lethality Test

The above results showed that toxicity of the extracts increased with increase in polarity of the solvent used. The bioactive compounds were likely to be polar hence; they were extracted easily as the polarity of the solvent increased. Lethal dose ($LD_{50}$) values of the plant extracts calculated assumed that the nauplii that survives a given dose would also have survived at any other lower doses and inversely nauplii that died in a certain dose would have died at any other higher doses (Sam, 1993).

Brine shrimp bioassay is a rapid preliminary screening method that determines the toxicity of the crude extracts. The test is based on the potential of the crude extracts of *E. leptostachya* and *R. rhododendroides* to become lethal to *A. salina* nauplii as a result of their toxicity. Crude extract derived from natural products with $LD_{50} \leq 0.1$ mg/ml are known to have toxic effects (Meyer *et al*., 1982).

The brine shrimp lethality test results of the plant extracts tested had $LD_{50}$ less than 100 µg/ml suggests that they were toxic according to Hodge and Sterner table (Singh, 2012). The result trend of brine shrimp toxicity concurs with the anthelmintic activity of the plant extracts.

### 5.4 Anthelmintic Activity

In this study, the anthelmintic activity of the solvent and aqueous extracts of *E. leptostachya* and R. *rhododendroides in* comparison to the reference drug-albendazole against adult *H.contortus* of sheep were evaluated *in vitro.*

### 5.4.1 Comparison of *in vitro* Anthelmintic Activity of *E. leptostachya* and *R. rhododendroides*

The main reason of using *in vitro* bioassays to screen the anthelmintic activity of plant extracts is low cost and rapid turnover which allow large scale screening of plant. The effect of anthelmintic is also measured directly on the process mortality of the parasite without interfering with internal physiological functions of the host (Molan *et al*., 2002; Githiori *et* al., 2006). In this study, the methanol extracts proved to be more potent (70 %) compared to the aqueous extract (60 %) and other solvent plant extracts Fig. 4.1. The result reveals that aqueous and solvent extracts of *Entada leptostachya* and *Rapanea rhododendroides* exhibited anthelmintic activity against *H. contortus* of sheep at different concentrations.

The findings above agree with Patil *et al.* (2009) where the anthelmintic activities of methanolic extracts of leaves of *Spinacia oleracae* (Linn.) were comparable with standard drug, Albendazole. All extracts of the plants showed dose-dependent activity ($P < 0.05$). Iqbal *et al.* (2004) who found alcoholic extracts of *Artemisia brevifolia* at 25 mg/ml to be more effective in causing mortality of *H. contortus* as compared to the aqueous extracts. Similarly, the hydro-alcoholic extract of *Coriandrum sativum* and *Hedera helix* also showed better anthelmintics activity against *H. contortus* as compared to aqueous extracts (Eguale *et al*., 2007). Studies have shown that transcuticular diffusion is a common means of entry for non-nutrient and non-electrolyte substances in nematodes. It has also been shown that this route is predominant for the uptake of major broad-spectrum anthelmintics as opposed to oral ingestion. Lipophilic anthelmintics such as albendazole have a greater capability to cross the external surface of the helminthes than the hydrophilic compounds. Hence the potency of albendazole compared to *R. rhododendroides* plant extracts. The findings also validate the traditional usage of *E. leptostachya* as anthelmintics.

The finding from the present study will contribute in the field of plant anthelmintics to develop sustainable, effective and safe alternatives to conventional anthelmintics. The natural compounds derived from plants are more stable as these are mostly plant secondary metabolites synthesized over a long period of time. Natural compounds also provide greater structural diversity than synthetic ones and therefore they are source of molecular weight structure active against a wide range of target agents and this property can preclude the occurrence of resistance.

Plant phytochemicals like condensed tannins and saponins have been found to have anthelmintics activities (Granun *et al.,* 2003; Molan *et al*., 2002). The plants in the present study were found to have tannins and saponins and these were thought to be responsible for the observed anthelmintic activities. This is reported here for the first time. However, aqueous extract of *Chenopodium ambrosioides,* has been shown to be effective against the adult *H. contortus* parasite (Equale and Gelay, 2009). This result agrees with that of Bachaya *et al.* (2009) where methanol extracts of *Terminalia arjuna* exhibited anthelmintic properties at different hour's exposure of *H. contortus.*

Plant phytochemicals like condensed tannins and saponins have been found to have anthelmintics activities (Granun *et al.,* 2003; Molan *et al*., 2002; Wasswa and Olila, 2006). Absence of tannins and saponins in the *n*-hexane extracts (Table 4.2) may have contributed to low anthelmintic activity. Aqueous extract of *Chenopodium ambrosioides,* has been shown to be effective against the adult *H. contortus* parasite. Bachaya *et al.* (2009) reported anthelmintic property of methanol extracts of *Terminalia Arjuna* (Roxb) where efficacy of the extract was evident by the mortality of *H. contortus* at different hours post exposure**.**

### 5.5 Characterization of *E. leptostachya*

#### 5.5.1 Column Separation of the Crude Saponins of *E. leptostachya*

Many triterpenoids and their glycosides have been isolated from the leaves of a South African *Combretum* species (Pegel and Rogers, 1985; Rogers and Verotta, 1996).

### 5.5.2 TLC of the Aglycone (Sapogenin) Mixture of Hydrolyzed Crude Saponins

The different spots indicated presence of different aglycone in the mixture.

### 5.5.3 PTLC of the Aglycone (Sapogenin) Mixture of Hydrolyzed Crude Saponins

### 5.5.4 TLC of Separated Sapogenin

The purple colour spot indicated the presence of a single terpenoid aglycone.

### 5.5.5 The IR (KBr) Spectrum of the Purified Sapogenin (Aglycone) from Hydrolyzed Methanol Extracts (*E. leptostachya*)

The IR (KBr disc) spectrum absorption band at 3444 and 1628$cm^{-1}$ indicated the presence of OH and C=C groups respectively.

### 5.5.6 The UV of the Sapogenin (Aglycone) form Hydrolyzed Methanol of *E. leptostachya*

The UV spectrum of the sapogenin suggests a double bond peak at 225.0 nm and 275.0 nm.

### 5.5.7 MS of the Sapogenin (Aglycone) form Hydrolyzed Methanol of *E. leptostachya*

The mass spectrum (Scheme 4.1) gave the highest peak at m/z 456 corresponding to the molecular ion [$M^+$] ion at m/z 457. A peak m/z 248 was due to loss of [$C_{16}H_{26}O_2$] $^+$ supported the presence of a carboxylic acid group. The peak at m/z 203 was due to the loss of [$C_{15}H_{23}$] $^+$. The peak at m/z 192 was due to [$C_{14}H_{25}$] $^+$. Scheme 4.2 shows the fragmentation pattern of the sapogenin (aglycone).

**Conclusions**

(i) Triterpenoids, saponins, flavonoids were among the phytochemicals which were detected in the crude extracts of *E. leptostachya* and *R. rhododendroides*. Alkaloids were absent in all extracts.

(ii) The low $LD_{50}$ values obtained from the different extracts of *E. leptostachya* root bark and *R. rhododendroides* stem bark support their medicinal uses in the treatment of gastrointestinal nematodes. Low $LD_{50}$ values indicate possibility of the presence of anthelmintics compounds in the extract.

(iii) Extracts of *E. leptostachya* showed the higher anthelmintics property than those of *R. rhododendroides.* Methanol extracts of both *E. leptostachya* and *R. rhododendroides* showed higher *in vitro* anthelmintics activity in a concentration dependent manner against *H. contortus.*

Acetone extracts showed moderate *in vitro* anthelmintics activities depended on concentration against *H. contortus*. *n*-Hexane extracts were the least active extracts.The higher saponins content in *E. leptostachya* was probably responsible for the observed anthelmintic property.

(iv) Triterpenoid aglycone was isolated from acid hydrolyzed saponins from the methanol extract of *E. leptostachya.*

(v) The result obtained in this study demonstrates a possible scientific rationale for the incorporation of the root bark of *E. leptostachya* into traditional medicine method of controlling gastrointestinal worms and are likely to have been selected after empirical demonstration of their efficacy over a long period of time.

**Recommendations**

(i) *In vivo* anthelmintic activity of methanol extract of *E. leptostachya* should be done to compare with the *in vitro* anthelmintics activity obtained in this study.

(ii) Purification of the crude saponins from *E. leptostachya* should be conducted and further structural elucidation should be done to identify the structure of these saponins.

(iii) Further studies on standardization of doses

(iv) Identify the mechanism(s) of mode of action(s) of these extract and the isolates

## REFERENCES

**Adam, K. T.; Amadou, N.; Man, G. B.; Isidore, B.; Valentin, T. H. H. and Gaston, B. M. A. M. (2012).** *In vitro* anthelmintic activity of *Leuceana leucocephala* (Lam) De Wit. (Mimosaceae) and *Gliricidia sepium* (Jacq.) Kunth ex Steud (Fabaceae) leaves extract on *Haemonchus contortus* ova and larvae. *Journal of Chemical Pharmacy. Research*. **4 (1):** 303 – 309.

**Ademola, I. O. and Idowu, S. O. (2006).** Anthelmintic activity of *Leucaena leucocephala* seed extract on *Haemonchus contortus* infective larvae. *Veterinary Records.* **158:** 485 - 486.

**Ahmed, E.; Malik, A.; Ferheen, S.; Afza, N.; Haq, A.; Lodhi, M. A. and Choudhary, M. I. (2006)**. Chymotrypsin inhibitory triterpenoids from *Silybum marianum*. *Chemical and Pharmacy. Bulletin***., 54(1):** 103 -106.

