FORMULATION AND EVALUATION OF A HERBAL ANTHELMINTIC PREPARATION FOR RUMINANT ANIMALS FROM A MIXTURE OF *ENTADA LEPTOSTACHYA AND PROSOPIS JULIFLORA*

David Kimani

A thesis submitted in partial fulfillment for the degree of Master of Science in Chemistry of Jomo Kenyatta University of Agriculture and Technology

2015

DECLARATION

This thesis is my original work and has not been presented in any university for an award of a degree.

Signature:……Date:……..

David Kimani (SC 331-0008/2008)

Declaration by supervisors

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

Signature:……Date:…….

Prof. Patrick Kareru,

J.K.U.A.T., Kenya

Signature:……Date:…….

2. **Prof. Gathu Nyagah**,

J.K.U.A.T., Kenya

Signature:……Date:……

3. **Dr. Francis Njonge**,

J.K.U.A.T., Kenya

Signature:……Date:……..

4. **Prof. Helen Kutima**,

J.K.U.A.T., Kenya

DEDICATION

I dedicate this work to God, my Lord and Savior and my first priority. He has built me on virtues of "work produced by faith", "labor prompted by love" and "endurance inspired by hope" (1 Thess. 1:1-3). I am not "self-made" and I owe everything that I am wholly to Him and His inspiration is constantly my strength. Secondly, to my wife Esther Kimani. She has had to endure the extremes of this long and difficult journey without complaining. She has supported my dream all through. Her prayers and encouragement meant a lot in this journey. My son Jeremy, who has taught me that nothing gets serious than it already is! He gave me the reason to work harder and faster. My daughter Deborah, who brought joy and promise in my life. She has taught me the virtue of God's promises come true. Her constant smile has brought this journey to an end, gracefully! Again, nothing gets serious than it already is! To my mother, Margaret Wachuka, for teaching me Godly endurance and constantly reminding me not to fall short of the finish line. My father, Fredrick Njaaga, my sisters, Christine Mutio and Elizabeth Njeri, for all the kind support and encouragement morally and materially.

ACKNOWLEDGEMENT

Firstly, honor and glory be to my Lord and Savior, Jesus Christ. He kept me alive through the journey and never looked away when I was utterly distressed and almost giving up. Thank you for providing miraculously and helping me support my dear family. Thank you for the strength You gave me at the point of caving in. I will never depart from You.

It is not enough to say thank you to my lead supervisor Prof. Patrick Kareru. He taught me to be independent-minded, generous with scientific knowledge to others and positive thinking. I will always be greatly indebted to you for your fatherly advice to come back to school and the financial offer you made when I almost lost my chance to do my exams and lose the entire year of study and hence, gave me another chance to be where I am today. God bless you. Prof. Gathu Nyagah, I can't forget the many teas and lunches you offered me with key interest to know how far I had gone in my laboratory work. I am forever indebted to you. Prof. Kutima for making my toxicity study a reality after you intervened and for the laboratory facilitation in the zoology department when you were at the helm of that department. Dr. Njonge, you always squeezed me whenever I lost the momentum and personally facilitated the initial *in vitro* work that propelled the whole study to where it is. God bless you all. I acknowledge the kind financial support of the Research, Production and Extension (RPE) division of Jomo Kenyatta University of Agriculture and Technology (J.K.U.A.T.) without which, this project would not have taken off.

My wife and kid, Esther and Jeremy, you saw a star in me and your unwavering love and support is indescribable. Jeremy, thank you for the night vigils! I now have the endurance of a horse! Deborah, thank you for learning my name fast! I needed such speed to be at the finish line. My dear mum, you never saw failure even when things were stuck but offered your weight behind every turn. I love you. Dad, your value for education and positive thinking never escaped me. Your virtues on life kept my thoughts

alive. My mother-in-law, Loise, your love and encouragement was vital. You never lost hope in me and always supported my course. My nephews Davy and Kiarie, sister and brother-in-law, Jane and Sammy-you journeyed with me. Liz (CTMDR, KEMRI), for encouraging me to go back to school and offering me her scripts to kick-off the journey. God bless you all.

I acknowledge the whole of chemistry department technical staff particularly, Mr. Mawili (Chief Technician), Mr. Tom Odera, Mr. Wafula, Mr. Njeru, Mr. Nderitu, Mr. Kung'u and Ms. Waithira. You extended long hours and odd days, making sure I was comfortable. Tom Odera, we have come a long way and you still baffle me with your resilience! Thank you for making the UV machine work and sacrificing your family time in order that I could get the UV results. The entire Zoology department technical staff, you did my 80%! I held you in the laboratory from Monday to Saturday till late and never complained a day. Your Godly humility is unmatched. I salute Mrs. Kiarugara, Evelyne, Eunice, Mary, Alice, Samuel and Christine for every great work you did for me including but not limited to helminth egg identification and dedicated facilitation during the egg-hatch inhibition assay and *in vivo* studies (toxicity and FECR test). I also acknowledge technical staff in the Botany department, Mr. Muthanga and Mr. Kamau, who helped me in plant sample identification and deposition and facilitation with the laboratory equipment our Chemistry laboratories didn't have. God bless you.

Lastly, to all my classmates who encouraged me when I came back to class very rusty and sleepy from my night shifts with my new-born son at the time! You were the best team I ever spent time with in a long time. My laboratory colleagues, Odhiambo, Magu, Mutembei and Waithaka, for taking me through the baby steps in the laboratory, Karanja for your journey with me and never quitting, Noor for your light-heartedness which lightened me and for every other person that walked me through this journey. God bless you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

EVM	Ethnoveterinary medicine
SHF	Small holder farmer
GIN	Gastrointestinal nematode
GIP	Gastrointestinal parasite
U.K.	United Kingdom
GDP	Gross domestic product
AR	Anthelmitic resistance
US$	United States dollar
FAO	Food and Agriculture Organization
OIE	Office Internationale des Epizooties
GIP	Gastrointestinal parasites
U.S.A.	United States of America
AADs	Amino-acetonitrile derivatives
EPG	Egg per gram
PCV	Packed cell volume
J.K.U.A.T.	Jomo Kenyatta University of Agriculture and Technology
WHO	World Health Organization
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
TLC	Thin layer chromatography
HT	Hydrolysable tannins
CT	Condensed tannins
PBS	Phosphate buffer saline
OECD	Organization for Economic Co-operation and Development
b.w.	Body weight
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
LBW	Live body weight

FECR	Feacal egg count reduction
nm	Nanometer

ABSTRACT

Helminthes infestation has for a long time been recognized as a major constraint to livestock production. Increasing anthelmintic resistance and the impact of conventional anthelmintics on the environment has led to increased interest on ethnobotanical approach to come up with new plant-based compounds. A number of plants have been investigated towards development of anthelmintic drugs. While some studies have provided confirmatory information on presence of anthelmintic properties in these plants, the bioactive elements in them have not been identified and formulated for commercial purposes. *Entada leptostachya* Harms (Mimosaceae), *Albizia anthelmintica* (Mimosaceae), and *Prosopis juliflora* (Mimosaceae) were investigated for anthelmintic activity and safety with the aim of formulating a cheaper, commercial herbal anthelmintic drug. The aqueous extracts of *E. leptostachya* (LC_{50}=0.323 mg/ml, 60.90±37.77%) and *P. juliflora* (LC_{50}=0.153 mg/ml, 73.81±41.42%) were found to be the most active using egg hatch inhibition assay. The extract mixture 1:7 (*E. leptostachya:P. juliflora*) showed the highest anthelmintic activity (LC_{50}=0.370, 59.33±43.34%) while ratio 2:1 showed the least activity (LC_{50}=2.052 mg/ml, 17.61±13.39%). Acute oral toxicity studies using OECD 425 guidelines to determine safety were performed on the ratio 1:7. The LD_{50} was estimated to be greater than 5000 mg/kg b.w. and the extract mixture determined to be non-toxic. The effectiveness and safety of the mixture ratio 1:7 was then determined using sheep by feacal egg count reduction assay. The extract mixture 1:7 generally showed a time-dependent but not dose-dependent *in vivo* anthelmintic activity. The 500mg/kg b.w. group gave the highest anthelmintic activity, giving a significant ($P<0.05$) maximum feacal egg count reduction of 84% on day 19 post-treatment while the group at 4500mg/kg b.w. gave the lowest reduction of -59% on day 19 post-treatment. Albendazole produced a maximum feacal egg count reduction of 51% on day 15 post-treatment, which was lower than that reported in most literature. The maximum FECR from the 500mg/kg b.w. group was considered biologically significant. The saponins in the mixture ratio 1:7 were quantified and IR and UV spectroscopic profiles determined. *E. leptostachya* gave the highest

percentage yield of 8.82%, followed by 1:7 mixture (5.11%) while *P. julifora* gave the least yield (4.41**%**). The FT-IR profile of *E. leptostachya*, *P. juliflora* and mixture 1:7 showed the presence of –OH, C-H, C=O, C=C, C=C-C, $RCOO^-$, Aryl-O and C-O-C functional groups. The UV absorbances (λ_{max}) ranging between 198.5 and 330.5 nm were recorded for *E. leptostachya*, *P. juliflora* and mixture 1:7. The findings of this study have confirmed that all the three plants were anthelmintically active. The formulated herbal preparation has also shown to be anthelmintically active, safe and on further determination, will likely be cheaper than the already available conventional synthetic drugs.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Background of the study

Diseases caused by helmintic parasites in livestock continue to be a major productivity constraint, especially in small ruminants in the tropics and subtropics. In the developed world, with exception of countries in the southern hemisphere, the greatest impact is found in the costs of control, mostly in the case of helminthes parasitoses while in the developing countries, the greatest impact is in the direct and potential productivity losses (Githiori, 2004).

Control of gastrointestinal nematode (GIN) parasitism is usually based on the use of chemical anthelmintics, whose effectiveness and consistent use has been limited by high levels of anthelmintic resistance and high cost of conventional, synthetic de-wormers, respectively. This has led to increasing popularity of herbal de-wormers for GIN control (Burke *et al.,* 2009; Zafar 2009).

1.1.1 Economic and social losses due to helminthes

Livestock are an important and integrated component of agricultural production system in developing countries. They are reared in production systems ranging from large-scale intensive commercial farms to traditional small-holder and village production systems (Satrija *et al.,* 2001). Small ruminants under intensive and extensive production systems are extremely susceptible to the effects of wide range of helminthes endoparasites (Abebe and Esayas, 2001). Helminthiasis has been shown to be one of the most common setbacks in production and reproductive performance of livestock (Dawo and Tibo, 2005; Váraday *et al.*, 2005; Agaie and Onyeyili, 2007;). Most of these effects go unnoticed because of sub-clinical or chronic nature of the diseases they cause unless the parasites caused death of the animal (Dawo and Tibo, 2005). Their impacts are characterized by lower outputs of animal products such as meat, milk, low quality hides and skins, manure and traction, which negatively impact on the livelihood of small

holder farmers (SHF). The economic impact lies not only in direct losses due to mortality but also indirect internal losses due to weaknesses, loss of appetite, decreased feed efficiency, reduced weight gain and decreased productivity (Váraday *et al.*, 2005; Agaie and Onyeyili, 2007). The greatest losses associated with nematode parasite infections are sub-clinical, and economic assessments show that financial costs of internal parasitism are enormous. A highly pathogenic nematode, *Haemonchus contortus,* is capable of causing acute disease and high mortality in all classes of stock (Githiori, 2004; Eguale *et al.*, 2006) and is the most prevalent and economically important (Kaplan *et al.*, 2003).

Over $4 billion is lost in animal productivity due to animal diseases, with over half of that loss being due to internal parasites such as helminthes with approximately over $1.7 billion being spent annually worldwide in control measures (Lanusse and Prichard, 1993). The total economic loss to the Kenya agricultural sector due to haemonchosis in small ruminants was estimated at US$ 26 million per year (Kareru, 2008); losses due to helminthes parasites constitute 11.8% of the total slaughter for cattle and 46.0% for sheep and goats (Olorunfemi *et al.*, 2006). In U.K., intestinal worms constitute the most important disease-related cost of sheep farming, leading to an annual loss of £83 million (Behnke *et al.*, 2008). In tropical countries, gastrointestinal nematodes are responsible for a 23-63% reduction in growth, up to 25% of pre-weaning mortality and 24-47% of reductions in annual off take from small ruminants (Kumsa *et al.*, 2010).

Livestock is a major source of food and direct income from sale of the animals and animal products like meat, milk, wool, hides and skins. Although livestock industry in Kenya contributes only 10% to the GDP, improved production can be realized with appropriate disease control. Goats are popular animals bred across Kenya by various communities. They are the second most popular livestock species in the Rift Valley area of Oromia, Ethiopia (Dawo and Tibbo, 2005). Livestock has traditionally been considered as a source of wealth to the pastoral communities in Kenya. Loss of livestock due to helminthes and helminthes-related ailments has contributed to community

conflicts as they try to restock their lost livestock. As a direct consequence of these losses, the family fabrics are weakened due to reduced income and food.

1.1.2 Anthelmintic resistance, availability and cost of conventional de-wormers

Anthelmintic resistance is the decrease in the efficacy of an anthelmintic against a population of parasites that is generally susceptible to that drug. The problem of anthelmintic resistance (AR) is world wide and has been reported in South Africa, Australia, New Zealand, Malaysia, Spain, France, Denmark, U.K., Brazil, and the United States of America. The earliest documentation of AR was to phenothiazine in 1957 followed by thiobendazole in 1964 (Fleming *et al.*, 2006). A recent survey carried out by FAO and the Office Internationale des Epizooties (OIE) in 77 out of 151 OIE member countries, revealed that over 50 per cent of countries are affected by parasite resistance (FAO, 2011).

High cost of modern anthelmintics and unavailability (or erratic availability patterns) to rural farmers has also limited the effective control of helminthes (Agaie and Onyeyili, 2007). Additionally, widespread and intensive use of sometimes low quality anthelmintics (Matthee, 2003; Githiori, 2004; Mohammed *et al.*, 2005) has led to resistance, leading to reduction in effectiveness of available anthelmintics (Matthee, 2003; Mohammed *et al.*, 2005; Maurer *et al.*, 2006). Chemical anthelmintics are also reported to be toxic and can pose side effects to the animal and the administrator (Kareru, 2008; Nalule *et al.*, 2011). Administration of chemical anthelmintics causes their existence as food residues and also leads to environmental pollution (Lalchhandama, 2009; Nalule *et al.*, 2011). In Kenya, adulteration of anthelmintics has become common practice. This class of anthelmintics has inadequate amounts of active ingredients which encourage anthelmintic resistance (Githiori, 2004). Emergence of resistant strains of pathogenic helminthes is an additional constraint to effectiveness of anthelmintics (Mohammed *et al.*, 2005). Gastrointestinal nematodes (GIN) of small ruminants have a number of genetic characteristics that promote the development of anthelmintic resistance. These helminthes have the genetic potential to respond rapidly

and successfully to chemical attack and the means to ensure dissemination of their resistant genes by host movement from farm to farm (Fleming *et al.*, 2006).

Various communities in Kenya have adopted low cost alternatives that are rarely documented in ethnobiological studies (Njoroge and Bussmann, 2006). Some of the plant species in frequent use in Kenya include *Asteraceae* and *Lamiaceae* plant families, *Synadenium compactum, Solanecio manii, Senna didymobotrya, Terminalia brownie, Vernonia lasiopus, Albizia anthelmintica* and *Tithonia diversifolia* (Njoroge and Bussmann, 2006; Kareru, 2008).

1.1.3 Helminth control

Besides use of anthelmintics, gastrointestinal helminthes are controlled in combination with credible farm management. In the developing world, this is impractical due to the high cost of the synthetic anthelmintics and expensive farm management procedures (Satrija *et al.*, 2001). As laid out by Padgham (2005), understanding parasite life cycles, pasture management, proper animal nutrition, breeding for parasite resistance and use of natural parasite controls are some of the other ways of maintaining control over ruminant parasites. Other broad approaches may include biological control, reduced frequency of anthelmintic treatments, parasite vaccines, and use of plants with anti-parasitic properties (Githiori, 2004).

Unmanageable load of helminthes may cause loss of immune function, decreased productivity and death in an infected animal. Young animals, whose immune system are not well developed, are more susceptible. Adult animals under stress or whose immunity has been compromised by disease, poor nutrition or other factors may also be affected by parasites. The way the soil, manure, feedstuffs and pasture are managed, may dictate the load of parasites such animals are exposed to. For example, most sheep graze close to the ground where the highest load of helminthes larvae exist as the larvae often don't climb higher than 5 inches from the ground. This puts them at high risk of endoparasite infection (Padgham, 2005).

1.1.4 Helminth chemotherapy

Chemotherapy has, for a long time, remained to be the primary strategy of helminthes control. In Nigeria, it is the major control measure against helminthiasis. Current strategies outside Africa involve reducing the level of pasture contamination through anthelmintic treatments and/or controlled grazing. In Africa, these methods are limited by the high cost of synthetic anthelmintics, their erratic availability, increased drug resistance and limited scope in many communal pastoral systems of grazing (Olorunfemi *et al.,* 2006). These constraints arising from the reliance on synthetic anthelmintics in most developing countries have presented difficulties in the management of GI parasite infections, calling for novel alternatives of helminthes control. As a result, pastoralists and SHFs continue to use indigenous plants as livestock de-wormers drawn from centuries of traditional beliefs. These herbal preparations are much cheaper and readily available than synthetic drugs and have been used for a long time by pastoralists and SHFs for treatment of livestock against helminthes parasites (Githiori, 2004). The most commonly used synthetic anthelmintic drugs in Kenya are Mebendazole (**1**), Levamisole (**2**) and Albendazole (**3**) (Kareru, 2008) as shown in **Figure 1.1**.

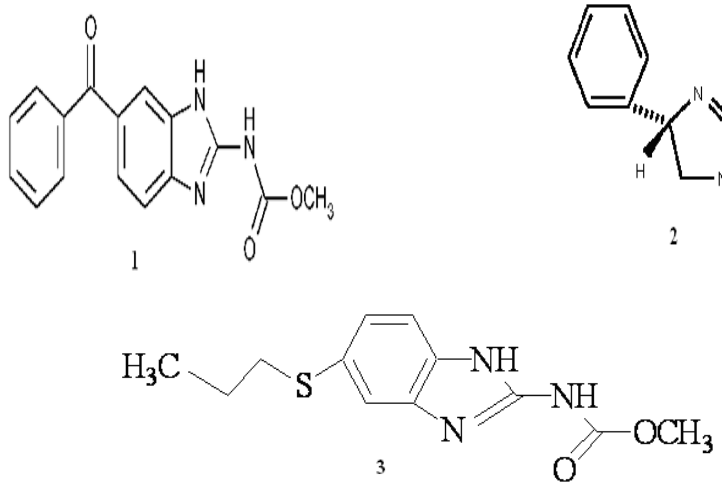


Figure 1.1: Structural representation of commonly used synthetic anthelmintics: Mebendazole (1), Levamisole (2), Albendazole (3) (Kareru, 2008)

1.1.5 Classes of synthetic anthelmintics and their resistance history

In the past 25 years, no new classes of anthelmintics have been developed for use in animals given the limited economic potential of small ruminant animals. Currently, there are 3 classes of

synthetic anthelmintics, each having different mode of action on the target nematodes (Fleming *et al.,* 2006; Behnke *et al.,* 2008):

i. Benzimidazoles (albendazole, fenbendazole)-group 1 anthelmintics,

ii. Cholinergic agonists (levamisole/morantel)-group 2 anthelmintics,

iii. Macrocyclic lactones or avermectins and milbemycins (ivermectin, moxidectin)-group 3 anthelmintics.