**Ahsan, R.; Islam, M.; Haque, E. and Mossaddik, A. (2009).** *In vitro* Anti-bacterial Screening and Toxicity Study of Some Different Medicinal Plants. *World Journal of Agricultural Science.* **5(5):** 617 - 620.

**Allonby, E. W. and Urquhart, M. (1975)**. The epidemiology and the pathogenic significance of Haemonchosis in Merino Folk in Eastern Africa. *Veterinary Parasitology.* **1:** 1001 - 1007.

**Anderson, R. C. (1992).** Nematode parasites of the vertebrates: Their development and transmission. *C.A.B International,* Wallingford, Oxon, UK, pp 578. ISBN 0851987990.

**Alawa, C. B. I.; Adam, A. M.; Gefu, J. O.; Ajanusi, O. J.; Abdu, P. A.; Chieezey, N. P.; Alawa, J. N.; and Bowman, D. D. (2003).** *In vitro* screening of two Nigerian medicinal plants (*Vernonia amygdalina* and *Annona senegalensis*) for anthelmintic activity. *Veterinary Parasitology*. **2542:** 1-9.

**Athanasiadou, U. (2000).** Consequences of long – term feeding with condensed tannins on sheep parasitized with *T. Colubriformis*. *International journal of Parasitology*. **30:** 1025 - 1033.

**Ayensu, E. S. (1978).** Medicinal plants of West Africa, Reference Publications Inc. Michigan, pp. 1 – 45.

**Bachaya, H.; Iqbal, Z.; Khan, M. N.; Jabbar, A.; Gilani, A. H. and Din, I. U. (2009).** *In vitro* and *in vivo* Anthelmintic Activity of *Terminalia Arjuna (*Bark). *International Journal of Agriculture and Biology*, **11(3):** 273 - 278.

**Baldo, R. C. (2001)**. Comparative efficacy of pineapple (*Ananas comosus*) leaves bolus and albendazole against gastrointestinal nematodes of sheep. DVM. *University of the Philippines, Department of Veterinary Parasitology*.38.

**Baoua, M.; Fayn, J. and Bassiere, J. (1976).** Preliminary phytochemical testing of some medicinal plants of Niger. *Plant Medica. Phytotherapy*. **10**: 251 – 266.

**Barbara, G.; Ramesh, T. and John, Y. (1991).** *Case study - Village Animal Healthcare: A community based approach to livestock development in Kenya*: Intermediate Technology Publication, London, UK.

**Barua, A. K.; Chakrabarty, M.; Datta, P. K. and Ray, S. (1988).** Phaseoloidin. A homogentisic acid glycoside from *Entada phaseoloides*: *Phytochemistry*. **27(10)**: 3259 – 3261.

**Beentje, H. J. (1994)**. Kenya trees, shrubs and lianas. National Museums of Kenya, Nairobi, pg 722.

**Behnke, J. M.; Buttle, G.; Lowe, A. and Duce, R. (2008).** Developing Novel Anthelmintic from Plant Cysteine Proteinases. *Parasites and Vectors*. 1:29.

**Birndorf, H. C.; D'Alossio, J. and Bagshaw, J. C. (1975**). DNA- dependent RNA- polymerases from Artemia embryos. Characterization of polymerases I and II from nauplii larvae. *Developmental Biology*. **45:** 34 - 43.

**Bishnu, C. and Zeev, W. (2005).** Larvicidal effects of aqueous extracts of *Balanites aegyptiaca* (desert date) against the larvae of *Culex pipiens* mosquitoes. *African journal of Biotechnology*. **4(11):** 1351-1354.

**Bizimenyenra, E. S.; Githiori, J. B.; Eloff, J. N. and Swan, G. E. (2006).** *In vitro* activity of *Peltophorum africanum* Sond. (Fabaceae) extract on the egg hatching and larval development of the parasitic nematode *Trichostrongylus colubriformis*, *Veterinary Parasitology.* **142:** 336 - 343.

**Bourdy, G. and Walter, A. (1992)**. Maternity and Medicinal plants in Vanuatu 1. The cycle of reproduction. *Journal of Ethnopharmacology*. **37 (3):** 179 - 196.

**Bowman, D. D.; Lynn, R. C. and Georgi, J. R. (2003).** Georgi's Parasitology for veterinarians. 8th. W. B. Saunders. pp 408.

**Brenner, S. (1974).** The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71 - 94.

**Bruneton, J. (1995).** Pharmacognosy, Phytochemistry, Medicinal Plants, Intercept, Hampshire.

**Bueding, E. (1971).** Chemotherapy of nematodes and cestodes. In: Drills Pharmacology in Medicine (Dipalma, J. R.ed). 4th Edition. MacGrill, New York, pp: 1822 - 1836.

**Butter, N. L.; Dawson, J. M.; Wakelin, D. and Buttery, J. (2000).** Effects of dietary tannin and protein concentration on nematode infection (*Trichostrongylus colubriformis*) in lamb. *Journal of Agricultural Science.* **134:** 89 - 99.

**Chandrawathani, P.; Brelin, D.; Nor Fasiha, A. S.; Adnan, M.; Jamnah, O.; Rehana, A. S.; Höglund, J. and Waller, P. J. (2002).** Evaluation of the neem tree (*Azadirachta indica*) as herbal anthelmintics for nematode parasite control in small ruminants in Malaysia. *Tropical Biomedicine.* **19:** 41 - 48.

**Choudhary, M. and Atta-Ur-Rahman, I. (2005)**. Medicinal plant biotechnology. A promise for global healthcare. The 7th scientific conference, National centre for research, Khartoum. Pg 40 - 51.

**Coe, F. G. and Anderson, G., J. (1996a).** Screening of Medicinal Plants used by the Garifuna of Eastern Nicaragua for bioactive compounds. *Journal of Ethnopharmacology.* 53**:** 29 - 50.

**Coe, F. G. and Anderson, G., J. (1996b**). Ethnobotany of the Garifuna of Eastern Nicaragua. *Economic Botany*. **50 (1):** 71 - 107.

**Coles, G. C.; Baucer, C.; Borgsteede, F. H. M.; Greets, S.; Klei, T. R.; Taylor, M. A. and Waller, P. J. (1992).** World association for advancement of veterinary Parasitology (WAAVP) methods for detection of anthelmintics resistance in nematodes of veterinary importance. *Veterinary Parasitology*. **44**: 35 - 44.

**Cox P. A. (1994).** The Ethnobotanical Approach to Drug Discovery: Strengths and Limitation. In: CIBA Foundation Symposium 185-Ethnobotany and the Search for New Drugs, Chadwick, D, J and J Marsh, (Eds.). John and Sons, Chichester. pp. 25 - 41.

**Croft, K. D. and Tutpulotu, P. (1980).** A Survey of the Tongan Medicinal Plants: *South Pacific Journal of Natural Science.* **1:** 45 - 57.

**Dahawi, H. O. A. (2009).** Phytochemical analysis, biological activity and cytotoxicity effects of selected Sudanese medicinal plants. Ph.D. Thesis, Unpublished, University of Gezira, Sudan.

**Dawo, F.; Assaye, Z. and Tibbo, M. (2001).** Comparative evaluation of crude preparations of *Azadirachta indica* leaf and Albendazole® in naturally infected goats with internal parasites. *Bulletin of animal health Production Africa*. **49:** 140 - 144.

**Debella, A.; Kunert, O.; Schmid, M. G.; Michl., B. F.; Abebe, D. and Haslinger, E, (2000).** A Di-terpene, A Flavonol Glycoside from *Securidaca longipedunculata* and *Entada abyssinica*. *Monatsh Chemistry*. **131(4):** 401 - 408.

**DeLaszlo, H. and Henshaw, P. S. (1954).** Plant Material used by primitive people to affect fertility: *Science*. **119:** 626 - 631.

**Derbesy, M. and Busson, F. (1968).** The lipids of certain West African Species. *Oleagineux*. **23:** 191 - 195.

**DeVaney, J. A.; Craig, T. M. and Row, L. D. (1992**). Effect of low levels of lice and internal nematodes on weight gain and blood parameters in calves in central Texas. *International Journal of Parasitology*. **22(3):** 369 - 376.

**Dhar, D. N.; Sharma, R. L. and Bansal, G. C. (1982).** Gastrointestinal nematodes in sheep in Kashmir. *Veterinary Parasitology.* **11:** 271 - 277.

**Dinnik, L. A. And Dinnik, N. N. (1958).** Observations on the development of the *Haemonchus contortus* larva under field conditions in the Kenya highlands. *Bulletin of Epizootic Disease of Africa.* **6:**11-12.

**Duke, J. A. (1975).** Ethnobotanical Observation on the *Cuna India*: *Economic Botany*. **29:** 278.

**Eguale, T.; Tilahun, G.; Debella, A.; Feleke, A. and Makonnen, E. (2007)**. *Haemonchus contortus: In vitro* and *in vivo* anthelmintic activity of aqueous and hydro-alcoholic extracts of *Hedera helix*. *Experimental Parasitology*. **116:** 340 - 345.

**Eguale, T. and Giday, M. (2009)**. *In vitro* anthelmintic activity of three medicinal plants against *Haemonchus contortus. International journal green Pharmacology*. **3(1):** 29-34.

**Farnsworth, N. R. (1984).** *The role of Medicinal Plants in Drug Development*, Krogsgaard-Larsen, P., S. B. Christensen and H. Kofod (Eds.). Munksgaard International Publishers Ltd., Copenhagen, Denmark, pp. 17 - 30.

**Fennel, C. W.; Lindsey, K. L.; Mc Gaw, L. J.; Sparg, S. G.; Stafford, G. I.; Elgorashji, E. E.; Grace, O. M. and Van Staden, J. (2004).** Assessing African medicinal plants for efficacy and safety pharmacological screening and toxicology. *Journal of Ethnopharmacology*. **94:** 205 - 217.

**Fleming, S. A.; Craig, T.; Kaplan, R. M.; Miller, J. E.; Navarre, C. and Rings M. (2006).** Consensus Statement on: Anthelmintic resistance of gastrointestinal parasites in small ruminants. *Journal of Veterinary Internal Medicine*. **20**:435 - 444.

**Freiburghaus, F.; Ogwal, E. N.; Knunya, M. H. H.; Kaminsky, R. and Brun, R. (1996).** *In – vitro* antitrypanosomal activity of Africa plants used in traditional medicine in Uganda to treat sleeping. *Trop. Med. Int. Health*, **1 (6):** 765 – 771.

**Gad, S.C.C. (2005).** Future of natural products: In drug discovery. John Wiley and Sons, Inc., New York.

**Gatongi, P. M.; Njoroge, J. M.; Scott, M. E.; Ranjan, S.; Gathuma, J. M.; Munyua, W. K.; Cheruiyot, H. and Prichard, R. K. (2003).** Susceptibility to IVM in a field strain of *Haemonchus contortus* subjected to four treatments in a closed sheep-goat flock in Kenya. *Veterinary Parasitology* **110:** 235 - 240.

**Gaudin, O. and Vacherat, R. (1938).** Rotenone and Ichthyotoxic power in some plants of Frence Sudan: *Bulletin Science and Pharmacology*. **45:** 385 - 394.

**Gee, J. M. and Johnson, I. T. (1988).** Interaction between haemolytic saponins, bile salts and small intestinal mucosa in rat. *Journal of Nutrition.**118*:** 1391 - 1397.

**Gefu, J. O.; Abdu, P. A. and Alawa, C. B. (2000).** Proceedings of workshop on Ethno-veterinary practice held 14-18 August 2000, Kaduna, Nigeria. Published by the National Animal Production. *Research Institute.* ABU, Zaria, Nigeria.

**Girach, R. D.; Aminuddi, N. and Siddioui, P. A. (1994).** The traditional plant remedies of among the Kondhi of District Dhenkanal (Orissa): *International. Journal of Pharmacognosy*. **32 (30):** 274 - 283.

**Giral, F.; Sotelo, A.; Lucas, B. and De La Vaga, A. (1978).** Chemical composition and toxic factors content in fifteen Leguminosae seeds. *Q. Journal of Crude Drugs Research.* **16:** 143 - 145.

**Githiori, J. B. (2004).** Evaluation of Anthelmintic Properties of Ethno-veterinary Plant Preparations Used as Livestock Dewormers by Pastoralists and Small Holder Farmers in Kenya. PhD. Thesis – Unpublished. Swedish University of Agricultural Sciences, Uppsala.

**Githiori, J. B.; Athanasiadou, S. and Thamsborg, S. M. (2006).** Use of plants in novel approaches in the control of gastrointestinal helminthes in livestock with emphasis on small ruminants. *Veterinary Parasitology.* **139**: 308 - 320.

**Gordon, Y.; Jackson, E.; Bartley, D. and Jackson, F. (2007)**. *In vitro* studies using bioassays to examine the effect of aqueous plant extracts on *Teladorsagia circumcincta. 21st AAVP* Conference Gent, 19th-23rd August, 2007. pp. 263.

**Goswami, S.; Pandey, A..; Triphathi, P.; Singh, A. and Rai, A. (2011).** *In vitro* evaluation of the anthelmintic activity of *Hedychium spichatum* rhizomes and *Zingiber zerumbet* rhizomes on the *Pheritima posthuma* model: A comparative study. *Pharmacognosy Research*. **3(2):** 140 -142.

**Granum, G.; Wanapat, M.; Wachiraparkorn, C. and Pakdee, P. (2003).** A comprehensive study on the effects of cassava hay in swamp buffaloes (*Bubalus bubalis*) and cattle (*Bos indicus*). M.Sc thesis, Unpublished. Department of Animal Science, Khon Kaen University, Khon Kaen, Thailand.

**Green, C. and Kremen, A. (2003).** U.S. organic farming in 2000–2001: adoption of certified systems. USDA, Economic Research Service, Resource Economics Division, Agriculture *Information Bulletin* No. 780.

**Hamilton, A. S. (2001).** Cooperia in cattle in New Zealand. Proceedings of the 30th Annual Seminar of the Society of Sheep and Beef Cattle Veterinarians of the new Zealand veterinary association, pp. 99 - 109.

**Hammond, J. A.; Fielding, D. and Bishop, S. C. (1997).** Prospects for plants in tropical veterinary medicine. *Veterinary Research Communication*. **21**: 213 - 228.

**Harborne, J. B. (1998).** Photochemical Methods: A guide to modern Techniques of plant Analysis.3rd edition. Chapman and Hall Ltd, London pg 279.

**Harder, A. (2002).** Chemotherapeutic approaches to nematodes: current knowledge and outlook. *Parasitology Research.* **88:** 272 - 277.

**Hariharan, V. K. (1975).** Prosapogenin-A, an alkaline hydrolytic product of *Entada scandens*: Indian Journal of Pharmacy. **37:** 67 - 72.

**Hariharan, V. K. and Rajagopalan, K. (1976).** Triterpenoid glycoside named Entamin isolated from seed kernels of *Entada scandens,* Indian – Patent pp. **140 - 384.**

**Hayashi, N.; Tabane, K.; Tsuge, T.; Nishimura, S.; Kohmoto, K. and Otani, H. (1990).** Determination of the host selective toxin production during spore germination of *Alternaria alterate* by High performance liquid chromatography. *Phytopathology.* **80:** 88 - 91.

**Holdsworth, D. K. (1990).**Traditional Medicinal Plants of Rarotonga, Cook Islands, part 1: *Internal Journal on Crude Drugs Research*. **23 (3):** 209 - 218.

**Hussain, H. and Deeni, Y. Y. (1991).** Plants in Kano Ethno botany; Screening for anti-microbials activity and alkaloids. *Internationa Journal of Pharmacognosy*., **29(1):** 51 - 56.

**Ikegami, F.; Ohmiya, S.; Ruangrungsi, N.; Sakai, S. I. and Murakoshi, I. (1987).** Entadamide B, a New Sulphur –Containing Amide from Entada phaseoloides: *Phytochemistry.***26 (5):** 1525 - 1526.

**Ikegami, F.; Sekine, T.; Duangteraprecha, S.; Matsushita, N.; Matsuda, N.; Ruangrungsi, N. and Murakoshi, I. (1989).** Entadamide C, sulphur – containing Amide from *Entada phaseoloides*: *Phytochemistry*, **28(3):**881 - 882.

**Iqbal, Z.; Lateef, M.; Ashraf, M. and Jabbar, A. (2004).** Anthelmintic activity of *Artemisia brevifolia* in sheep. *Journal of Ethnopharmacology*. **93**: 265 - 268.

**Jan, U.; Lanislav, K.; Iva, L. and Jana, U. (2008).** *In vitro* anthelmintic effect of medicinal plants used in Czech Republic. *Pharmaceutical Biology.*, **46**: 808 – 813.

**Parasites in Ruminants:** *Published by International Laboratory for Research in Animal Diseases' Nairobi, Kenya,* pp. 158 - 168.

**Kakar, S. A.; Tareen, R. B.; Sandhu, Z. U. D.; Kakar, M. A. Kakar, S. U. R.; Iqbal, Z. and Jabeen, H (2013).** *In vitro* and *in vivo* anthelmintic activity of *Ferula costata* (Kor.). Against gastrointestinal nematodes of sheep. *Pakistan journal of botany*. **45(I):** 263 - 268.

**Kabasa, J. D.; Opuda-Asibo, J. and Meulen, U. (2000).** The effects of oral administration of polyethylene glycol on feacal helminth egg counts in pregnant goats grazed on browse containing condensed tannins. *Tropical Animal and Production*, **32(2):** 73 - 86.

**Kamdem, L.; Messi, H. M.; Ndongo, N. A.; Mbi, C.; Njikam, A. P. and Elobo, S. (1986).** Ethnobotanical investigations carried out in Mouloundou (Eastern Province) and Zoetele (Southern Province): *Rev. Sci. Technol.* (*Health Science Services*). **3(3):** 59 - 68.

**Kamuhabwa, A. A.; Nshimo, C. and De Witte, P. (2000).** Cytotoxicity of some medicinal plant extracts used in Tanzania traditional medicine*: Journal of Pharmacology*. **70 (2):** 143 - 149.

**Kareru, P**. **G. (2008).** Ethno medicine practices, Analysis and Standardization of Herbal Anthelmintics used by the Embu and the Mbeere People of Kenya. PHD Thesis, Unpublished, Jomo Kenyatta University of Agriculture and Technology, Kenya. Pp 35 - 37.

**Kareru, P. G.; Kenji G. M.; Gachanja, A. N.; Keriko, J. M. and Mungai, G. (2007a).** Traditional Medicine and Healing Methods among Embu and Mbeere People of Kenya: *Journal for Africa Traditional, Complementary and Alternative Medicine,* **4(1):**75 - 86.