Benzimidazoles were introduced in the early 1960s but resistance to thiobendazole was detected only after 4 years of usage in the U.S.A. (Kaplan *et al.*, 2003; Behnke *et al.,* 2008). Group 2 anthelmintics were introduced in the early 1970s and resistance detected for the first time in 1977 in Australia. Group 3 anthelmintics were licensed in the early 1980s and resistance realized only after 7 years in South Africa. Although novel synthetic drugs seem to be coming up (such as nitazoxanide, cyclic depsipeptides, octadepsipeptides (for example emodepside), tribendimidine, diketopiperazines (for example paraherquamides), amino-acetonitrile derivatives (AADs)), no new drugs that operate through a different mode of action to that offered by the three present classes of anthelmintics have been developed (Behnke *et al.,* 2008).

In the United States, resistance to all the three classes of anthelmintics has been reported (Váraday *et al.*, 2005; Fleming *et al.,* 2006). Gastrointestinal nematodes of small ruminants include *Haemonchus contortus, Telodorsagia circumsita, Trichostrongylus axei, Nematodirus spp,* and *Cooperia spp.* Anthelmintic resistance is present in all of these parasites but prevalence is high for *Haemonchus contortus* (Fleming *et al.,* 2006).

1.1.6 Life cycle of Helminthes

The life-cycle of helminthes may be direct or indirect. The indirect cycle involves two or more hosts: a definitive/final host and an intermediate host. For instance, the Liver Fluke

spends part of its life cycle in certain snail intermediate host species before infecting the definitive ruminant hosts. The direct life cycle involves only one final host. This study was interested in those nematodes within the GI tract. **Figure 1.2** shows the direct cycle.

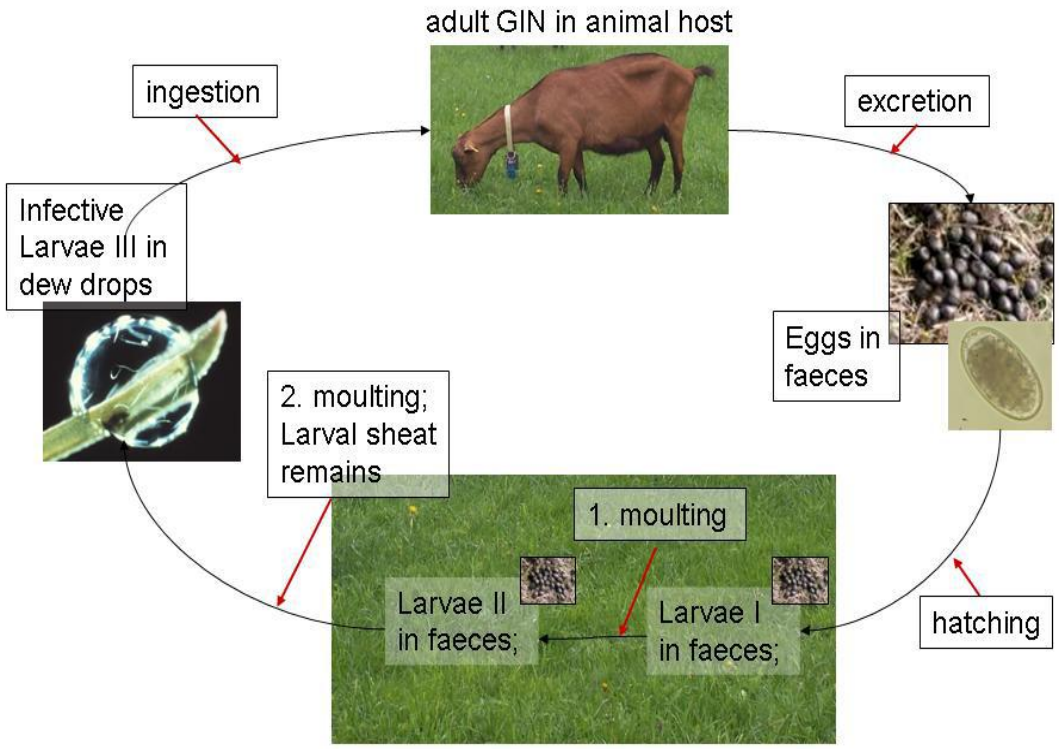


Figure 1.2: General life cycle of gastrointestinal nematodes of small ruminants (Scheuerle, 2009)

Parasite-infested animal (the host) harbors adult worms. The eggs produced by the female worm are deposited in pastures with feacal matter where they develop into various larvae stages. A host animal is contaminated by ingesting L_3 larvae (infective stage) with the grass. The larvae ex-sheath and make their way to the gastrointestinal

tract where they develop to L_4 stage and proceed to produce a new generation of adult male or female parasites (Scheuerle, 2009).

The major classes of worms include **round worms** (*Nemathelminthes strongyles*) and **flatworms** (*Platyhelminthes*). **Table 1.1** shows the general characteristics of main internal parasites found in cattle, sheep and goats.

Table 1.1: Characteristics of main internal parasites in cattle, sheep and goats (Andrews, 1969)

Parasite	**Description**	**Infected Organ**	**Life Cycle**	**Symptoms**
Haemonchus contortus	M: 10-20 mm red F: 18-30 mm red and white	Abomasum	IS: 4-6 days PP: 3 weeks	Anaemia, soft swelling under jaw and abdomen, weakness, no weight gain
Ostertagia circumcinta	M: 6-9 mm, brown F: 8-12 mm	Abomasum	IS: 4-6 days PP: 3 weeks	Same as *Haemonchus* and also lack of appetite, diarrhea
Trichostrongylus	M: 4-5.5 mm F: 5-7 mm light brown	Abomasum, duodenum	IS: 3-4 days PP: 2-3 weeks	Same as *Haemonchus* and also diarrhea and weight loss
Cooperia spp	red M: 5-7 mm F: 6-9 mm	Duodenum	IS: 5-6 days PP: 15-20 days	Same as *Haemonchus*
Bunostomum	10-30 mm	Duodenum	IS:? PP: 30-56 days	Edema, anaemia, weight loss, diarrhea
Strongyloides papillosus (young animals)	4-6 mm	Small intestine	IS: 1-2 days PP: 8-14 days	Anorexia, enteritis, diarrhea

Chabertia	M: 13-14 mm F: 17-20 mm	Large intestine	IS: 5-6 days PP: 42 days	Anemia, diarrhea with blood
Oesophagostomum columbianum	M: 12-17 mm F: 15-22 mm	Large intestine	IS: 6-7 days PP: 41-45 days	Dark green diarrhea, edema
Protostrongylus	M: 16-28 mm F: 25-35 mm	Lungs	IS: 12-14 days PP: 30-37 days	Pneumonia
Dictyocaulus	M: 30-80 mm F: 50-100 mm	Lungs	IS: 6-7 days PP: 3-4 weeks	Sticky nasal discharge, difficulty breathing, cough

Key: **M**=Males; **F**=Females; **IS**=Infectious stage i.e. Minimum number of days for parasite to reach infectious stage (L_3) after hatching of eggs; **PP**=Prepatent stage i.e. period up to appearance of first eggs in dung after host is infected.

1.2 Literature review

1.2.1 Ethnobotanical research and ethnoveterinary practices

Traditional medicine has a long history. World Health organization (WHO) has estimated that approximately 80% of the world's population depends on traditional medicines for meeting their primary health care needs (Deore and Khadabadi, 2010). WHO (2000) describes traditional medicine as the "sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness." Long historical use of many practices of traditional medicine, including experience passed on from generation to generation, has demonstrated the safety and efficacy of traditional medicine. However, scientific research is needed to provide additional evidence of its safety and efficacy (WHO, 2000). The most effective approach to obtain scientific

evaluation is the ethnobotanical approach, which assumes that indigenous uses of plants indicate the presence of biologically active compounds in the plants under study. The largest challenge in use of herbal medicine has been the lack of scientific evaluation (Salawu *et al.*, 2008; Sujon *et al.*, 2008).

In recent times, there has been an increasing interest in ethnomedical and ethnoveterinary practices globally in treating various ailments. In the developed world, this is in response to production of animals free from industrial chemical inputs and the need to discover new therapeutic substances of natural origin with potentially low toxicity to both humans and animals. Traditional veterinary practices are used in most developing countries as an integral part of people's culture and their use is not likely to significantly change to a significant degree in years to come (Komen *et al.*, 2005).

Helminth control programs, based on improvement of farm management and regular anthelmintic treatment, are often unrealistic in developing countries due to relatively high price of modern anthelmintics for SHF (Satrija *et al.*, 2001; Maurer *et al.*, 2006; Dawo and Tibbo, 2005), substantial levels of anthelmintic resistance (Githiori *et al.*, 2003), inadequate supply or unavailability, environmental pollution and food residues (Dawo and Tibbo, 2005). As a result of these challenges, pastoralists and SHF have continued to use indigenous plants as livestock dewormers drawn from traditional belief and use of EVM. These herbal preparations are much cheaper and readily available than the synthetic drugs (Githiori, 2004). In Nigeria, herbal treatment of helminthiasis is widely practiced by herbalists and nomadic Fulanis, who are the largest cattle rearers in Nigeria (Mohammed *et al.*, 2005).

The development of herbal anthelmintics can be initiated with collection of information concerning species of plants which have been used to treat parasites. An ethnoveterinary medicine survey involving questionnaire and/or interview with farmers who hold the information on traditional animal health care could be the most effective manner of revealing their beliefs, experiences and insights on parasite control (Satrija *et al.*, 2001).

Njoroge and Bussmann (2006) used semi-structured questionnaires and detailed discussions with SHFs and found out that 40 plant species in 26 families were useful in traditional management of various cattle ailments in the Central region in Kenya. The usage of some of these species is unfortunately unsustainable as some of the species are rare or endangered hence, the need for conservation measures to be taken. In Zimbabwe, ethnoveterinary medicine is gaining widespread recognition at the expense of conventional drugs especially because of its greater accessibility, lower costs and apparent effectiveness (Njoroge and Bussmann, 2006).

1.2.2 Anthelmintic bioactive principles

Plants and their derivatives have played a key role in universal health and have for a long time been known to possess biological activity (Falodun *et al.*, 2007; Khan *et al.*, 2010). Actually, 30% of all modern drugs are derived from plants (Khan *et al.*, 2010). Some of the problems deterring pharmaceutical companies from exploiting plant-derived medicines include variability in the concentration of active principles in relation to how the plants were grown, climate, soil quality, site (location), and season of the year (Behnke *et al.*, 2008). Tannins, which are naturally occurring secondary metabolites in plants, have the ability to reduce worm burdens (FAO, 2011). Recent explorations of members of the genus *Acacia* documented that they are generally rich in saponins and tannins. Two triterpenoid saponins, acaciaside A and B, isolated from *A. auriculiformis* have been found to possess significant activities against nematodes and cestodes. The mechanism is believed to be through destruction of the cell membranes by inducing peroxidation, increasing energy metabolism and inhibiting glucose uptake of the parasites (Lalchhandama, 2009). Condensed tannin-containing forages have the potential to help control anthelmintic-resistant gastrointestinal parasites. They have been shown to decrease fecal egg counts in sheep and goats and may decrease hatch rate and larval development in feaces. This reduces pasture contamination and ingestion of infective larvae and these may provide adequate control of gastrointestinal parasites (Min and Hart, 2003).

Transcuticular diffusion is a common means of entry into helminthes parasites for non-nutrient and non-electrolyte substances in nematodes and this route is predominant for uptake of major broad-spectrum anthelmintics such as Albendazole as opposed to oral ingestion (Eguale *et al.*, 2007; Keiser *et al.*, 2012). Condensed tannins can impair vital processes such as feeding and reproduction of the parasite or may bind and disrupt the integrity of the parasite's cuticle (Dave *et al.*, 2009; Zafar *et al.*, 2009). Tannins may also induce physiological changes in the gut of the host, resulting in secretion of mucous and chemicals harmful to the parasite (Min and Hart, 2003; Zafar *et al.*, 2009). The immune response to nematode parasites through rumen by-pass of proteins from feed intake (Zafar *et al.*, 2002; Min and Hart, 2003; Suleiman *et al.*, 2005) has shown to reduce reproductive activity of gastrointestinal parasites (Waghorn *et al.*, 1995; Athanasiadou *et al.*, 2000a,b; Suleiman *et al.*, 2005). It is reported that monodesmoside saponins have shown to destabilize membranes and increase cell permeability by combining with membrane-associated sterols (Geidam *et al.*, 2007; Ademola *et al.*, 2008; Ademola and Eloff, 2010) and producing changes in cell morphology leading to cytolysis (Geidam *et al.*, 2007). The hemolytic action of saponins is believed to be the result of the affinity of the aglycone moiety for membrane sterols, particularly cholesterol and this increases with increase in number of polar groups in the aglycone moiety (Becker *et al.*, 2002). Triterpenoid saponins, acaciaside A and B isolated from *Acacia auriculiformis*, were reported to cause destruction of cell membranes of nematodes and cestodes by inducing peroxidation, increasing energy metabolism and inhibiting glucose uptake of the parasites (Lalchhandama, 2009). Alkaloids may improve tonicity of the gastrointestinal tract and thus expel the worms or may have a direct effect on the nervous system of the nematodes (Ademola *et al.*, 2008; Lateef *et al.*, 2003). Albendazole works by interference with polymerization of microtubule, where the drug binds to the protein tubulin of the parasite leading to death by starvation (Kareru *et al.*, 2012; Lalchhandama, 2009). The other phytochemicals in the two plants like flavonoids and oleanane type triterpenes may have their independent or synergistic anthelmintic effects (Zafar *et al.*, 2009).

1.2.2.1 Saponins

Saponins get their name from the soapwort plant, whose root was traditionally used as soap. Saponins are glycosides of triterpenes (**a**), steroids (**b**), and sometimes alkaloids and occur primarily, but not exclusively in plants. They dissolve in water to form a soapy froth. The aglycone part of a saponin is referred to as a sapogenin while the glycone parts of the saponins are generally oligosaccharides.

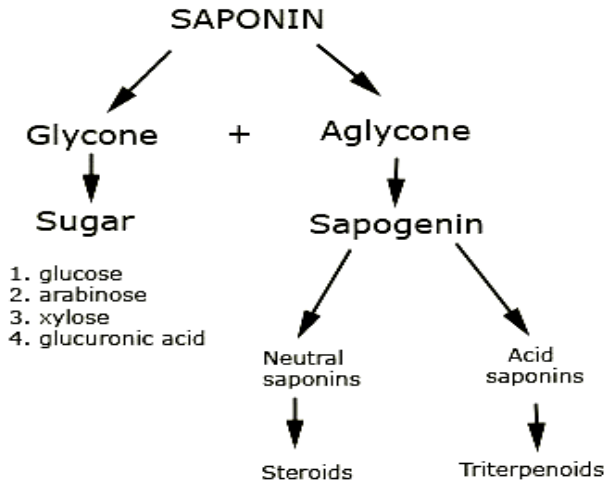


Figure 1.3: Composition and categorization of a saponin molecule

Oligosaccharides may be linked to the sapogenin through an ether or ester linkage at one or two glycosylation sites, giving a monodesmosidic and bidesmosidic saponin respectively. Many plants contain little or no saponin while in others, the triterpenoid saponins predominate. The base structure found in the largest variety of medicinal plants is the oleanane-type triterpene (Kareru, 2008).

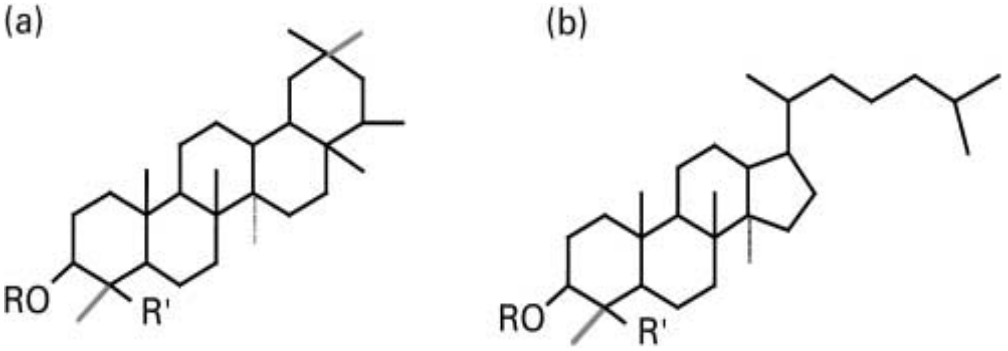
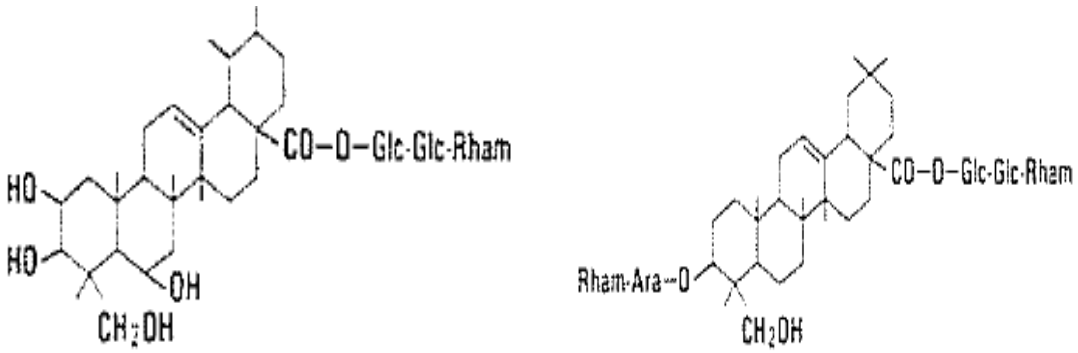


Figure 1.4: Basic structures of sapogenins: (a) triterpenoid and (b) steroid

Figure 1.5 represents glycosidic linkages through an ester linkage (**A**) and one ester and ether linkages in **B** to form a monodesmosidic triterpenoid saponin **A** and bidesmosidic triterpenoid saponin **B**. Saponins are reported to have anthelmintic property. Saponins were first reported to kill worms as early as 1962 using an extract from *Albizia anthelmintica* (Kareru, 2008).