**Kareru, P. G.; Kenji, G. M.; Gachanja, A. N.; Keriko, J. M. and Mungai, G. (2007b).** Direct detection of triterpenoids saponins in medicinal plants: *Journal of African Traditional, Complementary and Alternative Medicine,* **5**(1):51 -55.

**Khan, L. P. and Diaz-Hernandez, A. (2000).**"Tannins with anthelmintic properties" in Tannins in livestock and human nutrition, Brooker, *J.D(Ed.).*

**Kirmizigul, S. and Anil, H. (2002).** New Triterpenic saponins from *Celphalaria transsylvanica*: *Turkey Journal of Chemistry*: **26:** 947 - 954.

**Kokwaro, J. O. (2009).** Medicinal Plants of East Africa, (3nd Edition) University of Nairobi Press, Nairobi. Pg 90

**Lahlou, M. (2002).** Potential of *Origanum compactum* as a cercaricide in Morocco. *Annals of Tropical Medical Parasitology.,* **96:**587 - 593.

**Lans, C. and Brown, G. (1998)**. Ethno veterinary Medicines used for Ruminant in Trinidad and Tobago. *Preventive Veterinary Medicine.* **35:** 149 - 163

**Lans, C.; Turner, N.; Khan, T.; Brauer, G. and Boepple, W. (2007).** Ethno-veterinary medicine used for ruminants in British Columbia, Canada. *Journal for Ethnobiology and Ethnomedicine.* **3(11).67-69**

**Le Grand, A. (1989).** Anti-Infectious Phytotherapy of the Tree-savannah, Senegal (Western Africa) III: A Review of the phytochemical substances and anti-microbial activity of 43 species, *Journal of Ethnopharmacology*., **25(3):** 315 - 338.

**Lu, Y.; Luo, J.; Huang, X. and Kong, L. (2009).** Four new steroidal glycosides from *Solanum torvum* and their cytotoxic activities. *Steroids.***7 (4)**: 95 - 101.

**Macfoy, C. A. (1992).** Biologically active natural products of plant origin, Conservation Biology, A Training Manual for Biological Diversity and Genetic Resources. Editors: Pomila Kapoor-Vijay and James White, Commonwealth Secretariat, London. pp. 189 - 194.

**Magu, M. M. (2011).** Analysis of lipid contents, lipid classes, free fatty acids and heavy metals from common fishes found in Kenyan freshwaters. MSc. Thesis, Unpublished, Jomo Kenyatta University of Agriculture and Technology, Kenya. Pp 54 – 58.

**Maingi, N. (1991).** Resistance to thiabendazole, fenbendazole and levamisole in Haemonchus Trichostrongylus species in sheep on a Kenyan farm, *Veterinary Parasitology*, **39:**285 - 291.

**Manandhar, N. P. (1995).** Medicinal Folk-Lore about the plants used as anthelmintic agent in Nepal. *Fitoterapia*, **66(2):**149 - 155.

**Mann, J. (1987).** Secondary Metabolism. Claredon Press, Oxford. Pg 87 - 90

**Marley, C, L.; Cook, R.; Barrett, J.; Keatinge, R. and Lampkin, N. M. (2003).** The effect of bird foot trefoil (*Lotus corniculatus*) and chicory (*Cichorium intybus*) on parasite intensities and performance of lambs naturally infected with Helminth parasites. *Veterinary Parasitology.***112:** 147-155.

**Martinez, M. A. (1984).** Medicinal plants used in a Totonac Community of Seirra Norte De Puebla: Tuzamapan De Galeana, Mexico. *Journal of Ethnopharmacology*. **11 (2):** 203 - 221.

**Mascie-Taylor, C. G. N. and Karim, E. (2003).** The burden of chronic disease. *Science*, **30 (2):** 1921 - 1922.

**Mathias, M. E. (1982)**. Some medicinal plants of the Hehe (Southern Highlands Province, Tanzania): *Taxon* **31 (3):** 488 - 494.

**Maundu P. and Tengnas B. (2005).** Useful trees and Shrubs for Kenya. World Agro forest centre - Eastern and Central Africa Regional programme (ICRAF-ECA) Nairobi, Kenya.

**Max, R. A.; Kimambo, A. E.; Kassaku. A. A.; Mutenga, L. A. and Buttery, P. J. (2007).**Effects of *tanniferous browse* meals on nematode faecal egg counts and internal parasites burdens in sheep and goats. *South African Journal of Animal Science.* **37**: 97 – 106.

**Mc Allister, T. A.; Annett, C. B.; Cockwell, C. L.; Olson, M. E.; Wang, Y. and Cheeke, P. R. ( 2001).** Studies on the use of *Yucca schidigera* to control giardiosis, *Veterinary Parasitology*. **97:** 85 - 99.

**McLaughlin J. L.; Chang', C. J. and Smith, D. L. (1991).** Bench-top bioassay for the discovery of bioactive natural products. An update. *Journal of Natural Products*.**9:** 383 - 409.

**McLaughlin, J. K.; Rogers, L. L. and Anderson, J. E. (1998).** The use of biological assays to evaluate botanicals. *Drug Inform. J.,* **32:** 513 - 524.

**Mekarn, A. (2003).** Effects of grass or cassava foliage on growth and nematode parasite infestation in goat fed low or high protein diets in confinement. *Livestock Research for Rural Development.* **8(15).**

**Meyer, B. N.; Ferrigni, N. R.; Putnam, J. E.; Jacobsen, L. B.; Nichols, D. E. and McLaughlin, J. L.(1982**). A convenient general bioassay for active plant constituents. *Planta Medica.* **45:** 31 - 34.

**Min, B. R. and Hart, S. P. (2003).** Tannins for suppression of internal parasites: *Journal of Animal Science.* **81**:102 - 109.

**Molan, A, L.;Waghorn, G. C. and McNabb, W. C. (2002).**Effects of condensed tannins on egg hatching and larval development of *Trichostrongylus colubriformis In vitro*. *Journal of Ethnopharmacology.* **150(3):** 65 - 68.

**Mortensen, L.L.; Williamson, L.H. and Terrill, T.H. (2003).** Evaluation of prevalence and clinical implications of anthelmintic resistance in gastrointestinal nematodes of goats. *Planta Medica* **23(4)**: 495 - 500.

**Moshi, M. J.; Innocent, E.; Magadula, J. J.; Otieno, D. F.; Mbabazi, P. K.; Waisheit, A. P. K. and Nondo, R. S. O. (2010).** Brine shrimp toxicity of some plants used as traditional medicines in Kagera Region, North Western Tanzania. *Tanzania Journal of Health Research*. **12:**1.

**Murakami, C.; Myoga, K.; Kasai, R.; Ohtani, K.; Kurakawa, T.; Ishibashi, S.; Dayrit, F.; Padolina, W. G. and Yamasaki, K. (1993)**. Screening of plant constituents for effects on Glucose Transport Activity in Ehrlich Ascites tumour cells: *Chemical and Pharmacy, Bulletin*. **41 (12)**: 2129 - 2131.

**Nagaraju, N. and Rao, K. N. (1990).** A survey of the crude drugs of Rayalaseema, Andhra Pradesh, India: *Journal of Ethnophamacology*: **29 (2):** 137 - 158.

**Nakanishi, K. (1999)**. A Historical perspective of Natural Products Chemistry In Comprehensive Natural Products Chemistry. Ushio, S (Ed*), Elsevier*, Amsterdam, pp. 23 - 40.

**Natalie, J. L. and Peter, P. (1998).** Use of fingerprinting and marker compounds for the identification of botanical drugs: Strategies for applying pharmaceutical HPLC analysis to herbal plants. *Drug Information Journal*: **32:** 497-512.

**Natori S.; Ikekawa N. and Suzuki, M. (1981).** Advances in Natural Product Chemistry: Extraction and Isolation of Biologically Active Compounds, pp. 275 – 287.

**Nielzen, J. H.; Charleston W. A. G.; Hodgson, J.; Mackay, A. D. and Leathwick, D. M. (1996).** Controlling internal parasite I grazing ruminants without

recourse to anthelmintic: approaches, experience and prospects. *International Journal of Parasitology*, **26**: 983-992.

**Niezen, J. H.; Robertson, H. A.; Waghorn, G. C. and Charleston, W. A. G. (1998).** Production, faecal egg counts and worm burdens of Ewe lambs which grazed six contrasting forage. *Veterinary Parasitology***. 80:** 15 – 27.

**Okada, Y.; Shibata, S.; Javellana, A. M. J. and Kamo, O. (1988).** Entada saponins (ES) II and IV from bark of *Entada phaseoloides*: *Chemical and Pharmacy Bulletin*. **34(4):** 1264 – 1269.

**Okwu, D. E. and Josiah, C. (2006).** Nigerian Medicinal Plants: *Africa Journal of Biotechnology*, **5(4):**357-361.

**Onyeyili, P. A.; Amin, J. D.; Gambo, H. I.; Nwosu, C. O. and Jibike, G. I. (2001).** *Nigerian Veterinary Journal*.**22 (1):**74 - 82.

**Osamu, T. (1990).** Ginseng Saponins from Panax spcies. *Pure and applied Chemistry*. **62(7)**:1281-1284.

**Owuor, B. O. and Kisangau, D. P. (2006).** Kenya Medicinal Plants used as antivenins: a comparision of plant usage. *Journal of Ethnobiology and Ethnomedicine*: **27:**1-8.

**Ozipek, M.; Saracoglu, I.; Ogihara Y. and Callis, I. (2002).** Nautigenin- type steroidal saponins from *Veronica fuhsii* and *V multifida*: *Z Naturforsch*: **57:**603-608.

**Paolin, E. (2003).** Effects of sainfoin hay on gastrointestinal nematode infections in goats. *Veterinary Records*: **152:** 600-601.

**Patel, J.; Kumar, G. S.; Deviprasad S. P.; Deepika S. 1. and Harshavardhan R. A., (2011).** Phytochemical and anthelmintic evaluation of *lantana camara* (l.) var. aculeate leaves against *pheretima posthuma, Journal of Global Trends in Pharmaceutical Sciences,* **2(1):**1 – 20.

**Patil, U. K.; Dave, S.; Bhaij, A.; Baghel, U. S.; Yadav, S. K.; Vijay, K. and Sharma, V. K. (2009).** *In vitro* Anthelmintic Activity of Leaves of *Spinacia oleracae* Linn *International Journal of Toxicological and Pharmacological Research,* **1(1):** 21 - 23.

**Patnaik, T.; Dey, R. K. and Gouda, P. (2007)**. Isolation of Triterpenoid Glycoside from Bark of Terminalia Arjuna using Chromatographic Technique and Investigation of Pharmacological Behaviour upon Muscle Tissues. *E-journal of Chemistry* **4(4):** 474-479.

**Pegel, K. L. and Roger, C. B. (1985).** The characterization of mollic acid 3β-dxyloside and its genuine aglycone mollic acid and two novel 1α-hydroxycyloartenoids from *Combretum molle*. *J. Chem. Soc. Perkin. Trans.* **1:**1711–1715.

**Perry, B. D.; Randolph, T. F.; McDermott, J. J.; Sones, K. R. and Thornton, P. K. (2002).** Investing in animal health research to alleviate poverty. ILRI (International Livestock Research Institute), Nairobi, Kenya, 148 pp.

**Pessoa, L. M.; Morais, S. M.; Bevilaqua, C. M. and Luciano, J. H. (2002).** Anthelmintic activity of Essential oil of *Ocimum gratissimum* Linn. and eugenol against *Haemonchus contortus*. *Veterinary Parasitology*. **109:**59-63.

**Price, K. R.; Johnson, I. N. T. and Fenwick, G. R. (1987).** The chemistry and biological significance of saponins in food and feed stuffs, *Research Food, Science Nutrition.* **26:** 27–135.

**Rabel, B.; McGregor, R. and Douch, P. G. C. (1994).** Improved bioassay for estimate of inhibitory effects of ovine Gastrointestinal mucus and anthelmintic on Nematodes larval Migration. *International Journal of Parasitology*. **24:** 671- 676.

**Radostits, O. M.; Gay, C. C. and Blood, D. C. (2000).** Veterinary Medicine, 9th ed. London, England: WB Saunders; 1340.

**Rang, H. P.; Dale, M. M.; Ritter, J. M.; Flower, R. J. (2007).** Rang and Dale's Pharmacology, 6th ed. Churchill Livingstone Elsevier. London, England: 715

**Riguera, R. (1997)**. Isolating bioactive compounds from marine organisms. *Journal of Marine Biotechnology.* **:**187-193.

**Rogers, C. B and Verotta, L. (1996).** Chemistry and biological properties of the African Combretaceae. In: K. Hostettmann, F. Chinyanganya, M. Maillard and J-L. Wolfender, Editors, *Chemistry, Biological and Pharmacological Properties of African Medicinal Plants*, University of Zimbabwe Publications, Harare. 121- 141.

**Roos, M. H. (1997).** The role of drugs in the control of parasitic nematode infections: must we do without? *Parasitology* **114:** 137-144.

**Sabai, K. K.; Aung, W.; Nwe Ni Thin, N. N.; Shwel, K. and We, T. M. (2001)**. Evaluation of the Bioactivities of Some Myanmar Medicinal Plants Using Brine Shrimp *(Artemia salina)* Toxicity Test. *J. Myan. Acad. Tech.***107 (1):** 105-112.

**Saddiqi, H. A; Iqbal, M. N. and Muhammad, G. (2010).** Comparative resistance of sheep breeds to *Haemonchus contortus* in a natural pasture infection. *International Journal for Biology*.**12:** 739 – 743.

**Sam, T. W. (1993).** Toxicity testing using the brine shrimp: *Artemia salina*. In: Colegate S.M. and Molyneux, R. J. (Eds.), Bioactive Natural Products Detection, Isolation and Structural Determination. CRC Press, Boca Raton, Fl: 442-456.

**Sangster, N. C. and Gill, J. (1999).** Pharmacology of anthelmintic resistance. *Parasitology Today.* **15:** 141-146.

**Sanogo, R.; Germano, M. P.; D'Angelo, V.; Guglielmo, M. and De Pasquale, R. (1998).** Antihepatotoxic Properties of *Entada Africana* (Mimosaceae): *Phytotherapy. Research Suppliment*. **12 (1):** 157 - 159.

**Sengupta, A. and Basu, A. (1978).** Triglyceride composition of *Entada phaseoloides* seed oil: *Journal of Science Food and Agriculture*. **29:** 677 – 682

**Singh, D. K. (2012).** Pesticide Chemistry and Toxicology. (S.T) Bentham e Books© 2012 Series Toxicology, Agriculture and Environment.

**Solis, P. N.; Wright, C. W.; Anderson, M. M.; Gupta, M. P. and Phillipson, J. D. (1993**). A microwell cytoxicity assay using *Artemia salina*. *Planta Medica*., **59**: 250 - 252.

**Sorajini, N.; Manjari, S. A. and Kanti, C. C. (2011).** Phytochemical screening and anthelmintic activity study of *Saraca indica* leaves extracts**.** *International Research Journal of Pharmacy* **2 (5):** 194 - 197.

**Soulsby, E. J. L. (1982).** Helminthes, arthropods and protozoa of domestic animals. 7th Edition. W. B. Saunders, London. ISBN 070200896962.

**Tariq, K. A.; Chishti, M. Z.; Ahmad, F. and Shawl, A. S. (2009)**. Anthelmintic activity of extracts of *Artemisia absinthium* against ovine nematodes. *Veterinary Parasitology* **160:** 83–88.

**Trease, G. E. and Evans, W. C. (1989**). Trease and Evans, Pharmacognosy: A Physician's Guide to Herbal Medicine. 13$^{th}$ ed. Bailliere Tindall. London: 230 - 234.

**Uhlinger, C.; Fleming, S. and Moncol, D. (1992).** Survey for drug-resistant gastrointestinal nematodes in 13 commercial sheep flocks. *Journal of American Veterinary Medical Association.* **201:**77 - 80.

**Van Wyk, J. A.; Malan, F. S.; and Randles, J. L. (1997).** How long before resistance makes it impossible to control some field strains of *Haemonchus contortus* in South Africa of the modern anthelmintics. *Veterinary Parasitology*. **70(1-3):** 111 - 122

**Verpoorte, R. (1998).** Exploration of nature's chemo diversity: the role of metabolites as leads in drug development. *Drug Development Trends*, **3:** 232-238.

**Verpoorte, R. (2000).** Pharmacognosy in the millennium: Lead finding and biotechnology. *Journal for Pharmacy and Pharmacology.* **52:** 253 - 262.

**Vlientich, A.; Bergie, D. and Laekeman, G. (1997).** Chemotherapeutic and Pharmacological screening of biologically active plants. *The Bioassay Natural Products Seminar Manual* 21-24 October 1991, 1-27, University of Malaya, Malaysia.

**Waghorn, G. C. and McNabb, W. C. (2003).** Consequences of plant phenolic compounds for productivity and health of ruminants. *Proceedings of the Nutrition Society* **62,** 383-392.

**Waller, P. J. (1999).** Parasite epidemiology, resistance and the prospect for the implementation of the alternative control programs. *FAO Animal Health Paper 141*. FAO. Rome. Pp 1-10.

**Wamae, L. W.; Ngare, J. O.; Ihinga, M. A. and Mahaga, M. (1990).** Epidemiology of fasciolosis on a ranch in the Central Rift Valley, Kenya. *Tropical Animal Health and production* **22:** 132-134

**Wanapat, M.; Pimpa, O.; Petlum, A. and Boontao, U. (1997).** Cassava hay: A new strategic feed for ruminants during the dry season. *Livestock Research for Rural Development* **9(2):**60 - 62

**Wasswa, P. and Olila, D. (2006).** *In vitro* Ascaridal activity of selected indigenous medicinal Plants used in Ethno Veterinary practices in Uganda. *Journal of African Traditional and Complementary Medicine*.**3 (2):** 94 – 103.

**Wasuwat, S. (1967**). A list of Thai medicinal plants, A.S.R.C.T., Bangkok Report No. 1 on Res. Project. 17.

**Watt, M. and Brayer, N. (1962).** The medicinal and poisonous plant of Southern and Eastern Africa, E and Livingstone Ltd, 2nd edition, pp 554 – 555.

**WHO (1978).** The Promotion and development of traditional medicine, Technical report series, 622 Geneva.

**Wilcox, M. L.; Cosentino, M. J.; Pink, R.; Bodeker, G. and Wayling, S. (2001).** Natural products for the treatment of tropical diseases. *Trends Parasitology* **17:**58-60.