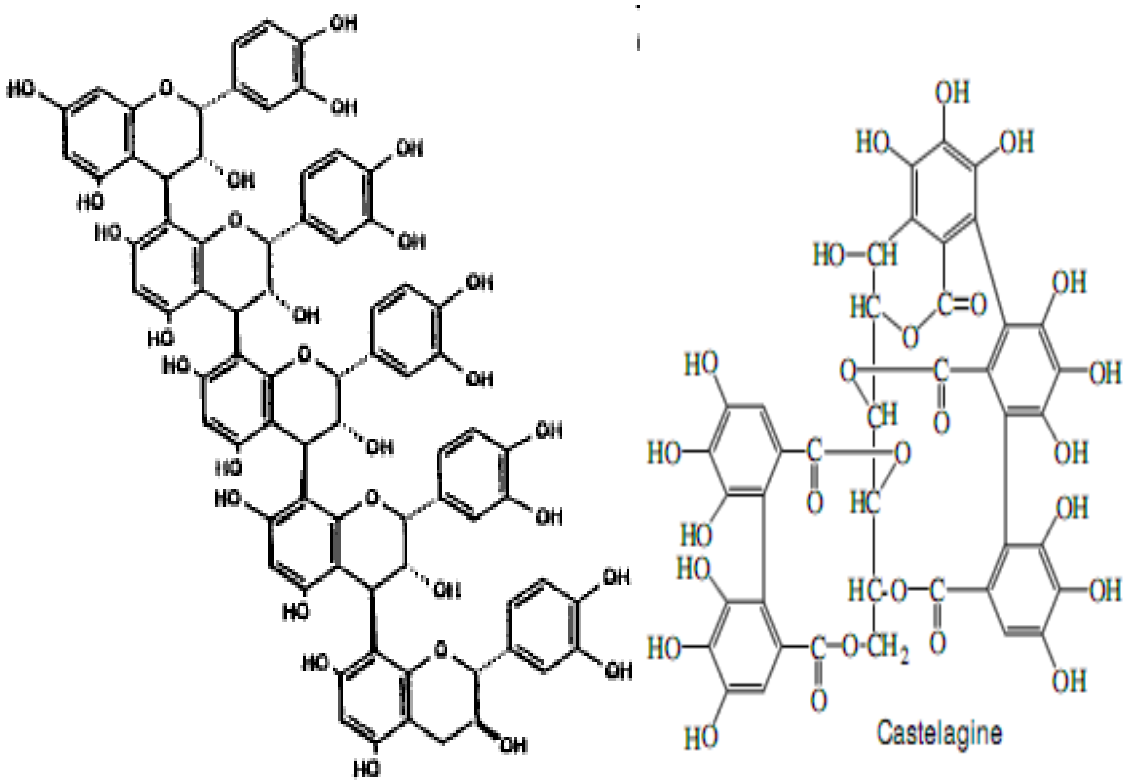
A-Madecassoside **B-Hederacoside**

Figure 1.5: Typical examples of monodesmosidic (A) and bidesmosidic (B) triterpenoid saponins: Glc=glucose, Rham=rhamnose, Ara=arabinose (Burnouf-Radosevich and Delfel, 1986)

1.2.2.2 Tannins

These are naturally occurring polyphenolic compounds of high molecular weight enough to form complexes with proteins. They are classified into two groups (typical examples in **figure 1.6**) based on their structural types: a) *hydrolysable tannins* (HT), and b) *condensed tannins* (CT) (Joint FAO/IAEA, 2000). Hydrolysable tannins are polymers esterified to a core molecule, commonly glucose or a polyphenol such as catechin. HTs are potentially toxic to ruminants. CTs are polymeric flavonoids (polyphenols) (Kareru, 2008). They are oligomers and polymers of polyhydroxyflavan-3-ol units (Umezawa, 2001). CTs are the most common types of tannins found in forage legumes, trees and shrubs (Min and Hart, 2003). Condensed tannins (proanthocyanidins) are relatively stable in the digestive tract of the animal and rarely have toxic effects (Githiori, 2004).

In spite of their difference in the basic structures, hydrolysable and condensed tannins have a similarity in that they have many phenolic units and therefore are often called plant polyphenols. Tannins have antioxidant, radical-scavenging, biological and pharmacological activities (Umezawa, 2001). High concentrations of CTs are reported to have anthelmintic activity against nematode parasites (Kareru, 2008). A new class of tannins, complex tannins, occurs widely in plants containing both condensed and hydrolysable tannins. They have shown to contain, in the molecules, a hydrolysable tannin moiety connected through a carbon-carbon linkage to flavan-3-ol (flavano-ellagitannin), procyanidin (procyanidino-ellagitannin) and flavonoid glucoside (flavono-ellagitannin) moieties and their classification has been based on their structural, rather than chemical properties (Nonaka, 1989).



C-Arecatannin **D-Castelagine**

Figure 1.6: Typical examples of CT (C) and HT (D)

While doing a study on the anthelmintic activity of the leaves of *Erythrina indica* against earth worms, Jesupillai and Palanivelu (2009) concluded that tannins produced anthelmintic activity by binding to free protein of the gastrointestinal tract of the host animal or the glycoprotein on the cuticle of the parasite. Research has also showed that inclusion in the diet of the condensed tannins in Quebracho extract reduces egg output and worm burden in sheep infected with *Trichostrongylus colubriformis*. Studies suggested that quebracho tannin was acting through a direct toxic effect against the nematodes (FAO, 2011).

Anthelmintic effect of condensed tannins (CT) against *Haemonchus contortus* in sheep has been evidenced by inhibited egg hatching while reduction in feacal egg count in sheep is through improved nutrient utilization (Zafar *et al.*, 2007). In another study on

the effect of condensed tannins in cassava hay, Netpana *et al.* (2001) concluded that feeding a supplement of cassava hay containing moderate levels of condensed tannins has the effect of reducing nematode egg counts in both cattle and buffaloes. A most recent research has led to formulation of an effective animal feed using *Lespedeza cuneata*, which has been patented in the U.S.A. The plant has been reported to contain 52 grams of CTs per kilogram of forage though the content varies with environmental conditions (Kareru, 2008).

Plant CT may have direct or indirect effects on gastrointestinal parasites: a) directly by mediation through CT-nematode interactions, affecting physiological functions of GIP. Condensed tannins extracted from various forages can markedly decrease the viability of the larval stages of several nematodes in sheep and goats. CT may also react directly by interfering with parasite egg hatching and development to infective stage larvae, and b) indirectly by improving protein nutrition by binding to plant proteins in the rumen and preventing microbial degradation, hence increasing amino acid flow to the duodenum. Several sheep studies have shown that improved protein nutrition decreases parasite infestation (Min and Hart, 2003; Zafar *et al.*, 2007).

1.2.3 Testing of anthelmintic activity of herbal plants

This is usually done to determine the anthelmintic activity and hence, the efficacy. This is done *in vitro* or *in vivo*. *In vitro* tests involve culturing anthelmintic eggs/larvae/worms from ruminant feaces either at room temperature or in an incubator. The eggs/larvae/worms are then inoculated with herbal extracts and eggs inhibited from hatching and dead larvae/worms are monitored with time (Zafar *et al.*, 2007; Kareru, 2008; Sujon *et al.*, 2008; Jesupillai and Palanivelu, 2009; Deore and Khadabadi, 2010). *In vivo* tests involve inoculation of test animals with pre-determined number of nematode eggs of a specific species (infection) and then feeding/treating the ruminant animal such as sheep with herbal extract followed by monitoring helminthes eggs in the animal feaces over time after administration. The experimental animals may also be left to aquire the nematode eggs naturally. Reduction of feacal egg counts with time is an

indication of *in vivo* anthelmintic activity (Githiori, 2004; Agaie and Onyeyili, 2007; Dawo and Tibbo, 2005; Krimpen *et al.*, 2008; Burke *et al.*, 2009; Deore and Khadabadi, 2010).

In vitro (biochemical or cellular) safety data is an indicator of potential toxicity but not an absolute marker and usually serves to verify the reported mechanism of action in animals or humans. On the other hand, *in vivo* data from animal studies are more indicative of toxicity and may be considered to be safety markers (WHO, 2000).

Such studies on numerous herbal plants have been reported. *In vitro* anthelmintic activity study on *Terminalia arjuna* bark showed a dose dependent anthelmintic activity against *Haemonchus contortus* ova with an LC_{50} value of 0.646 mg/ml (Zafar *et al.*, 2009). A similar trend was observed with the *in vitro* anthelmintic activity of ethanolic plant extracts from Northern Cameroon on inhibition of *Haemonchus contortus* eggs (Monglo *et al.*, 2006). *Combretum molle* acetone extract (LC_{50}=0.866mg/ml) and its fractions inhibited hatching of *Haemonchus contortus* eggs in a concentration-dependent manner where the chloroform fraction was significantly more active than the other fractions ($P<0.05$) (Ademola and Eloff, 2010). Crude aqueous and hydroalcoholic extracts of the leaves of *Chenopodium ambrosioides, Lawsonia inermis* and seeds of *Jatropha curcas* were studied for their possible anthelmintic activity against egg hatchability and mortality of adult *Haemonchus contortus*. Both *C. ambrosioides* and *J. curcas* inhibited the hatching of the eggs at a concentration of $\leq$ 2mg/ml while activity of *L. inermis* was not dose-dependent and did not inhibit hatching of eggs of *H. contortus* significantly at all tested concentrations (Eguale and Giday, 2009). *Aframomum danielli* hexane extract (yielding alkaloids, cardiac glycosides and glycosides) has demonstrated lower anthelmintic activity (LC_{50}=0.39mg/ml) compared to its ethanolic extract (LC_{50}=0.033mg/ml) (yielding alkaloids, saponins, cardiac glycosides, steroids and glycosides) using larval survival assay. The anthelmintic activity of this plant was attributed to presence of saponins. The possible mechanism was attributed to destabilization of the membrane, increasing cell permeability by

combining with membrane associated sterols leading to loss of osmotic control and causing cytolysis (Ademola *et al.*, 2008).

Several other studies have been done to characterize other plants believed to have anthelmintic activity. On the basis of saponin anthelmintic activity, Deore and Khadabadi (2010) undertook a study to prove the efficacy of *C. borivilianum* root tuber against selected worms. From the results, which confirmed presence of saponins from the TLC analysis of the crude extracts, they concluded that the tubers could be used as anthelmintics and further led to confirm that the anthelmintic activity of *C. borivilianum* was due to the presence of saponins. The values of time of paralysis and time of death of methanolic extracts confirmed anthelmintic activity of *Amorphophallus campanulatus.* The methanolic extract tested positive for saponins and tannins amongst other phytochemicals (Ramalingam *et al.*, 2010).

Some of the phytochemicals known to be active against gastro-intestinal nematodes include polythienyls, isothiocyanates, glucosinolates, cyanogenic glycosides, polyacetylenes, alkaloids, lipids, terpenoids, sesquiterpenoids, diterpenoids, quassinoids, triterpenoids, simple and complex phenolics (Chitwood, 2002), thymol (Lateef *et al.*, 2006), steroids (Badmanaban and Patel, 2010), saponins (Kareru, 2008), tannins (FAO, 2011; Githiori, 2004; Ramalingam *et al.*, 2010) amongst other classes of phytochemicals. Direct and indirect mechanisms behind anthelmintic effect of plants containing tannins actually depends on the type and content of tannins in the plant (Suleiman *et al.*, 2005). These may target viability of nematode eggs, survival or growth of larvae and/or fecundity of adult worms (Waghorn *et al.*, 1995; Athanasiadou *et al.*, 2000a,b; Min and Hart, 2003).

1.2.4 Toxicity testing of plant extracts

Herbal formulations as medicinal alternatives have continued to receive increased attention because of strong belief that these products are safe. It is this assumption, to a larger extent, that has influenced the indiscriminate use of these herbal formulations

leading to incidences of adverse effects and sometimes life-threatening conditions (Agbaje *et al.*, 2009; Mbaka *et al.*, 2010). The ethnomedical use of any plant for medicinal purposes does not warrant its safety, especially with regard to mutagenicity, carcinogenicity, embryotoxicity, nephrotoxicity and hepatotoxicity, where the effects are rather complex and not easily recognized by the local populations (Afolayan *et al.*, 2009).

Toxicity studies are conducted for safety assessment and to determine the possible adverse effects of a test substance. In the context of this study, OECD guideline Up-and-Down procedure (UDP) provides a more humanely acceptable animal use protocol. The Up-and-Down procedure (OECD 425 guideline) uses impending death as the only end-point and uses far less test animals than most guidelines (OECD, 2000). Organization of Economic Cooperation and Development's (OECD's) UDP uses a maximum of six animals in dose administration of test substances in a sequential manner after survival of previously dosed animal has been assured. Acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of test substance, or multiple doses given within 24 hours (OECD, 2001).

Toxicity testing has relied almost exclusively on the use of whole animal systems such as experimental rodents such as mice and rats to analyze pathological effects of chemical exposures, to elucidate mechanisms of action and to ascertain hazards to human and animal health. However, *in vitro* protocols have gained increasing popularity because of their better mechanistic investigations and lower costs and time of doing these experiments. These experiments fall into four categories: a) microorganism systems, b) mammalian cell culture systems, c) tissue preparations, and d) organ cultures (Tardiff, 1978). The animal tests investigate the effect of a test substance on the various body systems such as respiratory, nervous, and cardiovascular systems (Wamburu *et al.*, 2013).

Studies have been done using similar and other guidelines to establish toxicity of plant extracts in rat or mice models. Acute oral toxicity testing of *Hunteria umbellate* was done using the Up-and-Down procedure (OECD guideline 425).The study showed that the plant extract had an LD_{50} of 1020 mg/kg and was therefore slightly toxic (Adeyemi and Adeneye, 2009). Akanmu *et al.*, (2009) investigated acute and sub-acute oral toxicity of methanolic extract of *Bauhinia monandra* (used for treatment of diabetes). Acute administration of the extract up to a dose of 8000 mg/kg b.w. did not cause any deaths or any toxicity signs. The study concluded that *B. monandra* may possess relatively low toxicity. Acute toxicity study of the leaves of *Sphenocentrum jollyanum* showed no toxicity when administered up to 11000 mg/kg b.w. orally while intra-peritoneal (IP) administration produced dose dependent mortality with an LD_{50} of 1445.4 mg/kg b.w.. The results suggested that the leaves extract was potentially safe for oral consumption (Mbaka *et al.,* 2010). Acute and sub-acute toxicity of 95% ethanolic extract of aerial parts of *Cansjera rheedii* J. Gmelin (Opiliaceae) was evaluated in Swiss mice and Wistar albino rats. The acute toxicity study was conducted following the OECD 420 guideline where a limit test dose of 2000 mg/kg b.w. was used. No significant changes in the organ weights between the control and treatment groups were observed nor were there any gross pathological and histopathological changes observed after 28 days. There were no mortalities during the entire treatment period. In conclusion, the study presented strong evidence of non-toxic effects of the ethanol extract of *C. rheedii* and the extract was considered safe and could be extensively used (Mounnissamy *et al.*, 2010). Acute toxicity evaluation of an herbal extract mixture containing *Withinia somnifera*, *Tribulus terrestris*, *Mucuna pruriens* and *Argyreia speciosa* showed the combination product was safe up to the dose of 5000mg/kg b.w. on mice (LD_{50}>5000 mg/kg b.w.) within a 24 hours period (Khan *et al.,* 2010). Acute oral toxicity study of aqueous extract of *Artemisia afra* was done using the OECD 425 guidelines by Mukinda (2005). The estimated LD_{50} was >5000 mg/kg b.w. for both female and male mice. Fifty percent less animals were used using this guideline for both

oral and intraperitoneal administrations compared to the Litchfield and Wilcoxon protocol.

1.2.5 Ethnobotanical information on test plants reported to have anthelmintic activity

1.2.5.1 *Entada leptostachya* Harms (Mimosaceae)

Entada leptostachya is from *Fabaceae* family, genus *Entada.* It is a climbing shrub or tree, 3-10m tall. The leaves are stipulate, nearly always alternate. The flowers are usually bisexual. The plant is found in several parts of Kenya (Machakos, Embu and Mbeere districts) and other countries of Africa such as Somalia, Ethiopia and Tanzania. The communities in Embu and Mbeere districts of Eastern Province, Kenya use the root bark decoction to treat worms in humans and animals (Kareru, 2008). **Plate 1.1** shows the aerial parts of *Entada leptostachya*.

Plate 1.1: Aerial parts of *E. leptostachya* showing the foliage

Kareru (2008) reported the presence of triterpenes, tannins, saponins and glycosides in *Entada leptostachya*.

1.2.5.2 *Albizia anthelmintica* (Mimosaceae)

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

Saponins were first reported to kill worms as early as 1962, using an extract from *Albizia anthelmintica*. Phytochemical studies using extracts from *A. anthelmintica* has shown presence of triterpenes, saponins, tannins and anthraquinones (bound) (Kareru, 2008). Sesquiterpene and kosotoxins have also been isolated from the bark and root bark extracts of *Albizia anthelmintica* (Hussain, 2008).

1.2.5.3 *Prosopis juliflora* (Mimosaceae)

Prosopis juliflora is an evergreen tree with a large crown and an open canopy and can grow to a height of up to 14m. Its stem is greenish-brown, sinuous and twisted with axial and strong thorns. The barks are reddish-brown and rough and the root system has a deep tap root that allows the tree to reach deep water tables. The leaves are compound, dark bluish-green and are high in tannin (Pasiecznik *et al.,* 2001, Matthews and Brand 2004). Its fruits are pods, which are green when immature and yellow when mature and are high in sugar content (Talpada and Shukla 1988, Masilamani and Vadivelu 1997, Batista *et al.,* 2002). The tree is native to South America, Central America and the Caribbean (Pasiecznik *et al.,* 2004). It was introduced in Kenya in the early 1970s from Latin America and is locally known as ‘Mathenge’ (Ebenshade and Grainger 1980, Maghembe *et al.,* 1983). In Kenya, it is considered as a noxious weed. **Plate 1.2** shows the leaves and pods of *Prosopis juliflora*.

Phytochemical studies have revealed presence of saponins, tannins, flavonoids and alkaloids in leaf aqueous extracts of *Prosopis juliflora* (Wamburu *et al.*, 2013). Andersson (2005) has reported that *P. juliflora* leaves contain a high tannin content.

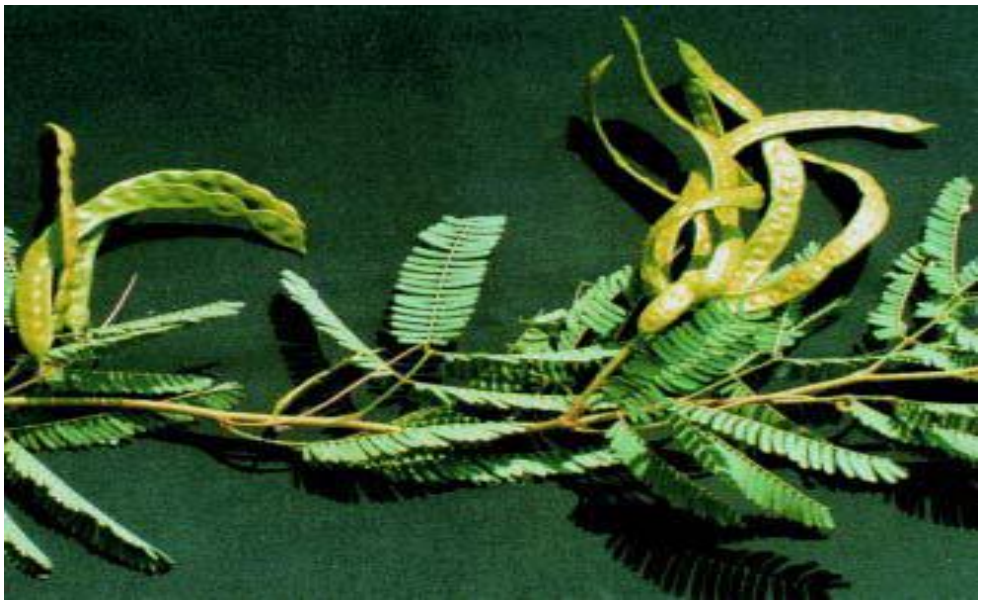


Plate 1.2: Aerial parts of *P. juliflora* showing the foliage and immature pods

1.2.6 Fingerprint profiles of plant extracts in quality control and standardization of herbal drugs

Standardization refers to the measures taken to ensure that there is a consistent quantity of a defined marker compound within a herbal material. This helps to achieve reproducible biological data in terms of safety and efficacy by standardizing the herbal material to the active ingredient(s) when they are known or to specific markers when the actives are not yet known (Saraf *et al.*, 2009; Giri, 2010). According to American Herbal Products Associations (AHPA), standardization refers to body of information and controls necessary to produce materials of reasonable consistency (Sasidharan *et al.*, 2010a).