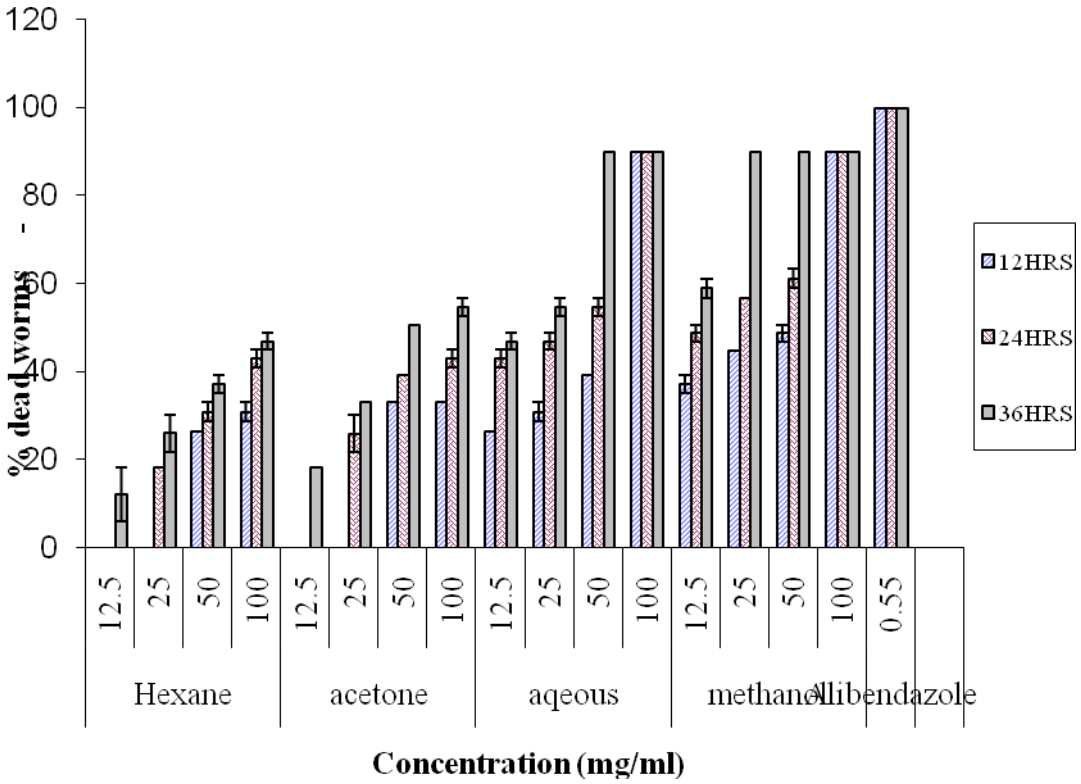
**Wu, L. J. and Yao, X. S. (2003).** Natural Pharmaceutical Chemistry, 4th Ed. Peoples Medical Publishing House: Beijing.

**Yates, D. M.; Portillo, V. and Wolstenholme, A. J. (2003)**. The avermectins receptors of *Haemonchus contortus* and *Caenorhabditis elegans*. *International Journal for Parasitology* **33:** 1183-1193.

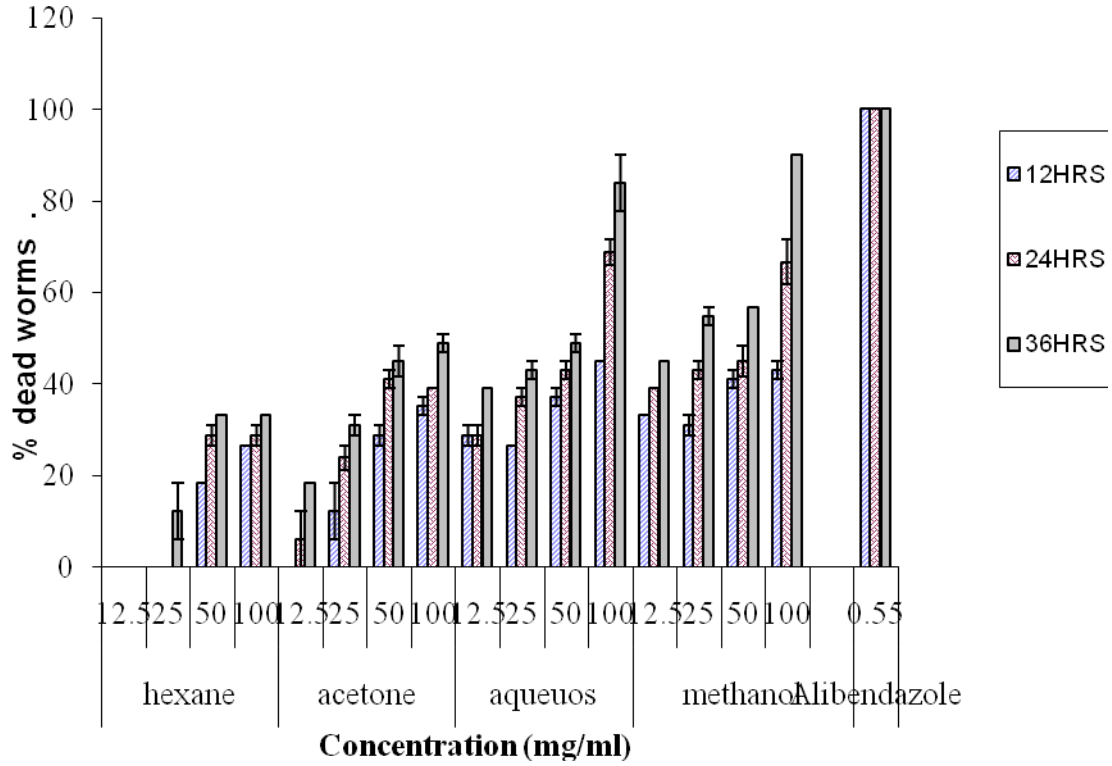
**Young, K. E.; Jensen, J. M. and Craig, T. M. (1997).** Verification of HDrenchRite for use in North America by comparison with FERT (faecal egg reduction tests). *America Association of Veterinary Parasitologist.* **42:**65.

## APPENDICES

**Appendix 1:** Activity of hexane acetone, aqueous and methanol extract of *E. leptostachya* compared to albendazole against *H. contortus*



**Appendix 2:** Activity of hexane acetone, aqueous and methanol extract of *R. rhododendroides*



**Appendix 3**: TLC fingerprints of the sapogenin mixture of hydrolyzed crude saponins of *E. leptostachya* from PTLC Solvent system B:A:W (4:1:5)

**Appendix 4**: Hodge and Sterner Toxicity

| Scale **Toxicity Rating** | **Description** | **Oral $LD_{50}$ (mg/kg)** | **Inhalation $LC_{50}$ (ppm)** | **Dermal $LD_{50}$ (mg/kg)** | **Probable Lethal Dose for Man** |
|---|---|---|---|---|---|
| 1 | extremely toxic | 1 or less | 10 or less | 5 or less | 1 grain, a taste, a drop |
| 2 | highly toxic | 1-50 | 10-100 | 5-43 | 1 teaspoon (4 mL) |
| 3 | moderately toxic | 50-500 | 100-1000 | 44-340 | 1 fluid ounce (30 mL) |
| 4 | slightly toxic | 500-5000 | 1000-10,000 | 350-2810 | 1 pint (600 mL) |
| 5 | practically non-toxic | 5000-15,000 | 10,000-100,000 | 2820-22,590 | 1 L or 1 quart |
| 6 | relatively harmless | greater than 15,000 | greater than 100,000 | greater than 22,600 | |

(**Source:** Singh, 2012)

**Appendix 5:** ANOVA Tables

Entada acetone (P=0.009)

**ANOVA Table**

| | | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|---|
| acetone * time | Between Groups | (Combined) | 3095.903 | 2 | 1547.951 | 5.429 | 0.009 |
| | Within Groups | | 9409.474 | 33 | 285.136 | | |
| | Total | | 12505.377 | 35 | | | |

Entada aqueous (P=0.048)

**ANOVA Table**

| | | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|---|
| aqueous * time | Between Groups | (Combined) | 3378.311 | 2 | 1689.155 | 3.335 | 0.048 |
| | Within Groups | | 16716.132 | 33 | 506.549 | | |
| | Total | | 20094.442 | 35 | | | |

Entada hexane (P=0.051)

**ANOVA Table**

| | | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|---|
| arcsine * time | Between Groups | (Combined) | 1584.855 | 2 | 792.427 | 3.258 | 0.051 |
| | Within Groups | | 8026.561 | 33 | 243.229 | | |
| | Total | | 9611.416 | 35 | | | |

Entada methanol (P=0.002)

**ANOVA Table**

| | | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|---|
| arcsine * time | Between Groups | (Combined) | 4533.971 | 2 | 2266.986 | 7.320 | 0.002 |
| | Within Groups | | 10220.318 | 33 | 309.707 | | |
| | Total | | 14754.289 | 35 | | | |

RAPA acetone (P=0.030)

**ANOVA Table**

| | | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|---|
| arcsine * time | Between Groups | (Combined) | 1682.961 | 2 | 841.481 | 3.909 | 0.030 |
| | Within Groups | | 7103.624 | 33 | 215.261 | | |
| | Total | | 8786.585 | 35 | | | |

Rapa aqueous (P=0.13)

**ANOVA Table**

| | | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|---|
| arcsine * time | Between Groups | (Combined) | 2250.175 | 2 | 1125.088 | 4.927 | 0.013 |
| | Within Groups | | 7535.185 | 33 | 228.339 | | |
| | Total | | 9785.360 | 35 | | | |