Fingerprint is a powerful tool for the quality control of herbal medicines (Xiao-he *et al.*, 2011) and has been widely accepted as a useful method for the evaluation and quality

control of herbal materials and their finished products (Liang *et al.*, 2004; Patra *et al.*, 2010; Demirezer *et al.*, 2011). The intuitive evaluation involves comparison of similarities and/or differences of the fingerprints' shape, more so for chromatographic fingerprints (Liang *et al.*, 2004). EMA (European Medicines Agency), FDA (Food and Drug Administration) (Demirezer *et al.*, 2011) and WHO (World Health Organization) (Saraf *et al.*, 2009) suggest the fingerprint method to evaluate the compatibility of herbal medicine to standard extract. Variances due to geographical source, cultivation and processing methods affect the chemical composition and clinical efficacy of these herbal drugs and it becomes necessary to establish a method to control their quality (Demirezer *et al.*, 2011).

Existence of numerous phytochemicals in plants makes it difficult to identify those that provide the main therapeutic effects since the total therapeutic effects are as a result of multiple interactions of these phytochemicals (Andola *et al.*, 2010a; Xiao-he *et al.*, 2011). However, it is impossible and unnecessary to qualitatively and quantitatively study every component (Xiao-he *et al.*, 2011). A lot of emphasis is being laid on the need to use modern scientific techniques to ensure herbal products contain specified amounts of marker compounds in order to guarantee accurate identification, safety, efficacy and stability (Andola *et al.*, 2010a; Patil and Shettigar, 2010). To ensure this, it is necessary to combine chemical constituent analysis with bioassay (Xiao-he *et al.*, 2011). Standardization methods should take into consideration all aspects contributing to the quality of herbal drugs (Patil and Shettigar, 2010).

Several analytical techniques have been utilized in order to obtain fingerprint profiles of herbal drugs, qualitatively and quantitatively, and are a valuable tool for proving constant composition of herbal preparations by establishing relevant criteria for uniformity. These methods provide ways of identifying authentic drugs, in excluding adulterants and in maintaining the quality and consistency of the drug (Patil and Shettigar, 2010). Chemical, chromatographic, spectroscopic and hyphenated methods have been utilized. Techniques such as thin layer chromatography (TLC), gas

chromatography (GC), capillary electrophoresis (CE), infrared spectroscopy (IR), high performance liquid chromatography (HPLC), ultraviolet spectroscopy (UV), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) have been applied for fingerprinting (Demirezer *et al.*, 2011). FT-IR fingerprinting for herbal products tends to focus on identification and assessment of the stability of the chemical constituents' functional groups. FT-IR fingerprint can be used to ensure that the functional groups present in a herbal extract are present in a reproducible manner and this can assist a manufacturer in controlling and assuring the consistency and the standard quality of an extract in each phase of an extraction (Sasidharan *et al.*, 2010b). Hyphenated techniques provide simultaneous separation as well as identification of phytochemicals in a mixture. For example, for GC-MS and LC-MS, GC and LC separate the components while MS identifies components in the mixture on the basis of molecular mass and fragmentation pattern (Saraf *et al.*, 2009). HPLC is now being extensively used to establish chromatographic fingerprints for quality control of raw herbal products (Xiao *et al.*, 2011).

Hyphenated methods such as HPLC-DAD and HPLC-MS have been used for fingerprint analysis of *Coptidis rhizome* (Sheng *et al.*, 2006; Tong *et al.*, 2008). Infrared spectroscopy (IR) is also an important technique as it is rapid and reasonably cost-effective (Xiao-he *et al.*, 2011) and Kareru *et al.* (2008) have detected saponins directly in crude root powder of *Entada leptostachya* using FT-IR without prior chemical preparation. Wedge *et al.* (2009) determined phthalides and monoterpene hydrocarbons to be good systematic chemical fingerprints for *Angelica sinensis* and *Angelica archangelica* root oils and by chemical profiling using GC-MS of *A. sinensis*, they were also able to confirm the misidentification of one *A. archangelica* sample sold in the Chinese market. HPTLC fingerprints have been used to compare and estimate the berberine contents in root and stem barks of three *Berberis* species (Andola *et al.*, 2010b).

1.3 Statement of the problem

Diseaes caused by helmintic parasites in livestock have continued to be a major productivity and reproductivity constraint. This has led to huge economic losses all over the world due to the sub-clinical or chronic nature of diseases they cause. Loss of livestock due to helminth and helminth-related ailments continue to be a major cause of inter-communal conflicts as they try to recover such losses. Helminthes have also shown a great deal of resistance towards the three classes of synthetic anthelmintic drugs worldwide leading to reduction in effectiveness of the available anthelmintics. The synthetic drugs are also toxic towards the animals and the drug administrators, expensive and sometimes beyond reach of most rural SHF and pastoralists. In Kenya, adulteration and drugs with inadequate amounts of active ingredients have also encouraged drug resistance. Emergence of drug-resistant strains of nematodes is an additional constraint to effectiveness of anthelmintics. Herbal alternatives to the synthetic drugs could lead to novel herbal formulations which could help eradicate this problem.

1.4 Justification of the study

Livestock production is one of the main economic activities for rural people both in the arid and semi-arid areas in Kenya. The economic loss due to helminthes infestation in goats alone was estimated at US$ 26 million (Kareru, 2008). In other parts of the world, for example U.S.A., helminthiasis has also been reported to contribute to economic losses. The major hindrance to economic goat production in this region is reported to be due to gastrointestinal nematodes (GIN), particularly *Haemonchus contortus* (Prichard, 1994). The average prices of the conventional anthelmintics in the market are too expensive for the SHFs and pastoralists to sustain subsequent treatments. Besides, synthetic anthelmintics are known to be toxic and long use and poor administration leads to resistance. The synthetic drugs have also been shown to interfere with the immunity mechanism of ruminant animals (Kareru, 2008). The conventional drugs are not availed in the market from time to time at the most needed time, causing an erratic drug administration schedule to the farmer. Additionally, in Kenya, adulteration of synthetic drugs has become common practice (Githiori, 2004).

Long historical use of many practices of traditional medicine, including experience passed on from generation to generation, has demonstrated the safety and efficacy of traditional medicine (WHO, 2000). However, this ethnomedical knowledge has not been adequately validated scientifically while commercial utilization of data from such research is almost non-existant. Ethnoveterinary use of *Entada leptostachya* and *Albizia anthelmintica* as an anthelmintic is evident but this knowledge has not been scientifically validated. Ethnoveterinary use of *Prosopis juliflora* has not been established and it presents new scientific work on this plant in validating it as an anthelmintic. Due to the challenges such as high cost, drug resistance, erratic availability, adulteration and high toxicity profiles experienced with chemical anthelmintics, investigation of these three plants as anthelmintics will help come up with equally (if not better) herbal alternatives with minimal, if any, challenges experienced by chemical anthelmintics. Tapping into this knowledge can give novel herbal alternatives that are cheaper and readily available.

1.5 Hypothesis

The herbal anthelmintic preparation from *Entada leptostachya*, *Prosopis juliflora* and *Albizia anthelmintica* does not have better anthelmintic properties than Albendazole. It is also not safer and more affordable.

1.6 Objectives

1.6.1 General objective

To formulate and evaluate a herbal anthelmintic mixture preparation from two most active plants of the three plants, *Entada leptostachya*, *Prosopis juliflora* and *Albizia anthelmintica* and to assess its anthelmintic activity in ruminant animals.

1.6.2 Specific objectives

1. To determine *in vitro* anthelmintic activities of *Entada leptostachya*, *Prosopis juliflora* and *Albizia anthelmintica* plant extracts and their extract mixtures using egg hatch inhibition assay (EHI).

2. To determine acute oral toxicity of the most active extract mixture from two of the plants, *Entada leptostachya*, *Prosopis juliflora* and *Albizia anthelmintica*, using Wistar albino rats.
3. To determine the anthelmintic activity and safety of the most active extract mixture through *in vivo* studies using sheep.
4. To determine the chemical and spectroscopic profiles (IR and UV) of most active individual plant extracts and the formulated herbal anthelmintic preparation.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study site

The plant materials were collected from Embu and Mbeere areas (*Entada leptostachya, Albizia anthelmintica*) and Marigat area (*Prosopis juliflora*) of Baringo County. The research was conducted in JKUAT Chemistry, Zoology, Botany and Biochemistry laboratories.

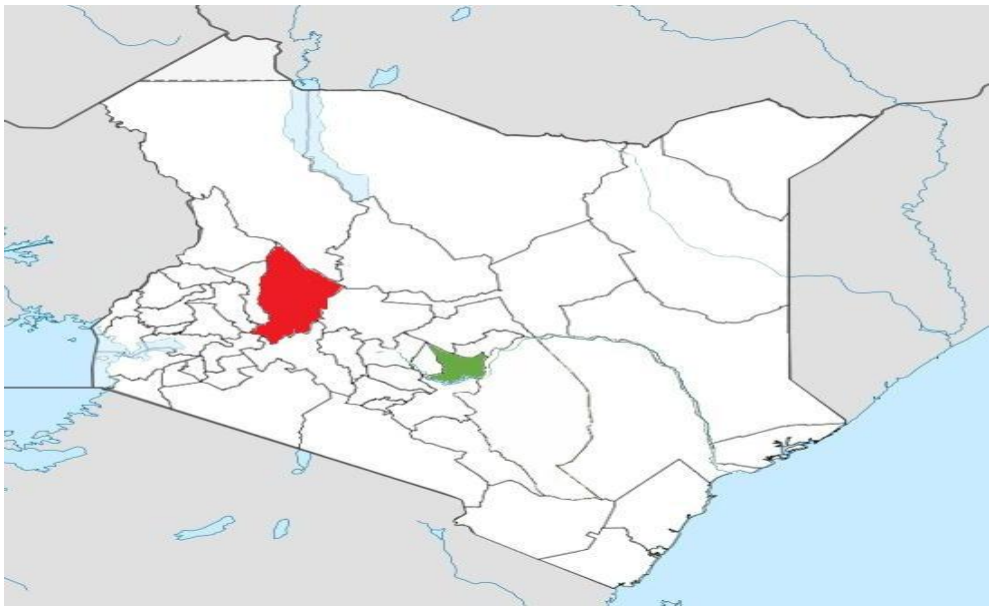


Plate 2.1: Map of Embu County (green) at latitude 0˚31′52″S, longitude 37˚27′02″E and Baringo County (red) at latitude 0.47˚N, longitude 35.97˚E (Source: Google maps)

2.2 Ethical clearance

No human subjects were used. The intended end user of the herbal anthelmintic product (sheep) was not put into the study until toxicity tests using OECD UDP guidelines were conducted on the herbal extract mixture of interest to avoid unnecessary suffering and mortalities of the animals. The research was conducted in accordance with JKUAT guidelines on animal use and care (see ethical approval letter in **Appendix V**) and the internationally accepted guidelines (OECD guidelines) on laboratory animal use and

care. All the rats used in the experiment were humanely handled before, during and after the tests. They were humanely sacrificed by euthanizing using carbon dioxide gas inhalation in a closed chamber before pathological procedures.

The community, through the traditional healers (THs), were involved through informal verbal interviews while collecting information on the plants and during collection of the plant material. They were also informed of the expected benefits from the research so that they can make full use of the invention to benefit the society as a whole. All herbal plant material was authenticated by a taxonomist and voucher specimen numbers deposited at JKUAT Chemistry and Botany laboratories.

2.3 Experimental design and procedures

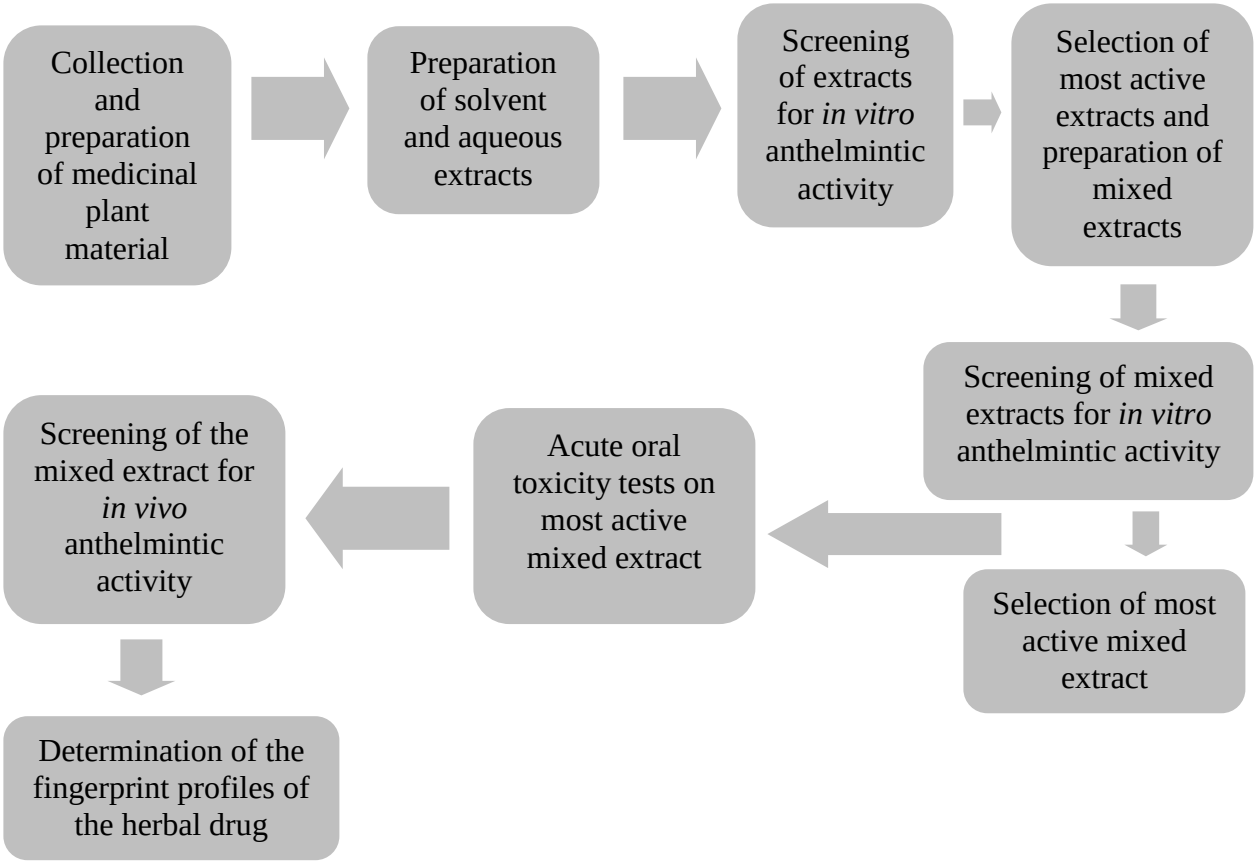


Figure 2.1: Schematic representation of the experimental design

2.3.1 Collection and preparation of medicinal plant material

Root barks of *Entada leptostachya* Harms and stem barks of *Albizia anthelmintica* were collected from Embu and Mbeere areas of Kenya while *Prosopis juliflora* leaves were collected from Marigat area of Baringo County, Kenya. The plants were identified by a herbalist in the field and authenticated by a plant taxonomist from Botany laboratory in J.K.U.A.T.. They were then given correct botanical names. Authentic voucher specimen numbers **En-jkuat/092010, Al-jkuat/092010** and **Pro-jkuat/092010** (for *E. leptostachya*, *A. anthelmintica* and *P. juliflora* respectively) were given to the plant samples and voucher specimens deposited at the J.K.U.A.T. Botany laboratory. The applicable sample specimen parts were sorted, cleaned using tap water to remove adhering soil and other foreign matter and air dried at room temperature with intermittent turning for about three weeks on the laboratory benches away from direct sunlight. Once dry, the sample specimens were ground to a powder using an electric mill (manufactured by J.K.U.A.T. Mechanical Engineering Department) and the various specimen parts separately stored in air-tight plastic bags for further use to avoid contact with moisture.

2.3.2 Preparation of solvent and aqueous plant extracts

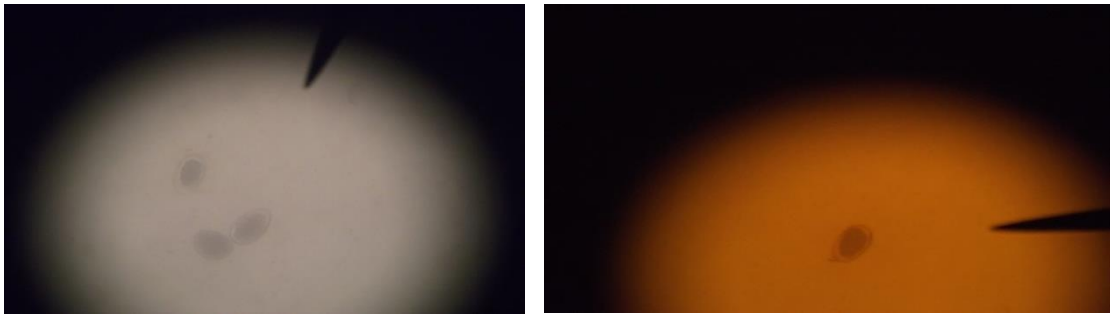
Ethanol extracts were prepared by soaking 50g of each of the three plant powders in 500ml of distilled ethanol for 72 hours. The ethanol extracts were then filtered under vacuum using a Buchner funnel and concentrated under vacuum using a rotary evaporator (BUCHI Rotavapor R-200) at 40°C. The concentrates were then dried under vacuum and their weights determined. The extracts were then stored at -4°C until needed for bioassay. For aqueous extracts, 2g of each of the three plant powders were stirred in 100ml of hot, distilled water for one hour and then left to cool at room temperature and filtered through cotton wool. The aqueous solutions were used fresh and were not crystallized and were prepared moments before bioassay. The concentrations and doses of the aqueous extracts were based on weight of crude plant powders per volume.

2.3.3 Screening of the plant extracts and their mixtures for *in vitro* anthelmintic activities using egg hatch inhibition assay (EHI)

2.3.3.1 Nematode egg recovery

Nematode egg recovery was carried out using simple salt floatation method (Hansen and Perry, 1994) with a few modifications. Fresh droppings from a herd of grazing sheep and goats, initially determined to be naturally infected with mixed nematode species using salt floatation method, were collected and put in an opaque, air-tight polythene bag and taken to the laboratory. The droppings were well mixed by shaking inside the polythene bag and 60g weighed into a 5-liter bucket. One liter of brine solution (floatation fluid) was prepared using normal table salt and distilled water. A little brine solution was added into the weighed feacal sample and the mixture homogenized into a smooth paste using a wooden puddle. The rest of the brine solution was added with thorough mixing and the feacal suspension stirred for about five minutes. The suspension was then sieved through two tea strainers into another 5-liter bucket and this done again for the second time to reduce as much feacal particles as possible. The feacal suspension was swirled and transferred into two half-liter glasses to form a convex meniscus on both. Two glass panes were gently placed on top of the glasses and left to stand for about 20 minutes. Goodwin's solution was prepared by dissolving 4.25g of NaCl and 0.5g of glucose in 50ml of distilled water and this was used as egg diluent and physiological solution. After 20 minutes, the glass pane covers were carefully lifted so that the first drop adhering to them was transferred into a 50ml measuring cylinder. A teat-dropper was used to carefully wash-off the adhering nematode solution from the glass panes into the measuring cylinder using the Goodwin's solution. The solution was topped up to 10ml using the Goodwin's solution. The solution was swirled well and 5µL of the egg solution transferred onto three separate microscope slides using a micropipette and cover slips placed on each of them. The eggs on each slide were carefully identified (see **Plate 2.2**) and counted and the average number of nematode eggs recorded. The average egg count was equated to the stock egg solution (10ml) and the concentration of the nematode eggs in the stock solution adjusted accordingly by

diluting using Goodwin's solution. The nematode egg solution was used while fresh for the *in vitro* studies. The nematode species present were identified and generally determined to be a mixture of *Haemonchus spp., Trichostrongyle spp.* and *Oesophagostomum spp.*.



a **b**

Plate 2.1: a-mixed nematode *Paramphistomum* and *Strongyle* eggs and b-iodine-stained *Strongyle* egg seen under a microscope (X10) using a 14mp camera (Fujifilm, 3X zoom)

2.3.3.2 Testing of ethanolic and aqueous plants extracts for *in vitro* anthelmintic activities

The nematode egg solution was adjusted to 16 eggs per 5µL of egg solution using Goodwin's solution. Egg hatch inhibition assay was done following a method by Thoithi *et al.* (2002) with slight modifications. The stock egg solution was homogenized by swirling, each time before drawing, and five microliters (5µL) of the egg solution measured into each well of a well labelled 96-well microtitre plate using a micropipette. Hundred microliters (100µL) of the plant extracts at the various graduated concentrations (0.5, 1.0, 2.0 and 6.0mg/ml) were then added. Distilled water and 5% ethanol/DMSO solution (v/v) were used as negative controls for aqueous and ethanolic extracts respectively. The anthelmintic activities of the plant extracts were compared to albendazole at the same concentration range. The experiment was done in triplicate. The hatched eggs were then counted in each of the wells under a microscope (X10) after 48 hours and the anthelmintic activities of aqueous and ethanolic extracts as egg hatch inhibitions were reported. The LC_{50} of the individual plant extracts in the two solvents

were determined and these were used to determine the two most active plant extracts from each of the solvents. A test of significance between the mean activity parameters was done between each of the individual plants across the solvent media and the most active extract between aqueous and ethanolic extracts determined and reported. The aqueous extracts of *E. leptostachya* and *P. juliflora* were found to be the most active extracts from their anthelmintic activities and these were used to prepare extract mixtures.

2.3.3.3 Preparation of plant extract mixtures

Fresh aqueous extracts of *E. leptostachya* and *P. juliflora* were separately prepared as done in section 2.3.2. The fresh extract solutions were used to prepare extract mixtures of the following *E. leptostachya:P. juliflora* ratios: 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 and 1:1 (see **Plate 2.3**). The stock solutions of the various mixture ratios were used to prepare serially diluted concentrations of 0.4, 0.8, 1.6 and 3.2 mg/ml. The fresh mixture extracts were then tested for anthelmintic activity using the egg hatch inhibition assay.

c

d

Plate 2.2: Extraction of individual plant aqueous extracts (c) before mixing into various ratio mixtures of stock solutions (d)

2.3.3.4 Screening of the aqueous plant extract mixtures for *in vitro* anthelmintic activity

Fresh nematode eggs were recovered as done in section 2.3.3.1. The nematode egg solution was adjusted to 24 eggs per 5µL of egg solution using Goodwin's solution. The egg hatch inhibition assay was done following a method by Thoithi *et al*. (2002) with slight modifications. Five microliters (5µL) of the egg solution was measured into each well of a 96-well microtitre plate. Hundred microliters (100µL) of the plant extract mixtures at the various graduated concentrations (0.4, 0.8, 1.6 and 3.2mg/ml) were then added. Distilled water was used as negative control. The anthelmintic activities of the plant extract mixtures were compared to albendazole at the same concentration range. The experiment was done in triplicate. The hatched eggs were then counted in each of the wells under a microscope (X10) after 48 hours and the anthelmintic activities of aqueous plant extract mixtures as egg hatch inhibitions were reported. The LC_{50} of each plant extract mixture ratio was determined and reported and the most active plant extract mixture ratio determined.

2.3.4 Safety determination of most active plant extract mixture using acute oral toxicity tests

The most active extract mixture was taken through acute, oral toxicity test to establish its safety based on single dose administration. Standard procedures using OECD 425 guidelines (Up-and-Down procedure) were followed (OECD, 2001) with minor, selective additional data on biochemical analysis and relative organ weights. The main test was performed at a starting dose of 175 mg/kg b.w. as no toxicity data for the herbal mixture existed in literature or was documented.

Seven adult female Wistar albino rats (including one control), 10-12weeks old and weighing 165-196g, were used in the study. The rats were procured from Zoology Department animal house of Kenyatta University at the age of between 7-9 weeks. The rats were left to acclimatize in their cages (bedded with wood shavings) for three weeks with *ad libitum* access to food (standard commercial rat pellets from Unga Feeds

Limited, Kenya) and tap water. Twelve hours artificial lighting and 12 hours darkness sequence was followed. The rats were uniquely marked on their tails for easy individual identification using permanent ink and fasted overnight with *ad libitum* access to water and weighed prior to dosing with the extract mixture. A constant volume of 2 ml per rat based on a single dose was maintained while varying the concentration of the extract mixture using the equation:

$$Concentration\ (mg/ml) = \frac{Dose\ rate\ (mg/kg\ b.w.) \times Body\ weight\ (kg)}{Volume\ (ml)}$$

A dose progression factor of half log (equivalent to 3.2) was followed with a starting dose of 175 mg/ml. Food, but not water, was withheld for a further three hours after dosing and any toxicity signs noted during the first 30 minutes and then hourly for the next six hours. Individual observations were made daily for the next 48 hours for any signs of pain such as crouching or writhing or enduring distress, being moribund or mortality before dosing the next animal. Each rat was then observed on a daily basis for 14 days and each rat weighed once weekly and at the end of the study. The dose progression sequence and LD_{50} estimate in toxicity testing was done using AOT425statpgm (version 1) program software.

Each rat was humanely sacrificed at the end of the study by euthanizing it using carbon dioxide gas in a closed desiccator. Fresh blood samples were obtained by cardiac puncture (see **Plate 2.4**) using a needle and syringe into blood sample tubes containing EDTA (anticoagulant) and the biochemical parameters recorded using an autoanalyzer using assay kits from Roche diagnostics, GmbH, Mannheim, Germany. The lungs, spleen, kidneys and liver were immediately weighed on an electronic balance and their weights recorded. The toxicity results of the mixture ratio were then reported.

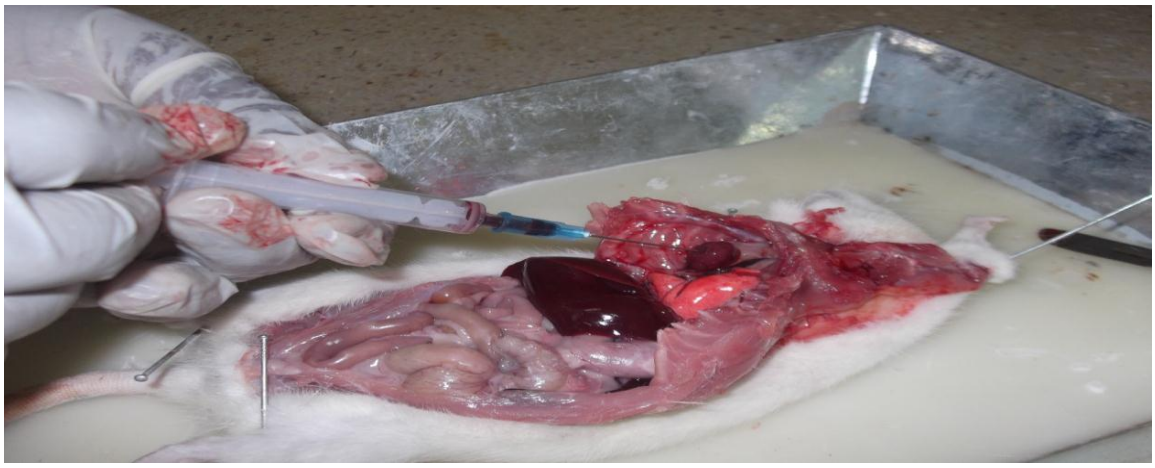


Plate 2.3: Drawing blood for biochemical screening from one of the experimental rats by cardiac puncture

2.3.5 Screening of the most active plant extract mixture for *in vivo* anthelmintic activity and safety

Following the results of the toxicity tests, the *in vivo* anthelmintic activity of the most active plant extract mixture was determined to evaluate its efficacy using feacal egg count percent reduction (FECR) test. Naturally infected sheep were used for these tests. Documented standard procedures were used (Lateef *et al.* 2003; 2006; Sujon *et al.*, 2008; Krimpen *et al.*, 2008) with a few modifications.

2.3.5.1 Sourcing, housing and grouping of animals

Twenty sheep (male and female stock of 6-12 months) weighing between 13-26.5 kg were bought from Kariobangi livestock market in Nairobi. They were transported to J.K.U.A.T. and left to graze and acclimatize for one month. The shed that was used for housing the sheep is shown in **Plate 2.5**.

Plate 2.4: Outside view of the shed used to house the experimental sheep at J.K.U.A.T.'s Zoology department animal farm

The sheep were housed in a pre-designed animal shed in the university's animal farm consisting of five cubicles and a feed storage cubicle with each cubicle having a meshed window for ventilation and raised wooden floor, a wooden feeding trough and watering bucket. The sheep were then grouped according to the various test concentrations of 500mg/kg, 1,500mg/kg and 4,500mg/kg b.w. for the extract treatment groups, 10mg/kg b.w. for the treated control group (Albendazole) and untreated control group. Each group consisted of two males and two females. The sheep were randomly selected and assigned to the various groups so that the individual weights in each group were as close as possible. There was no physical contact between sheep from different cubicles (**Plate 2.6**).

Plate 2.5: Some of the experimental sheep grouped in one of the cubicles (note the partitions used to avoid physical contact between animals in adjacent cubicles)

2.3.5.2 Pre-treatment procedure

The sheep were left to acclimatize in the cubicles for about 2 days. The sheep were fed on commercial feed supplemented with grass and they had *ad libitum* access to tap water. Feacal samples from each group were collected and egg count per gram (EPG) of their fresh feacal samples determined to confirm that the sheep were naturally infected with mixed species of worms. The infected sheep were then regrouped again so that each group had low, medium and highly infected sheep. The sheep were then given unique individual identifications on laminated paper wound on their necks (**Plate 2.7**).

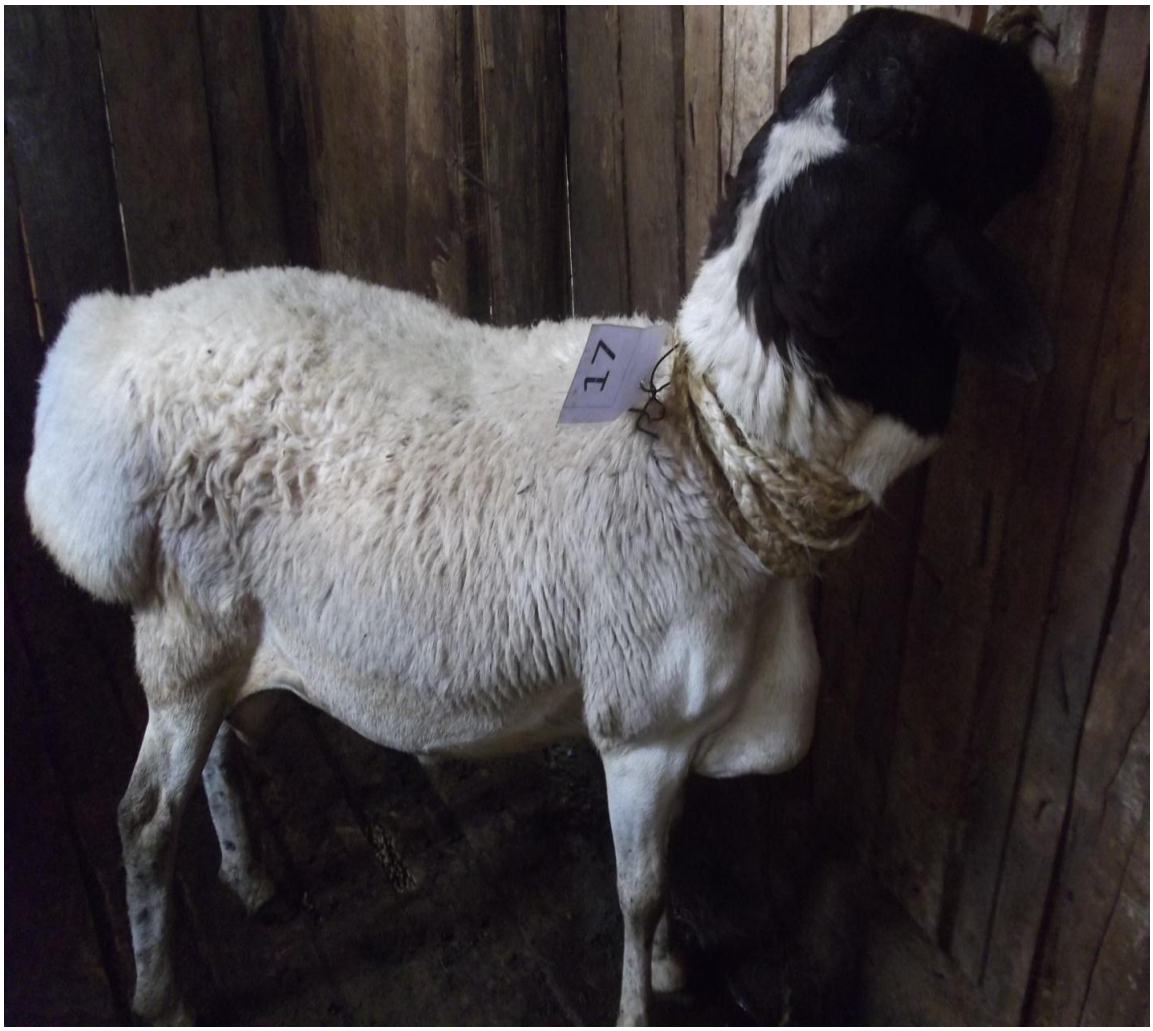


Plate 2.6: Individual identification on experimental sheep

2.3.5.3 Treatment procedure

The fresh crude aqueous extract mixture and positive control (albendazole) were diluted in distilled water in graduated doses of 500mg/kg, 1,500mg/kg and 4,500mg/kg b.w. for the aqueous extract mixture and 10mg/kg b.w. for the positive control. The sheep were fasted overnight with *ad libitum* access to water. On day 0, the sheep were weighed using an overhead spring balance (0-50kg scale), fresh feacal samples collected directly via rectum into clean, capped, air-tight plastic sample containers (**Plate 2.8**) ready for EPG counting. Fresh blood sampling was done directly from the sheep's ear capillary

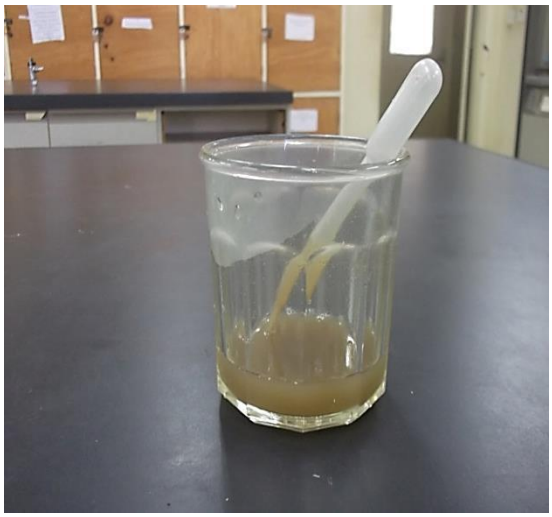
into heparinized microhaematocrit tubes ready for packed cell volume (PCV) determination (Hansen and Perry, 1994). The sheep were then treated with single doses of the crude extract mixture according to the animals' live body weights (LBWs) by varying the volume using the following equation:

$$Volume\ (ml)\ = \frac{Dose\ rate\ (mg/kg\ b.w.) \times Body\ weight\ (kg)}{Concentration\ (mg/ml)}$$

The sheep dosing was followed by further withdrawal of food for about 3 hours with *ad libitum* access to water and then fed with commercial feed supplemented with grass with *ad libitum* access to water.

Fresh feacal samples were obtained from the sheep each morning via rectum on day 3, 7, 11, 15 and 19 post–treatment and screened for presence of nematode eggs by salt floatation technique and the EPG determined (Hansen and Perry, 1994).

e



f

Plate 2.7: e-Fresh feacal samples in air-tight sample bottles; f-Freshly prepared nematode egg solution ready for EPG counting

The eggs were observed and counted using a modified McMaster egg counter with a sensitivity of 50 eggs per gram of feaces and feacal egg count percent reduction (FECR) calculated using the following formula:

$$\%FECR = \frac{\mathrm{Pr}e - treatment\ egg\ count\ per\ gram - Post - treatment\ egg\ count\ per\ gram}{\mathrm{Pr}e - treatment\ egg\ count\ per\ gram}$$

Individual live body weights of the animals were taken on day 3, 7, 11, 15 and 19 using an overhead spring balance (0-50kg scale) and PCV also determined on day 0 and day 19. **Plate 2.9** shows the centrifuged blood samples with the dark red section showing the packed cell content while the clear part showing the plasma.