Rapa hexane (P=0.325)

**ANOVA Table**

| | | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|---|
| arcsine * time | Between Groups | (Combined) | 535.457 | 2 | 267.729 | 1.163 | 0.325 |
| | Within Groups | | 7596.117 | 33 | 230.185 | | |
| | Total | | 8131.574 | 35 | | | |

Rapa methanol (P=0.000)

**ANOVA Table**

| | | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|---|
| arcsine * time | Between Groups | (Combined) | 3617.624 | 2 | 1808.812 | 10.910 | 0.000 |
| | Within Groups | | 5470.973 | 33 | 165.787 | | |
| | Total | | 9088.597 | 35 | | | |

**Afri. j. ethnomed. pract.** 1(1):36-43


**Mortality of *Heamonchus contortus* adult worms in *Entada leptostachya* Harms. and *Rapanea rhododendroides* (Gil) Mez. extracts**

Omingo O.[1] , Kareru P. G. [1*], Rukunga G.[2], Mbaria J.[3], Keriko J. M.[1], Njonge F. M.[1], Owuor B. O.[4]

[1]Jomo Kenyatta University of Agriculture and Technology, P.O Box 62000-00200, Nairobi, Kenya
[2]Kenya Medical Research Institute, Nairobi, P.O Box 54840-00200, Nairobi, Kenya
[3]College of Agricultural and Veterinary Sciences, University of Nairobi, P.O Box 29053-00625, Nairobi, Kenya
[4]Catholic University of Eastern Africa, P.O Box 62157-00200, Nairobi, Kenya
E-mail: pgkareru@yahoo. Com
Telephone: +254 6752223, Fax: +254 6752164
*Corresponding athour

**Abstract**

This study was on evidence based information that *Entada leptostachya* Harms and *Rapanea rhododendroides* (Gil) Mez were used by the herbalists in Mbeere County, Kenya, for the treatment of gastrointestinal worms. The plants' aqueous and solvent extracts were tested for their *in -vitro* antihelmintic activity against *Heamonchus contortus* adult worms. Of the eight plant extracts investigated, four extracts exhibited adult worm mortality less than 50% while the other four afforded mortality ranging between 60-77%. *E. leptostachya* methanol extract was the most active (77%). Albendazole was used as a positive control drug while Goodwin's physiological solution was used as negative control. Methanol extracts for both plants exhibited the highest anthelmintic activity at the test concentration of 25mg/ml. Although *R. rhododendroides* was ranked third in general usage by the herbalists, *E. leptostachya* was solely used for the treatment of intestinal worms. Both plants contained saponins and tannins which have been established to exhibit antihelmintic activity. The present results demonstrated that *E. leptostachya* plant extracts had antihelmintic agents, and could be developed as an alternative drug for the treatment of heamonchosis.

**Keywords**: heamonchosis, herbalists, tannins, saponins, *Entada leptostachya, Rapanea rhododendroides*.

## Introduction

Gastrointestinal nematodes are a major threat to sheep productivity and endanger animal welfare worldwide particularly in developing countries (Dhar *et al.,* 1982). Haemonchosis has been identified as one of the top ten constraints to sheep and goat rearing in East Africa (Perry *et al.*, 2002). *Haemonchus contortus* is the leading pathogenic and resistant helminth parasite in small ruminants that causes acute and high mortality (Allonby and Urquhart, 1975) .Control of gastrointestinal helminths is mainly by use of synthetic anthelmintics which are invariably expensive, inaccessible or inadequately available to the poor farmers of developing countries ( Marley *et al.*, 2003; Waller, 1998). Furthermore, usage of conventional anthelmintics has led to development of resistance, presence of residues in meat and milk and high environmental impact (Echevarria *et al,*). Resistance of *H. contortus* to ivermectin has been reported, the parasite being a problem and occurrence significantly higher in sheep than in goats. Anthelmintic resistance and other associated pitfalls of conventional drugs has necessitated search for alternative potent herbal remedies (Oliveira *et al.*, 2009). *In-vitro* activity of plant extracts against *H. contortus* adult worms, larvae development and hatching has been demonstrated (Eguale *et al.*, 2009). Tannins (Athanasiadou *et al.,* 2001; Min and Hart, 2003; Poalini, *et al.* 2003) and saponins (Bishnu and Zeev, 2005) are some of the plant secondary metabolites established to have anthelmintic activity. Studies from various parts of the world have shown that certain plant species effectively reduce the degree of parasite infestation in ruminants (sheep) and are promising alternatives to conventional anthelminthics (Githiori *et al.,* 2006.; Max *et al.,* 2007; Jan *et al.*, 2008).

*Entada leptostachya* and *Rapanea rhododendroides* are among the plants used by the traditional healers as dewormers and have been previously cited in ethno botanical reports (Kareru *et al.*, 2007). Hence the aim of the present study was to determine the potency of *E. leptostachya* Harms and *R. rhododendroides* (Gil) Mez. extracts against *H. Contortus* adult worms from infected sheep. *Rapanea rhododendroides* and *Entada leptostachya* are found in East Africa, including Ethiopia, Somalia and Mayotte (Dale *et al*, 1961; Brena, 1959).

## Materials and methods

*Collection of indigenous knowledge, plant materials and extraction*

Over 30 herbalists from Mbeere County,

Kenya were interviewed for their indigenous knowledge on common plants used to treat worm infestation in humans and ruminant animals. From this information two plants were selected for the present study.

The root bark of *E. leptostachya* and stem bark of *R. rhododendroides* were collected after identification and authentication by a taxonomist in the Department of Botany, Jomo Kenyatta University of Agriculture and Technology (JKUAT). Voucher specimens OOM/1 and OOM/2 were deposited in the herbarium of that department. The collected plant parts were dried under the shade and ground to a powder. The powders were then subjected to hot water maceration and successive solvent extraction using methanol, acetone and hexane in a soxhlet extractor. Extracts were dried under vacuum, placed in dry vials and stored at 4°C.

*In-vitro antihelmintic activity assay*

Mature *H. contortus* worms were collected from the abomasums of freshly slaughtered sheep at a local abattoir (Ruiru), identified by a meat inspector and later confirmed by a parasitologist at Zoology department, JKUAT. The worms were washed in distilled water and suspended in phosphate buffered saline (PBS) made by dissolving 0.85 g of sodium chloride and 1 g glucose in 1 litre distilled water. They were then transported to the laboratory in an air tight can and then left for 2hrs to acclimatize.

Plant extracts were dissolved in DMSO and made to the mark using distilled water to make 25mg/ml solutions. Filter paper discs (6mm diameter) were dipped into each solution. The discs impregnated with the above extracts were dried at room temperature to evaporate the DMSO, leaving only the test compounds. The discs for water extracts were not dried.

Ten (10) adult *H. contortus* worms were placed into a sterile Petri dish containing 10ml of PBS. The filter paper disc containing the aqueous extract was added and agitated. After 24 hours, the worms were removed from the Petri dish and the parasites suspended in PBS for 30min for possible recovery of the parasite motility. The number of motile (alive) and immotile (dead) worms were counted using a hand lens and recorded. Death or paralysis of worms was ascertained by absence of motility for an observation period of 5–6 seconds (Rabel *et al.*, (1994).

The above procedure was repeated for all the other plants' extracts. Three replicates were performed for each treatment. Albendazole (purchased from Kobian Ltd) at a concentration of 0.55 mg/ml was used as the reference drug (positive control) while PBS was used as a negative control. Worm motility and mortal-

ity were the rationale for anthelmintic activity.

**Results and discussion**

Table 1 shows the list of plants reported to treat worm infestation in ruminants as well as in humans. The plants were ranked 1-9 on the basis of the indigenous knowledge and potency. There were claims, however, that plants with the highest ranking were used to treat stomach troubles in humans due to amoeba infections. There was unanimous agreement that *E.leptostachya*, *R. rhododendroides* and *Albizia anthelmintica* were used for deworming ruminant animals. The latter plants have been reported to have anthelmintic activity (Kareru *et al*, 2006, 2007).

**Table 1: Plants reported to treat worms (Human/animals)**

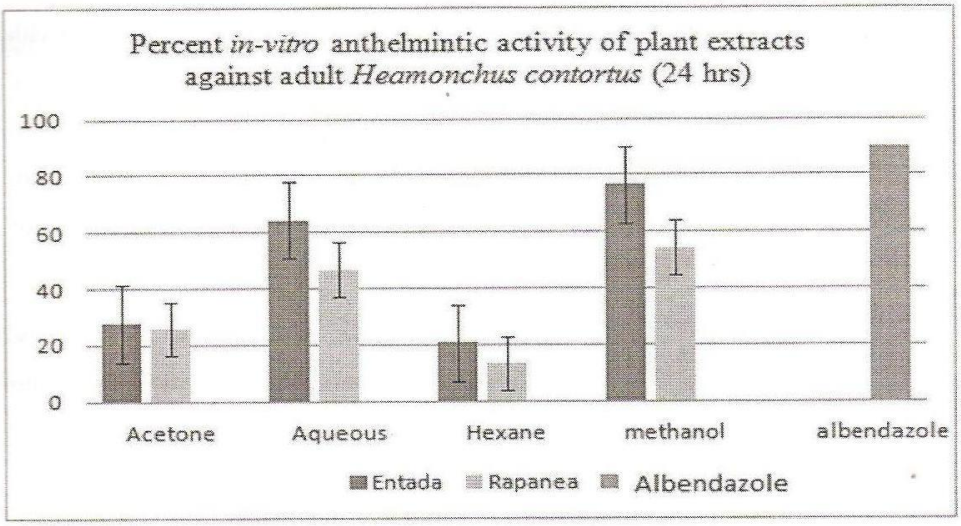
| Local names | Scientific names | Ranking | Parts used |
|---|---|---|---|
| Mubarwa | *Albizia anthelmintica** | 3 | Bark/roots |
| Mwinu | *Senna didymobotrya** | 8 | Leaves |
| Muvovo | *Leonotis mollissima* | 5 | Leaves |
| Mucaritha | *Entada leptostachya** | 1 | Roots |
| Mugeeta | *Rapanea rhododendroides* | 2 | Bark |
| Mururuku | *Terminalia brownii* | 6 | Bark |
| Terere | *Amaranthus hybridus* | 9 | Leaves |
| Mubera | *Psidium guajava* | 4 | Leaves |
| Mubiru | *Vangueria* madagascariensis | 7 | Leaves |

*Plants reported to treat only worms

*In-vitro anthelmintic activity*

Anthelmintic activity of the *E. leptostachya* and *R. rhododendroides* extracts against *H. contortus* adult worms were presented in Figure 1. The samples were tested at a concentration of 25 mg/ml and compared to Albendazole positive control, whose concentration was maintained at 0.55mg/ml as directed on the product label.