Plate 2.8: Centrifuged fresh blood samples (at 12,000 r.p.m.) from the sheep in labelled microhaematocrit tubes resting on a centrifuge plate for PCV determination

2.3.6 Determination of chemical and spectroscopic profiles of the individual plants and formulated herbal anthelmintic drug

Fingerprint profiles of crude saponin extracts from *E. leptostachya* and *P. juliflora* and their formulated active drug mixture were determined. Standard documented methods were used to get the chemical and spectroscopic profiles of the crude saponin extracts of the individual plants and their mixtures (Kareru, 2008; Obadoni and Ochuko, 2001; Sasidharan *et al.*, 2010a,b) with a few modifications. Each experiment was carried out in triplicate.

2.3.6.1 Extraction of crude saponins

Two sets each of 5g of *E. leptostachya* and *P. juliflora* crude plant powders were cold macerated in 50ml n-hexane for at least 72hrs until the filtrate was clear. The plant powder residues were then each soaked in 40ml of 20% aqueous ethanol. The extraction was done with frequent shaking in a water bath at 55°C for 1 hour and this was repeated thrice until the filtrate was clear. The residues were discarded and the combined ethanolic extracts evaporated in a water bath up to 20ml. At this point, one set of the *E. leptostachya* and *P. juliflora* aqueous ethanolic extracts was used to prepare the formulated mixture ratio. After cooling, the aqueous ethanolic extracts were transferred into 50ml and 100ml separating funnels and 5ml of ethyl acetate added into each of the funnels, corked and well shaken and left to settle overnight. The aqueous layer from each funnel was recovered into 50ml beakers, the organic layer discarded and the aqueous layer put back into the separating funnels. The aqueous layers were each partitioned using 15ml of n-butanol for four successive times, each n-butanol layer being recovered into a corked 100ml conical flask until the aqueous layer became clear. The combined n-butanol extracts were each washed with 2.5ml of 5% aqueous NaCl. Two hundred and fifty millilitres beakers were cleaned and dried in the oven at 105°C for 1 hour and left to cool in a desiccator. Each of the beakers was then weighed on an analytical balance. Each of the remaining organic solution was recovered into these beakers and labelled. The extracts were heated on a water bath (with a shaker) at 90°C to dryness (Obadoni and Ochuko, 2001; Kareru, 2008).

2.3.6.2 Gravimetric quantification of the crude saponin extracts

The crude saponin extracts were dried in the oven at 105°C for 1 hour and left to cool in a desiccator before recording the combined weights of the extracts and the beakers. The extracts were re-dried in the oven at 105°C, each time for one hour, and the combined weights of extracts and beakers recorded until a constant weight was achieved and the percentage crude saponin content of the samples in triplicate computed. The crude saponins were then confirmed. One milligram of dry, crude saponin extract was dissolved in 5ml 20% ethanol in a clean conical flask and topped up with distilled water to 10ml. The flask was shaken. Formation of foam confirmed they were saponins. A drop of olive oil was added in the saponin solution and the mixture vigorously shaken. Formation of an emulsion further confirmed they were saponins (Kareru, 2008).

2.3.6.3 Infrared (FT-IR) fingerprints (spectra) of the crude saponin samples

The crude saponin extracts were dried in the oven at 105°C for 1 hour and left to cool in a desiccator. Each of the crude saponin extracts (1mg) was mixed with 25mg of dry spectroscopic grade potassium bromide and mixed by grinding using mortar and pestle and compressed into a thin pellet. This was done in triplicate and the infrared spectra then recorded using a Shimadzu infrared spectrophotometer, 8000 series as KBr pellets (Kareru, 2008; Sasidharan *et al.*, 2010a,b).

2.3.6.4 Ultraviolet (UV) fingerprints of the crude saponin samples

The crude saponin extracts (1mg) were dissolved in 5ml of 20% aqueous ethanol. The UV spectra were recorded at between 190 and 600nm using a Shimadzu (UV-1800 series) UV recording spectrophotometer equipped with a UV detector (Kareru, 2008).

CHAPTER THREE

3.0 RESULTS AND DISCUSSION

3.1 Extraction yields

The mean percentage extraction yields of crude ethanolic extracts from *Entada leptostachya, Prosopis juliflora* and *Albizia anthelmintica* are recorded in **Figure 3.1**. Each of the yields was derived from a mass of 50g of crude plant powder and a sample size, n=3 for each plant extract.

Entada leptostachya gave the highest crude extract yield (11.39%) while *Albizia anthelmintica* gave the lowest yield (5.24%). The higher yield of *Entada leptostachya* compared to *P. juliflora* and *A. anthelmintica* could possibly be as a result of its roots having more phytochemical principles whose polarity corresponds to that of ethanol.

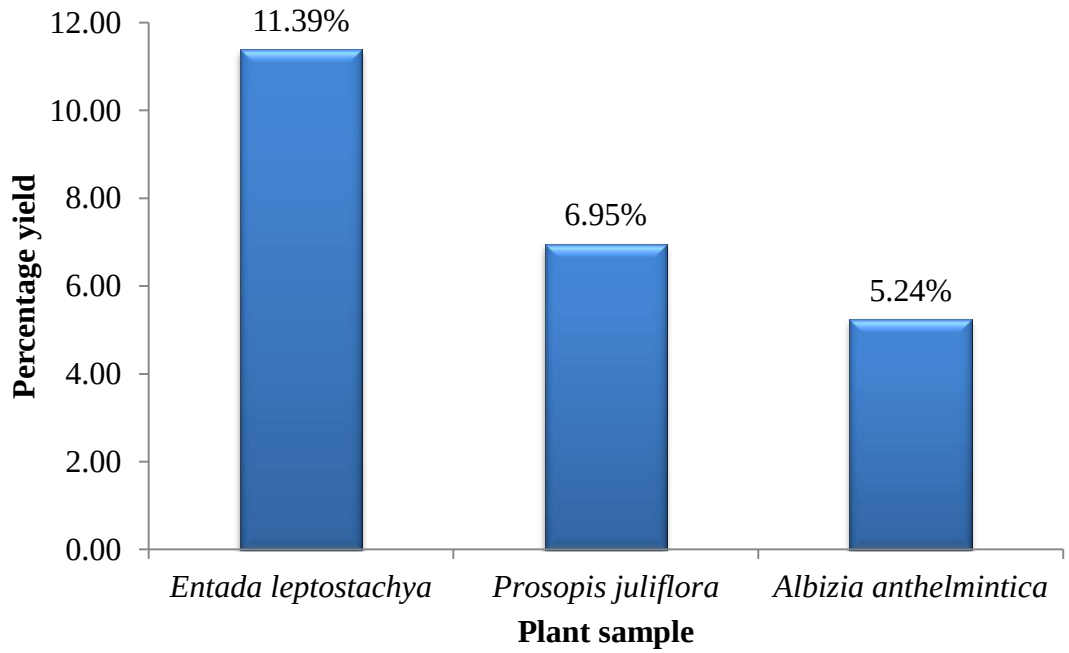


Figure 3.1: Mean percentage (w/w) yields (n=3) of ethanolic extracts of *E. leptostachya, P. juliflora* and *A. anthelmintica*

Kareru (2008) got a methanolic extract yield of 5.30% (w/w) from the roots of *E. leptostachya.* Wamburu *et al.* (2013) got an ethanolic extract yield of 6.94% from the leaves of *P. juliflora.*

3.2 *In vitro* anthelmintic activities of ethanolic and aqueous plant extracts and their mixtures using egg hatch inhibition assay

3.2.1 *In vitro* egg hatch inhibition results of *Entada leptostachya* and *Prosopis juliflora*

Mixed nematode eggs containing *Haemonchus spp., Trichostrongyle spp. a*nd *Oesophagostomum spp.* were recovered from fresh feacal samples and used for EHI assay.

The plants' ethanolic and aqueous extracts generally showed concentration-dependent anthelmintic activities in inhibiting egg hatching (**Figure 3.2** and **Figure 3.3**). From **Table 3.1**, of the three plants, aqueous extract of *Prosopis juliflora* showed the highest anthelmintic activity (LC_{50}=0.153 mg/ml) while aqueous extract of *Albizia anthelmintica* showed the least activity (LC_{50}=0.594 mg/ml).

There were no significant differences ($P>0.05$) in the activities of the plant extracts compared to albendazole (LC_{50}=0.245 mg/ml) except with aqueous extract of *Prosopis juliflora* which showed significantly higher activity ($P<0.05$) than albendazole (**Table 3.2**). *P. juliflora* aqueous extract showed a consistently higher percentage egg hatch inhibition compared to the other plant extracts and control at all concentration levels except at 6 mg/ml.

There was also high positive correlation (R^2) of the activities of the extracts when compared to albendazole as shown in **Table 3.2**. This shows that the *in vitro* anthelmintic activities of the test plants compared very well with albendazole. A comparison between the anthelmintic activities of both ethanolic and aqueous extracts of *E. leptostachya* (0.317mg/ml, 63.53±39.16%) and *A. anthelmintica* (0.330mg/ml,

62.35±37.52%) (**Table 3.2**) showed significantly higher anthelmintic activities (P<0.05) of their ethanolic extracts.

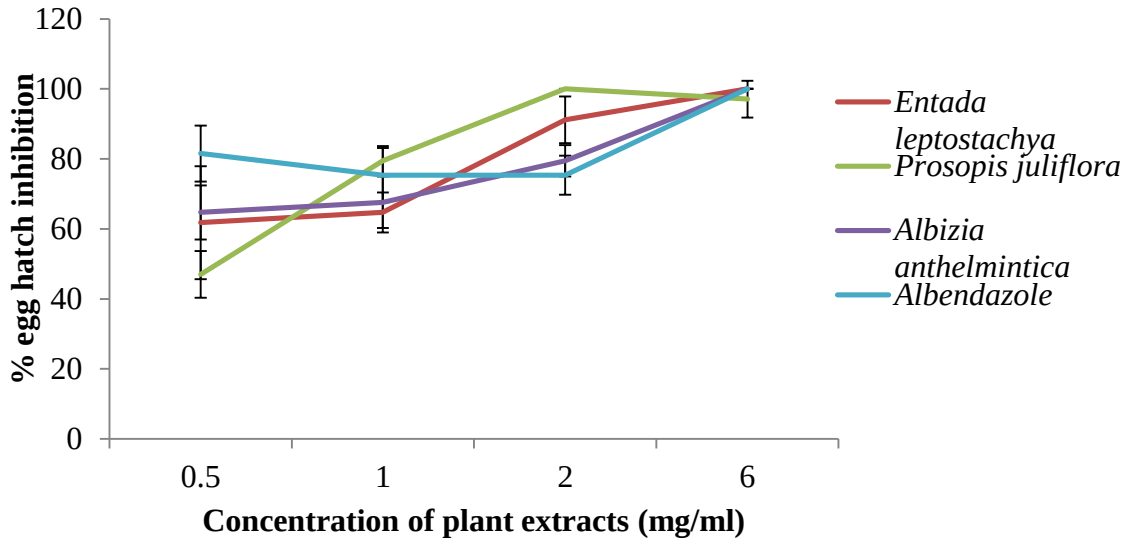


Figure 3.2: *In vitro* egg-hatch inhibition assay of ethanolic extracts from *Entada leptostachya, Prosopis juliflora and Albizia anthelmintica* at various concentrations (mg/ml)

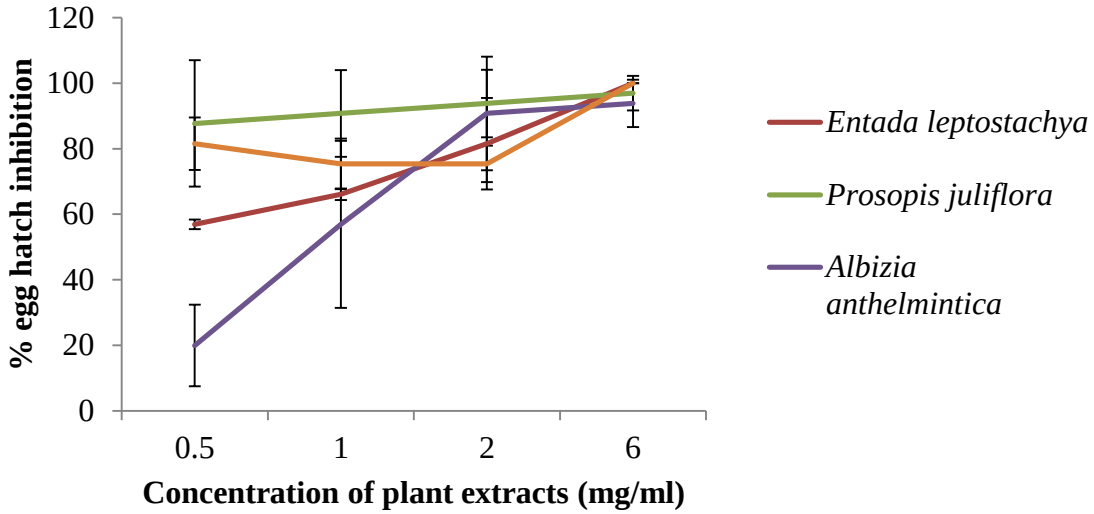


Figure 3.3: *In vitro* egg-hatch inhibition assay of aqueous extracts from *Entada leptostachya, Prosopis juliflora and Albizia anthelmintica* at various concentrations (mg/ml)

Table 3.1: LC_{50} values of ethanolic and aqueous extracts from *Entada leptostachya, Prosopis juliflora and Albizia anthelmintica* from EHI assay

Plants and control	95% confidence limits for concentration (mg/ml)		
	LC_{50}	Lower boundary	Upper boundary
E. leptostachya (Et)	0.317	0.168	0.498
P. juliflora (Et)	0.251	0.127	0.401
A. anthelmintica (Et)	0.330	0.178	0.509
E. leptostachya (Aq)	0.323	0.194	0.489
P. juliflora (Aq)	0.153	0.071	0.284
A. anthelmintica (Aq)	0.594	0.378	0.881
Albendazole	0.245	0.119	0.400

Key: Et=ethanolic, Aq=aqueous

A comparison between both aqueous and ethanolic extracts of *E. leptostachya* and the other plants (**Table 3.3**) showed that no significant differences in the activities ($P>0.05$) were observed except with aqueous extract of *A. anthelmintica* where *A. anthelmintica* aqueous extract exhibited lower anthelmintic activity ($P<0.05$).

From **Table 3.3**, both aqueous and ethanolic extracts of *A. anthelmintica* were significantly less active ($P<0.05$) compared to aqueous extract of *P. juliflora.* Anthelmintic activity of ethanolic extract of *P. juliflora* was higher than that of the aqueous extract of *A. anthelmintica* ($P<0.05$). Anthelmintic activities of ethanolic extracts of *E. leptostachya* and *P. juliflora* were not significantly different ($P>0.05$) when each of them was compared to *A. anthelmintica* ethanolic extract.

Based on their LC_{50} values seen on **Table 3.1** and test of significance of their mean egg inhibitions on **Tables 3.2 and 3.3**, the aqueous extracts of *E. leptostachya* (0.323mg/ml, 60.90±37.77%) and *P. juliflora* (0.153mg/ml, 73.81±41.42%) were determined as the most active in inhibiting hatching of the mixed nematode eggs. The justification of choosing the aqueous extract of *E. leptostachya* to its ethanolic extract despite the

anthelmintic activity of its ethanolic extract being higher (P<0.05) was based on the fact that from **Table 3.3**, the activity of aqueous extract of *E. leptostachya* was comparable to that of aqueous extract of *P. juliflora* (P>0.05). The aqueous extract of *P. juliflora* was comparable to its ethanolic counterpart (P>0.05) as seen earlier. However, significantly higher activity (P<0.05) of aqueous extract of *P. juliflora* compared to Albendazole (**Table 3.2**) justified the choice of the aqueous extract of *P. juliflora* and although there were no significant differences (P>0.05) with its ethanolic analogue, *P. juliflora* ethanolic extract was only comparable to Albendazole (P>0.05).

Table 3.2: EHI assay correlations of ethanolic and aqueous extracts from *Entada leptostachya, Prosopis juliflora and Albizia anthelmintica*

	Sample/Control comparison	% mean eggs inhibited (±S.D.)	Total eggs used (Mean)	(R^2)
Pair 1*	*Entada leptostachya* ethanolic	63.53±39.16	12.65	0.994
	Entada leptostachya aqueous	60.90±37.77	13.27	
Pair 2	*Prosopis juliflora* ethanolic	64.71±41.86	13.25	0.899
	Prosopis juliflora aqueous	73.81±41.42	10.40	
Pair 3*	*Albizia anthelmintica* ethanolic	62.35±37.52	12.98	0.925
	Albizia anthelmintica aqueous	55.36±39.37	11.86	
Pair 4	*Entada leptostachya* ethanolic	63.53±39.16	12.65	0.941
	Albendazole	66.45±38.49	12.73	
Pair 5	*Prosopis juliflora* ethanolic	64.71±41.86	13.25	0.863
	Albendazole	66.45±38.49	12.73	
Pair 6	*Albizia anthelmintica* ethanolic	62.35±37.52	12.98	0.976
	Albendazole	66.45±38.49	12.73	
Pair 7	*Entada leptostachya* aqueous	60.90±37.77	13.27	0.951
	Albendazole	66.45±38.49	12.73	
Pair 8*	*Prosopis juliflora* aqueous	73.81±41.42	10.40	0.975
	Albendazole	66.45±38.49	12.73	
Pair 9	*Albizia anthelmintica* aqueous	55.36±39.37	11.86	0.824
	Albendazole	66.45±38.49	12.73	

* Paired means are significantly different (P<0.05)

A. anthelmintica aqueous extract was significantly less potent (P<0.05) than both aqueous and ethanolic extracts of *E. leptostachya* and *P. juliflora* and was eliminated on

that basis (**Table 3.3**). While ethanolic extract of *A. anthelmintica* was comparable (P>0.05) to ethanolic extracts of *E. leptostachya* and *P. juliflora* (both of which were also comparable, P>0.05) in **Table 3.3**, the aqueous extract of *P. juliflora* was significantly more active than ethanolic extract of *A. anthelmintica* P<0.05) and hence *P. juliflora* aqueous extract was still a more potent choice considering it was also significantly active (P<0.05) than Albendazole (**Table 3.2**). Ethanolic extracts of *E. leptostachya* and *P. juliflora* were, however, not significantly more active than Albendazole (P>0.05).