*Entada. leptostachya* and *R. rhododendroides* methanol extracts were more potent against *H. contortus* adult worms with mortalities of 77 and 54% respectively, when compared to other extracts. Hexane extracts were the least potent exhibiting mortalities of 21 and 13% for *E. leptostachya* and *R. rhododendroides* respectively. Aqueous extracts of both *E. leptostachya* and *R. rhododendroides* showed moderate potency against *H. contortus* adult worms. Acetone extracts were the second least potent, compared to other extracts. There were no significant differences ($p = 0.165$) in anthelmintic activity between acetone extracts




of *E. leptostachya* and *R. rhododendroides*. However, aqueous, hexane and methanol extracts had significant differences ($p<0.05$). The reference drug albendazole showed 90% mortality at a concentration of 0.55mg/ml. The worms which were kept in the negative control

PBS showed no mortality($p=0.00$).The high mortality values exhibited by the methanol extracts of *E. leptostachya* root bark and *R. rhododendroides* stem bark support their medicinal uses in the treatment of gastrointestinal nematodes.

The present study evaluated *in-vitro* anthelmintic property of crude extracts acetone, aqueous, hexane and methanol of *E. leptostachya* and *R. rhododendroides* plants in comparison to the reference drug - albendazole against *H. contortus* adult worm. The acetone, aqueous, hexane and methanol root bark extracts of *E. leptostachya* and stem bark extracts of *R. rhododendroides* showed anthelmintic activity especially at higher concentration of 100 mg/ml comparable to the reference drug albendazole 0.55mg/ml. The high activity of methanol extract confirms the findings from other studies of hydro-alcoholic extract of *Coriandrum sativum* and *Hedera helix* at 25mg/ml showed a higher anthelmintic activity against *H. contortus* adult worm (Eguale *et al.,* 2007a). Similarly, ethanolic extract of *Artemisia absinthium* at 25 mg/ml was more efficacious anthelmintic than aqueous extract against *H. contortus* adult worm (Tariq *et al.*, 2009). The crude extract showed a dose and time dependent anthelmintic activity against *H.*

*contortus* adult worm. Albendazole works by interference with the polymerization of microtubule (Harder, 2002). The drug binds to the protein tubulin of the *H. contortus* hence causing death by starvation (Roos, 1997). The phytochemical screening of these extracts exhibited the presence of saponins, tannins, and steroid. These phytochemicals are known to exhibit anthelmintic activity (Wasswa and Olila, 2006). It is possible that the phytochemicals found in the extracts of *E. leptostachya* and *R. rhododendroides* are responsible for the observed anthelmintic activity. Tannins are reported to cause anthelmintic activity by binding to the proteins found in the gastro intestinal of the host or to glycoprotein on the cuticle of the parasite and may cause death (Kane, 2009).

## Conclusion

The traditional use of root bark of *E. leptostachya* and stem bark extracts of *R. rhododendroides* having anthelmintic activity is confirmed as extracts displayed anthelmintic properties at concentration and doses tested against GI nematodes as determined by

## Acknowledgements

The authors are grateful to the financial support received from Research, Production and Extension (RPE) Division of JKUAT. Thanks go to the Director of Culture, Mbeere-Embu Counties, for facilitating interviews with the Mbeere Herbal Practitioners Association. We are indebted to one of the herbalists, Mr. Mwaniki and taxonomist, Geoffrey Mungai, for assisting in identification and collection of the plant materials.

## Conflict of Interest

The authors wish to declare that there are no commercial/financial conflicts of interest.

## References

**Eguale, T., Tilahun, G., Debella, A., Feleke, A., Makonnen, E. (2007a).** *Haemonchus contortus*: *In vitro* and *in vivo* anthelmintic activity of aqueous and hydro-alcoholic extracts of *Hedera helix*. *Exp. Parasitol.* **116:** 340 - 345

**Githori, J. B. Athanasiadou, S. and Thamsborg, S. M., (2006).** Use of plants in novel approaches in the control of gastrointestinal helminthes in livestock with emphasis on small ruminants. *Veterinary Parasitology,* **139:** 308 - 320.

**Harder, A. (2002).** Chemotherapeutic approaches to nematodes: current knowledge and outlook. *Parasitology Research* **88:** 272 - 277.

**Jan, U., Lanislav, K., Iva, L and Jana, (2008).** *In vitro* anthelmintic effect of medicinal plants used in Czech Republic,

*Pharmaceut. Biol.*, **46**: 808 – 813.

**Kane S. R., Mohite, S. K.,Rajarambapu, J. S. S., (2009).** Anthelmintic activity of aqueous and methanol extracts of *Euphorbia tymifolia. International Journal of PharmTech Research.***1 (3)**666-669

**Kareru P. G, Gachanja A. N KerikoJ. M Kenji G. M.(2006).** Screening of indigenous medicinal plants for anthelmintic activity. Journal of Pharmacy and Pharmacology, Science Proceedings 2006, Supplement 1, British Pharmaceutical Conference.

**Kareru P.G, Gachanja A. N Keriko, Mungai G (2007).** Traditional Medicines among the Embu and Mbeere Peoples of Kenya. *African Journal of Complementary and Alternative Medicine, 4(1): 75-86*

**Marley, C, L., Cook, R., Barrett., J., Kea tinge, R. and Lampkin, N. M., (2003).** The effect of bird foot trefoil (*Lotus corniculatus*) and chicory (*Cichorium intybus*) on parasite intensities and performance of lambs naturally infected with Helminth parasites. *Vet. Parasitol.* **112,** 147-155.

**Max, R. A., Kimambo, A. E., Kassaku. A. A., Mutenga, L. A and Buttery, P. J., (2007).** Effects of tanniferous browse meals on nematode faecal egg counts and internal parasites burdens in sheep and goats. *S. Afr. J. Anim. Sci.* **37**: 97 – 106.

**Min, B.R and Hart, S.P (2003).** Tannins for suppression of internal parasites: *J. Anim. Sci.* **81:** (E Supp. 2) E102-E109.

**Oliveira, L.M.B., Bevilaqua, C.M.L.Costa, C.T.C.Macedo, I.T.F Barros, R.S..Rodrigues, A.C.M.Camurc¸a-Vasconcelos A.L.F,.Morais S.M,.Lima, Y.C.Vieira L.S,.Navarro A.M.C (2009).** Anthelmintic activity of Cocos nucifera L. against sheep gastrointestinal nematodes, Veterinary Parasitology 159 55–59

**Perry, B. D., Randolph, T. F., McDermott, J. J., Sones, K. R. and Thornton, P. K., (2002).** Investing in animal health research to alleviate poverty. ILRI (International Livestock Research Institute), Nairobi, Kenya, 148 pp.

**Paolini, V., Bergeaud, J.P, Grisez, C Prevot, F.,Dorchies, P. H., Hoste, H.,(2003).** Effects of condensed tannis on goats experimentally infected with Haemonchus contortus. *Parasitol.* **113**: 253-261.

**Rabel, B., McGregor, R., Douch, P. G. C., (1994).** Improved bioassay for estimate of inhibitory effects of ovine gastrointestinal mucus and anthelmintic on Nematodes larval Migration. *Int. J. Parasitol.* **24**:671- 676.

**Roos, M. H., (1997).** The role of drugs in the control of parasitic nematode infections: must we do without? *Parasitology* **114:** 137-1

**Tariq, K. A., Chishti, M. Z., Ahmad, F. and Shawl, A. S., (2009).** Anthelmintic activity of extracts of *Artemisia absinthium* against ovine nematodes. *Veterinary*

*Parasitology* **160:** 83–88.

**Waller, P. J., (1998).** Parasite epidemiology, resistance and the prospect for the implementation of the alternative control programs. *FAO Animal Health Paper 141*. FAO. Rome. Pp 1-10.

**Wasswa, P. and Olila, D. (2006).** *In vitro* Ascaridal activity of selected indigenous medicinal Plants used in Ethno Veterinary practices in Uganda. *J African .Traditional and Complementary Medicine*. **3(2):** 94-103.

**World Fund for Nature WWF. (1993).** Vital wealth plants, Gland, Switzerland