Table 3.3: EHI assay correlations of ethanolic and aqueous extracts from *Entada leptostachya* and *Prosopis juliflora* with extracts of *Albizia anthelmintica*

Sample/Control comparison		% mean eggs inhibited (±S.D.)	Total eggs used (Mean)	R^2
Pair 1	*Entada leptostachya* ethanolic	63.53±39.16	12.65	0.963
	Prosopis juliflora ethanolic	64.71±41.86	13.25	
Pair 2	*Entada leptostachya* ethanolic	64.71±41.86	12.65	0.988
	Albizia anthelmintica ethanolic	62.35±37.52	12.98	
Pair 3	*Entada leptostachya* aqueous	60.90±37.77	13.27	0.934
	Prosopis juliflora aqueous	73.81±41.42	10.40	
Pair 4*	*Entada leptostachya* aqueous	60.90±37.77	13.27	0.957
	Albizia anthelmintica aqueous	55.36±39.37	11.86	
Pair 5	*Entada leptostachya* ethanolic	63.53±39.16	12.65	0.937
	Prosopis juliflora aqueous	73.81±41.42	10.40	
Pair 6*	*Entada leptostachya* ethanolic	63.53±39.16	12.65	0.962
	Albizia anthelmintica aqueous	55.36±39.37	11.86	
Pair 7	*Prosopis juliflora* ethanolic	64.71±41.86	12.65	0.938
	Albizia anthelmintica ethanolic	62.35±37.52	12.98	
Pair 8*	*Prosopis juliflora* ethanolic	64.71±41.86	12.65	0.979
	Albizia anthelmintica aqueous	55.36±39.37	11.86	
Pair 9*	*Prosopis juliflora* aqueous	73.81±41.42	10.40	0.833
	Albizia anthelmintica aqueous	55.36±39.37	11.86	
Pair 10*	*Prosopis juliflora* aqueous	73.81±41.42	10.40	0.955
	Albizia anthelmintica ethanolic	62.35±37.52	12.98	

* Paired means are statistically significantly different (P<0.05)

Ethanolic extract of *A. anthelmintica* was eliminated based on that criterion. Water, as an extraction medium, is unarguably a cheaper solvent for commercial application and hence, the choice of aqueous extracts for onward formulation of the extract mixtures between *E. leptostachya* and *P. juliflora*.

The anthelmintic activities of the plants under investigation in this study can be attributed to the presence of phytochemicals antagonistic toward gastro-intestinal nematodes. The polar phytochemicals in the plants could have been responsible for the observed *in vitro* anthelmintic activity (Ademola and Eloff, 2010).

3.2.2 *In vitro* egg hatch inhibition results of the aqueous plant extract mixtures of *Entada leptostachya* and *Prosopis juliflora*

Mixed nematode eggs containing *Haemonchus spp., Trichostrongyle spp. a*nd *Oesophagostomum spp.* were determined and used for EHI assay. The plant extract mixtures generally showed concentration-dependent anthelmintic activities in inhibiting egg hatching as shown in **Figure 3.4** and the table in **Appendix III**. From **Table 3.4**, the ratio 1:7 showed the highest anthelmintic activity (LC_{50}=0.370 mg/ml) while the ratio 2:1 showed the least activity (LC_{50}=2.052 mg/ml). Generally, the ratios where *P. juliflora* was in higher proportion exhibited better activity. However, there was no trend to indicate a rise in anthelmintic activity with rise in *P. juliflora* proportion in a mixture.

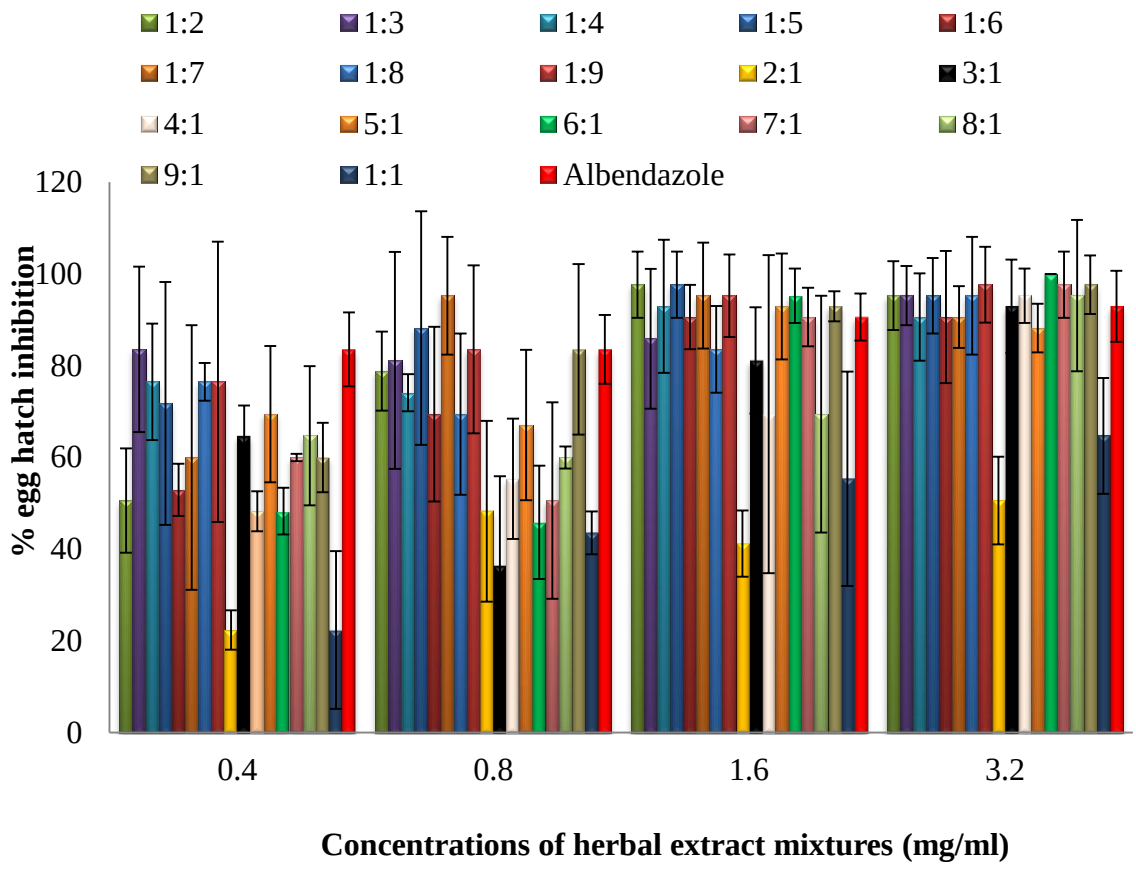


Figure 3.4: *In vitro* egg-hatch inhibition assay of the various *E. leptostachya:P. juliflora* aqueous mixture ratios at various concentrations (mg/ml)

The ratio 1:7 showed comparable anthelmintic activity to ratios 1:3 (LC_{50}=0.743 mg/ml, 60.27±38.31%), 1:4 (LC_{50}=0.545 mg/ml, 55.91±33.77%), 1:5 (LC_{50}=0.398 mg/ml, 59.54±40.33%), 1:9 (LC_{50}=0.448 mg/ml, 57.65±40.34%) and 5:1 (LC_{50}=0.638 mg/ml, 53.04±35.94%) but was significantly higher than the rest of the ratios as shown in **Table 3.5**.

Table 3.4: LC_{50} values of various *E. leptostachya: P. juliflora* mixture ratios from EHI assay

Mixtures and control	95% confidence limits for concentration (mg/ml)		
	LC_{50}	Lower boundary	Upper boundary
1:2	0.595	0.371	0.942
1:3	0.743	0.454	1.195
1:4	0.545	0.345	0.843
1:5	0.398	0.231	0.664
1:6	0.884	0.583	1.334
1:7	0.370	0.227	0.585
1:8	0.712	0.445	1.124
1:9	0.448	0.262	0.747
2:1	2.052	1.407	3.048
3:1	0.741	0.514	1.054
4:1	0.832	0.574	1.200
5:1	0.638	0.415	0.962
6:1	0.775	0.512	1.165
7:1	0.877	0.566	1.352
8:1	1.093	0.713	1.675
9:1	0.517	0.315	0.832
1:1	0.954	0.712	1.270
Albendazole	0.248	0.158	0.374

There was no significant difference ($P>0.05$) in the anthelmintic activity of the ratio 1:7 (LC_{50}=0.370 mg/ml) compared to Albendazole (LC_{50}=0.245 mg/ml). There was also a high positive correlation (R^2=0.808) of the activity of the ratio 1:7 when compared to Albendazole as shown in **Table 3.5.**

The extract mixture formula 1:7 did not show better anthelmintic activity than anthelmintic activity of any of the individual constituent plants in the mixture. The concentration range of the individual plant extracts during the *in vitro* studies ranged between 0.5 and 6.0mg/ml while the extract mixture ranged between 0.4 and 3.2mg/ml. The lower concentration range of the mixture could explain the reason for the observed anthelmintic activity. However, synergistic, additive or antagonistic forces may have played a part in the observed anthelmintic activity of the mixture ratio 1:7 although this

may have been difficult to determine under the differing concentrations. It was, however, expected that the extract mixture would give better anthelmintic activity than the individual plant extracts at these lower concentrations and perhaps, future work could try this at the same concentrations used with individual plant extracts.

The high standard deviation values observed on **Table 3.5** are attributed to the wide range of the nematode egg count of the hatched eggs in each well of the microtitre plate. It is not practically possible to have nematode eggs equally distributed in each well at any particular concentration of the plant extract. The S.D. of the EHI values would be expected to be much lower if equal number of eggs in each well from a single nematode species were to be used. The observed S.D. were therefore, within the normally expected range under these particular experimental conditions.

Other similar work on anthelmintic activity of plant extract mixtures has been reported. An aqueous mixture of *Chebulum myrobalans*, *Belleric myrobalans* and *Emblic myrobalans* in equal parts (locally known in India as Triphala) was found to possess better anthelmintic activity than the individual plant extracts alone. This was attributed to the synergistic action of the three extracts in combination (Zafar *et al.*, 2005; Tandon *et al.*, 2011).

Table 3.5: Paired sample correlations between the percentage mean egg inhibition of mixture ratio 1:7 (*E. leptostachya: P. juliflora*) and other mixture ratios and albendazole

Sample mixtures/control		% mean eggs inhibited (±S.D.)	Total eggs used (Mean)	(R^2)
Pair 1*	1:2	53.07±40.32	9.58	0.884
	1:7	59.33±43.34	10.65	
Pair 2	1:3	60.27±38.31	9.18	0.947
	1:7	59.33±43.34	10.65	
Pair 3	1:4	55.91±33.77	9.85	0.832
	1:7	59.33±43.34	10.65	
Pair 4	1:5	59.54±40.33	8.98	0.973
	1:7	59.33±43.34	10.65	

Sample mixtures/control		% mean eggs inhibited (±S.D.)	Total eggs used (Mean)	(R^2)
Pair 5*	1:6	49.29±36.29	10.65	0.904
	1:7	59.33±43.34	10.65	
Pair 6*	1:7	59.33±43.34	10.65	0.788
	1:8	49.34±33.67	9.32	
Pair 7	1:7	59.33±43.34	10.65	0.956
	1:9	57.65±40.34	8.92	
Pair 8*	1:7	59.33±43.34	10.65	0.934
	2:1	17.61±13.39	12.38	
Pair 9*	1:7	59.33±43.34	10.65	0.697
	3:1	48.64±38.25	13.32	
Pair 10*	1:7	59.33±43.34	10.65	0.748
	4:1	46.05±34.04	12.32	
Pair 11	1:7	59.33±43.34	10.65	0.827
	5:1	53.04±35.94	10.98	
Pair 12*	1:7	59.33±43.34	10.65	0.776
	6:1	48.33±45.27	10.98	
Pair 13*	1:7	59.33±43.34	10.65	0.681
	7:1	45.23±41.49	10.12	
Pair 14*	1:7	59.33±43.34	10.65	0.673
	8:1	39.86±33.16	10.05	
Pair 15*	1:7	59.33±43.34	10.65	0.922
	9:1	53.79±37.47	9.38	
Pair 16*	1:7	59.33±43.34	10.65	0.800
	1:1	41.13±28.84	17.25	
Pair 17	1:7	59.33±43.34	10.65	0.808
Albendazole		69.43±39.16	14.58	

* Paired means are significantly different ($P<0.05$)

3.3 Results of toxicity studies

3.3.1 Clinical toxicological signs

During the entire study, no signs of toxicity were observed after administration of a single oral dose of aqueous mixture ratio 1:7. No signs of pain or enduring distress were evident during the 14 days (long-term) of observation and no mortalities were observed

as seen in **Table 3.6**. The study had to be stopped when no deaths were observed after three consecutive rats were dosed at the upper bound (5000 mg/kg b.w.). The median lethal dose (LD_{50}) was, therefore, estimated at >5000 mg/kg b.w. following the stopping rule procedure. No delayed behavioural toxicity signs were observed.

Table 3.6: Toxicity test mortality data for the mixture ratio 1:7 (*E. leptostachya: P. juliflora*) on rats

Test Sequence	Animal ID	Dose (mg/kg b.w.)	Short-term result (48hrs)	Long-term result (14days)
1	A	175	O	O
2	B	550	O	O
3	C	1750	O	O
4	D	5000	O	O
5	E	5000	O	O
6	F	5000	O	O

Key: O = Survived

3.3.2 Biochemical data

The effects of the herbal extract mixture 1:7 on the biochemical parameters of the rats are shown on **Table 3.7**. The creatinine values obtained from all the animals treated and the control were similar at <44.2 µmol/L which was within the normal range of between 15-61 µmol/L for experimental rats (Wamburu *et al.*, 2013). This implies that none of the rats suffered from impaired renal functioning. Creatinine is a major by-product (chemical waste) of energy usage in the muscles (metabolism) through a biological system involving creatine, phosphocreatine (creatinine phosphate) and adenosine triphosphate (ATP), which is the body's immediate energy supply. Creatinine is chiefly filtered out of the blood by the kidneys, with little or no tubular reabsorption of creatinine. Creatinine blood levels rise if the kidney filtering process is deficient and this is a reliable indicator of kidney malfunction/impaired function.

The alanine aminotransferase (ALT) values for all the animals apart from the control were within range of 35-80 U/L (Research Animal Resources, University of Minnesota, 2013) for experimental rats. Low ALT levels in the control may indicate a normal healthy liver or a low/non-functioning liver which may fail to release a lot of ALT into the blood. A high level of ALT in the blood usually signifies liver cell damage (Agbaje *et al.*, 2009). ALT is found mainly in the liver, but also in smaller amounts in the kidneys, heart, muscles and pancreas (Penlap *et al.*, 2010).

Aspartate aminotransferase (AST) values were within range of 65-203 U/L for experimental rats (Giknis and Clifford, 2008) for all the rats apart from two rats in the 5000mg/kg b.w. category. AST is also a hepatic health/function indicator but may also be used to assess damage in the heart and cell necrosis of many tissues (Agbaje *et al.*, 2009). A mild elevation of AST level has been associated with liver injury or myocardial infarction. The amount of AST in the blood is directly related to the extent of the tissue damage.

An AST/ALT ratio is sometimes useful in differentiating causes of tissue damage. A typical myocardial infarction gives an AST/ALT ratio greater then 1 while an AST/ALT ratio less than 1 is due to liver injury and AST/ALT of more than 2 indicates alcoholic hepatitis or cirrhosis (Mounnissamy *et al.*, 2010). The AST/ALT ratio (>2) for the rats in upper-bound doses indicate possible plant extract-induced liver cirrhosis as the cause of elevated AST levels. However, macroscopic observation of the organs from the two animals did not show any form of inflammation, colour consistent with cirrhosis or abnormal texture. The AST levels were consistently higher than the ALT levels which was expected since the body cells contain more AST than ALT. Since ALT is localized mainly in cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocellular damage than AST (Aniagu *et al.*, 2005; Mounnissamy, 2010; Penlap *et al.*, 2010). Toxin-induced hepatocellular damage is caused by leakage of cytosolic enzymes out of the cells due to increase in cell permeability, membrane damage and cell necrosis (Kumar and Mishra, 2009). Cell permeability, and hence the cellular leakage,

could be caused by saponins which combine with membrane-associated sterols reducing the membrane integrity (Geidam *et al.*, 2007; Ademola *et al.*, 2008; Ademola and Eloff, 2010).

Table 3.7: Effect of treatment of rats with mixture ratio 1:7 (*E. leptostachya:P. juliflora)* on biochemical parameters

		Dose (mg/kg b.w.)					
Parameter	**Control**	**175**	**550**	**1750**	**5000**	**5000**	**5000**
Creat. (µmol/L)	<44	<44	<44	<44	<44	<44	<44
ALT (U/L)	18.5	66.6	38.1	63.3	44.4	73.5	36.6
AST (U/L)	110	67	152	82.1	423	336	148
AST/ALT ratio	5.9	1	4	1.3	9.5	4.6	4

3.3.3 Changes in organ and live body weights

There were no major differences in the gross pathological parameters in the study. In **Figure 3.5**, the lung weights of the rat in control and one rat in upper bound were the only conspicuously visible outliers. There were no major deviations in the other organ weights compared to control. The slight differences in liver and spleen weights could not be directly attributed to any internal pathological processes. Macroscopic examination of the organs showed no changes in colour and texture or any visible inflammation compared to the control.

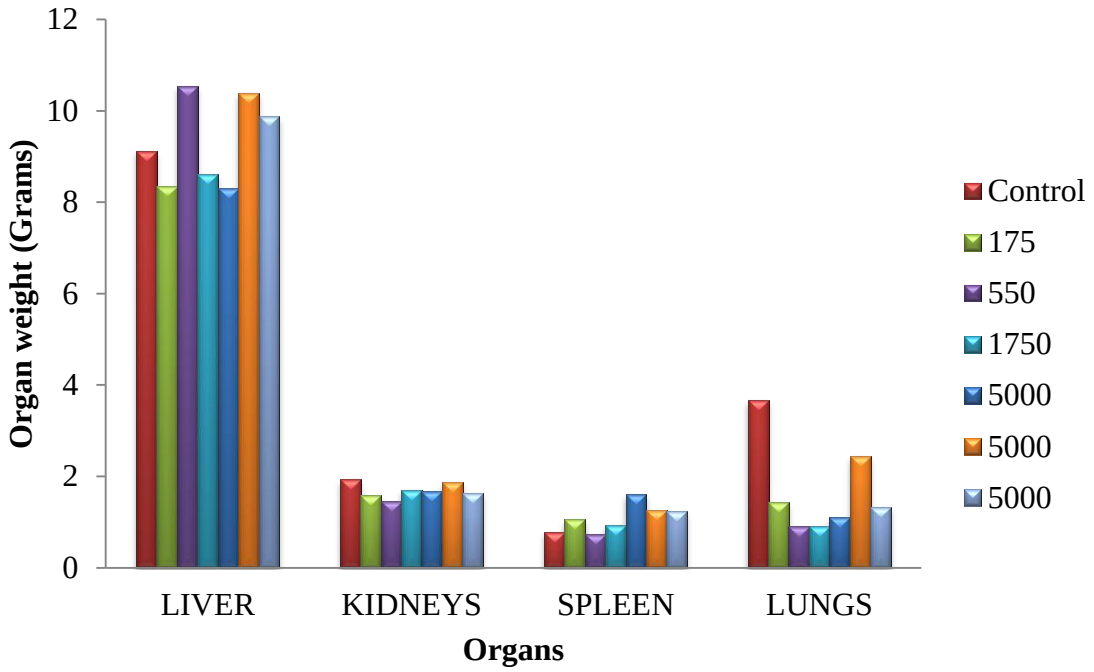


Figure 3.5: Internal organ weights of rats after acute oral administration of herbal extract mixture ratio 1:7 (*E. leptostachya:P. juliflora)*

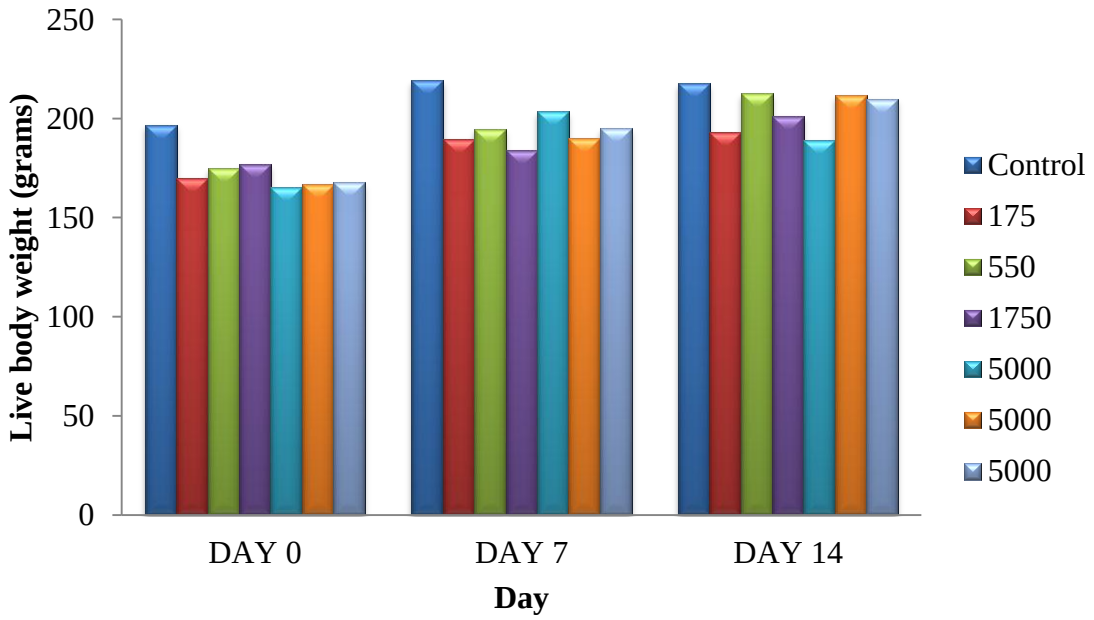


Figure 3.6: Effect of herbal extract mixture ratio 1:7 (*E. leptostachya:P. juliflora)* on live body weights of rats in grams between day 0 and day 14 after treatment

Generally, the animals had progressive weight gains (**Figure 3.6** and Table in **Appendix IV**). The progressive weight gains indicate positive growth response. The treated rats generally showed higher percentage weight gains from day 0 compared to control (Table in **Appendix IV**).

In this study, the saponins determined in this study and other constituent phytochemicals determined in literature in the extract mixture may not have had any or may have had very minimal negative effects on the internal vital organs to produce any noticeable physical toxicity signs. Another reason could have been due to poor absorption and bioavailability of the herbal extract mixture from the gastrointestinal tract (Mukinda, 2005). Perhaps other methods of administration like intraperitoneal administration could have validated that argument if severe toxicity signs and mortality were eventually observed. According to the American Society for Testing and Materials (ASTM) (1987), any chemical substance with an LD_{50} estimate less than 2000 mg/kg/oral route but greater than 1000 mg/kg/oral route could be considered to be slightly toxic although Clarke and Clarke (1977) consider any compound with an estimated LD_{50} greater than 1000 mg/kg/oral route to be safe. A scale proposed by Lorke (1983), roughly classifies substances according to their LD_{50} as follows: very toxic (LD_{50}<1.0 mg/kg), toxic (LD_{50} up to 10.0 mg/kg), less toxic (LD_{50} up to 100.0 mg/kg) and only slightly toxic (up to 1000.0 mg/kg). Substances with LD_{50} values greater than 5000 mg/kg are practically non-toxic (Salawu *et al.*, 2008). The extract mixture formula 1:7 qualified as a safe substance (non-toxic) using the proposed toxicological scales (Clarke and Clarke, 1977; Lorke, 1983; ASTM, 1987; OECD, 2001).

3.4 Results of *in vivo* anthelmintic activity of the herbal extract mixture using sheep

3.4.1 Feacal egg count results

Feacal egg counts (FEC) and the percentage reduction/increase are recorded in **Table 3.8**. These *in vivo* anthelmintic results for extract mixture 1:7 are being reported for the first time by the time of conducting this study. The high standard deviations recorded in

Table 3.8 are attributed to the large range of EPG counts within the groups. The larger the range of EPG, the higher the S.D. The wide range of EPG count was partly due to the unequal level of nematode infection, with unequal numbers of certain highly reproductive nematode species in the sheep and faecal volumes that were being collected from each sheep within the group which have a direct effect on aggregation of nematode eggs leading to a virtual high EPG.

Table 3.8: Eggs per gram (EPG) results after single oral administration of the herbal extract mixture 1:7 to experimental sheep, n=4

Day PT	Controls		Herbal extract mixture dose (mg/kg b.w.)		
	Untreated	Albendazole (10mg/kg b.w.)	500	1500	4500
0	1325±1024.3 (0%)	1800±2079.7 (0%)	7963±6713.7 (0%)	6038±5861.9 (0%)	1513±904.9 (0%)
3	12250±15033.4 (-825%)	3188±2131.7 (-77%)	18513±18896.7 (-132%)	8750±6559.1 (-45%)	6338±8877.3 (-319%)
7	3050±2949.3 (-130%)	2225±1274.4 (-24%)	12263±8479.0 (-54%)	10537±7243.8 (-75%)	3825±1756.2 (-153%)
11	6413±5906.3 (-384%)	2350±1931.3 (-31%)	6250±3946.1 (22%)	10587±10575.6 (-75%)	3750±1239.6 (-148%)
15	1613±1438.4 (-22%)	883±800.5^{e} (51%)	1775±885.5^{a} (78%)	4938±4659.5^{b} (18%)	2663±832.0^{c} (-76%)
19	7288±7530.1 (-450%)	1333±548.5 (-26%)	1313±438.5^{a} (84%)	4863±3336.8^{b} (19%)	2413±2148.8 (-59%)

PT=Post treatment; **Untreated**=Naturally infected but untreated control group; **b.w.**=body weight; values with same lettered superscripts are significantly different ($P<0.05$) from day 0 FEC in the same column; Negative percentage values indicate the extent of increase in FEC value compared to day 0 PT

There were no signs of toxicity such as salivation, diarrhoea, skin reaction, amongst other related toxicity signs in all the groups throughout the study period apart from reduced feed intake in the initiation stage of the experiment. The herbal extract mixture 1:7 generally showed a time-dependent but not dose-dependent *in vivo* anthelmintic activity for 500mg/kg b.w. and 4500mg/kg b.w. The general expectation was to have a reduction on EPG with increase in dosage of the plant extract mixture. The dose preparation of the aqueous extract mixture was based on crude plant powders and not on crystalized crude extracts i.e. .

In general, the herbal extract mixture reduced the EPG count with time at the dosage levels used. This general trend was consistent for 500mg/kg b.w. and 4500mg/kg b.w. while the 1500mg/kg b.w. group had an increase on day 7 PT but eventually gave a significant decrease ($P<0.05$) in EPG on day 19 PT. A similar trend was observed with Albendazole and untreated group on day 11 PT with a significant decrease (maxima) on day 15 PT for Albendazole. Albendazole and the herbal extract mixture 1:7 showed a general positive *in vivo* anthelmintic activity. The high faecal egg counts could be attributed to the high presence of adult parasites in reproductive stages in the host (Worku *et al.,* 2009). There was a general increase in FEC in all the animal groups on day 3, with a general drop from day 7 except for treatment group at 1500mg/kg b.w.. This increase in FEC could have been due to reduced faecal output hence, a virtually higher faecal nematode egg concentration (Githiori, 2004). This was caused by general reduction in the animals' feed intake by day 0 post treatment. This may be attributed to a shorter period of acclimatization to the commercial feed leading to poor palatability despite supplementing the commercial feed with grass. However, the feed intake had improved by day 5 post-treatment after mineral supplementation was introduced on day 4 post-treatment.

The peak (maxima) anthelmintic effects of the herbal extract mixture 1:7 doses show a decreasing anthelmintic activity with increasing dosage with a similar trend having been observed by Lateef *et al.* (2006) with crude methanolic extract of *Carum copticum*. The

peak FEC reductions for Albendazole and extract mixtures at 500mg/kg b.w. and 1500mg/kg b.w. were significant ($P<0.05$). The 500mg/kg b.w. group produced a maximum faecal egg count reduction (FECR) of 84% on day 19 post-treatment (PT) followed by 1500mg/kg b.w. group which gave 19% on day 19 PT while 4500mg/kg b.w. group gave the least reduction of -59% on day 19 PT while Albendazole gave 51% on day 15 PT. This, however, should not be construed to mean that the 1500mg/kg and 4500mg/kg doses were ineffective as there was a consistent FEC reduction from day 7 post-treatment for both dose levels though the herbal drug at 4500mg/kg b.w. was not able to reduce the FEC below the pre-treatment EPG by day 19 PT. Probably, drug metabolism/breakdown could have taken longer with increase in dosage. Feed intake was restricted overnight as restricting feed intake for 24 hours before drug administration slows digesta flow and this helped to increase drug availability and efficiency (Fleming *et al.,* 2006; Kaplan *et al.*, 2003). However, the 500mg/kg b.w. dose may not have been affected by slow drug metabolism and this could provide direction on the maximum dose necessary to produce better *in vivo* anthelmintic effects. The other reason for this observation could have been due to early saturation of the aqueous solutions of the individual plants during preparation of the extracts.

Albendazole produced a maximum faecal egg count reduction of 51% on day 15 PT, which was still lower than that achieved by the herbal mixture (78%) at the dose of 500mg/kg b.w. on the same day. The *in vivo* anthelmintic activity of Albendazole was lower than the reported values in most literature (Agaie and Onyeyili, 2007; Gathuma *et al.*, 2004; Githiori, 2004) which produced FECR of 98.4%, 100% and >90%respectively at the same dosage level of 10mg/kg b.w..This could be attributed to adulteration of the drug or low drug quality such as insufficient active ingredients (Githiori, 2004) as dosing of the animals was followed using the product label instructions. Kumsa *et al.* (2010) did *in vitro* and *in vivo* anthelmintic tests against naturally acquired gastrointestinal nematodes to determine the efficacy of seven chemical anthelmintic drugs based on Albendazole. The results of the study indicated that two of the brands

showed low level of *in vitro* and *in vivo* efficacies. He attributed this to low quality of the brands rather than to the doses of the drugs administered.

Another study done by Monteiro *et al.* (1998) in Kenya found that of seven anthelmintic drugs marketed in Kenya as containing Levamisole, two contained none, while two others had levels of 11.8% and 78.7% of the amount stated on the label. With a peak faecal egg count reduction of 84%, the 500mg/kg b.w. dose passed the threshold FEC reduction. Wood *et al.* (1995) reports that any anthelmintic product that reduces FEC by less than 80% during FECR test trial should be considered insufficiently active as a curative agent. Githiori (2004) considered FEC and total worm count (TWC) reductions greater than or equal to 70% biologically significant based on the same guideline. Furthermore, Kagira *et al.* (2003) outlines that resistance is declared for any anthelmitic if FECR is less or equal to 80%. The 500mg/kg b.w dose produced a maximum reduction effect from -132% to 84%, 1500mg/kg b.w. from -45% to 19%, 4500mg/kg b.w. from -319% to -59% while albendazole reduced from -77% to 51% form day 3 PT. Significant faecal egg count reductions (FECR) were realized ($P<0.05$) by Albendazole on day 15 PT (51%), herbal extract mixture at 500mg/kg b.w. on day 15 and day 19 PT (78% and 84% respectively), herbal extract mixture at 1500mg/kg b.w. on day 15 and day 19 PT (18% and 19% respectively) and herbal extract mixture at 4500mg/kg b.w. on day 15 PT (-76%). Maximum faecal egg count reductions for the treatment groups were all significant ($P<0.05$) except for the 4500mg/kg b.w. dose of the extract mixture.

Studies have shown that correct interpretation of FECR test is not always possible which can be due to study design, level of excretion and aggregation of parasite eggs within the host population (Hussain, 2008; Levecke *et al.*, 2012).

The herbal extract mixture formula exhibited *in vivo* anthelmintic activity against mixed gastrointestinal nematodes. This could be attributed to the mixture of polar phytochemicals present in *E. leptostachya* and *P. juliflora* which are soluble in aqueous medium. Phytochemical studies done on the two plants confirm the presence of alkaloids, flavonoids, saponins, tannins and sterols/triterpenes from the leaf aqueous

extracts of *P. juliflora* (Wamburu *et al.*, 2013) while root aqueous extracts of *E. leptostachya* have tested positive for sterols/triterpenes, glycosides, saponins and tannins (Kareru, 2008; Kareru *et al.*, 2012).

In sheep and goats in Kenya, GI parasites are prevalent and especially the nematode *Haemonchus contortus* and to a lesser extent the nematode species *Trichostrongylus colubriformis* and *Oesophagostomum* spp., with occasional infections of *Strongyloides* and *Trichuris* spp. (Githiori, 2004). *Haemonchus* spp., *Trichostrongyle* spp. and *Oesophagostomum* spp. were predorminant in the egg feacal cultures derived from the sheep used in this study.

3.4.2 Results of live body weights of the experimental sheep

Changes in the live body weights of the sheep were recorded in **Table 3.9**. There were no significant changes (P>0.05) in the live body weights (LBWs) of the sheep treated with the herbal extract mixture and Albendazole on day 19 PT compared with day 0. All the groups had an increase in their mean LBWs by day 19 PT except the untreated group. However, none of the increase was significant (P>0.05).

Table 3.9: Effect of herbal extract mixture ratio 1:7 (*E. leptostachya:P. juliflora)* on mean±S.D. (n=4) live body weights of sheep

	LIVE BODY WEIGHTS (KG)				
DAY PT	**UNTREATED (10mg/kg)**	**ALBENDAZOLE**	**500mg/kg**	**1500mg/kg**	**4500mg/kg**
0	19.4±4.2 (0%)	21.0±3.7 (0%)	19.6±5.1 (0%)	22.3±4.4 (0%)	21.0±5.5 (0%)
3	18.5±3.2[a] (-4.6%)	20.3±4.4[b] (-3.3%)	19.6±5.4 (0%)	21.0±3.0[d] (-5.8%)	21.0±6.5 (0%)
7	18.5±3.5[a] (-4.6%)	22.2±2.3 (5.7%)	19.6±5.2 (0%)	20.6±3.0[d] (-7.6%)	19.6±6.3[e] (-6.7%)
11	18.4±3.2[a] (-5.2%)	20.8±2.4 (-1%)	19.0±4.7[c] (-3.1%)	20.6±4.1[d] (-7.6%)	20.4±6.4[e] (-2.6%)
15	18.9±3.6 (-2.6%)	21.7±2.5 (3.3%)	20.1±4.5 (2.6%)	21.0±4.2[d] (-5.8%)	21.4±6.1 (1.9%)
19	19.0±2.5 (-2.1%)	25.0±2.6 (19.0%)	20.5±4.6 (4.6%)	22.8±3.4 (2.2%)	24.5±6.2 (16.7%)

PT=Post treatment; Mean LBWs with same lettered superscripts are significantly different (P<0.05) from day 0 LBW in the same column; **Untreated**=Naturally infected but untreated control group; Negative percentage values (in parentheses) indicate percentage mean LBW decrease from value on day 0 PT

Nematodes have been reported to cause severe damages to the gastrointestinal tract (GIT) and the host therefore, expends energy and protein repairing these damages caused by these parasites other than for growth (Agaie and Onyeyili, 2007). This could have been responsible for the insignificant changes (P>0.05) in LBWs of the sheep despite anthelmintic treatment. Another possible reason could have been due to confinement of the animals to dry commercial feeds as well as leakage of blood and plasma protein to the gastrointestinal tract by the parasites (Eguale *et al.*, 2007). Presence of tannins in the leaf and root aqueous extracts of *P. juliflora* and *E. leptostachya* respectively is reported (Kareru *et al.*, 2012; Wamburu *et al.*, 2013).

Because of their reactivity with plant proteins as they get chewed by ruminant animals, condensed tannins partially protect animals against rumen degradation of proteins, and so increase the flow of amino acids to the small intestines hence, increasing their absorption (FAO,2011; Min and Hart, 2003; Zafar *et al.*, 2002). This increase may help to counteract the losses of protein attributed to gastrointestinal nematode infection and for immune response to nematode parasites (Barry *et al.*, 2001; Niezen *et al.*, 2002; Zafar *et al.*, 2002; Min and Hart, 2003).

Condensed tannins bind to proteins by hydrogen bonding at near neutral pH of 6.0-7.0 in the rumen to form CT-protein complexes, but dissociate and release bound protein at pH less than 3.5 in the abomasum and eventual availability of amino acids to the small intestines (Barry *et al.*, 2001; Min and Hart, 2003). The herbal extract mixture treated groups could have benefitted from better utilization of proteins in the presence of tannins or the weight gains could have been as a result of improved feeding or both or due to better nutrient utilization due to lower worm load. Weight gains in the Albendazole-treated group could have been due to maximization of nutrient utilization as a result of lower worm load. However, determination of total worm count will be essential in future work with this extract mixture to validate weight gains as a result of lower worm loads as EPG counts cannot reliably be used to make conclusions on worm loads.

3.4.3 Results of packed cell volumes (PCV) of the experimental sheep

There were no significant changes ($P>0.05$) in the PCV by day 19 PT compared to day 0 PT except for the extract mixture dose of 1500mg/kg b.w. whose PCV significantly dropped ($P<0.05$) as recorded on **Table 3.10**. All the animals recorded pre- and post-treatment PCV values that were within the permissible range of between 24-45% (Research Animal Resources, University of Minnesota, 2013; Worku *et al.*, 2009) for experimental sheep. All the groups recorded improved PCV values (thus, reduced degree of anaemia) except the doses at 1500mg/kg b.w. and 4500mg/kg b.w.. Anaemia, following administration of an agent, could be as a result of lysis of blood cells and/or inhibition of blood cell synthesis by the active constituents of the extract (Orisakwe *et*

al., 2003). The higher doses of the extract mixture (1500 and 4500mg/kg b.w.) most likely had this effect on the experimental animals thus, recording decreased PCV values.

Table 3.10: Effect of the herbal extract mixture on the mean±S.D. (n=4) percentage packed cell volume (PCV) of the sheep

	%PCV				
DAYS PT	**UNTREATED**	**ALBENDAZOLE (10mg/kg)**	**500mg/kg**	**1500mg/kg***	**4500mg/kg**
0	28.71±2.61	25.97±4.52	30.29±2.55	25.17±2.09	26.33±2.30
19	31.74±4.34 (10.55%)	30.60±3.39 (17.83%)	30.63±3.95 (1.12%)	24.21±3.67 (-3.81%)	25.51±3.00 (-3.11%)

*Significant difference (P<0.05) between day 0 and day19; Negative percentage values (in parentheses) indicate percentage decrease in PCV from value on day 0 PT

Besides the negative effects worms have on increasing anaemia, the lower percentage increase on mean PCV of the treated animals at 500mg/kg b.w. compared to the controls and percentage PCV drops at 1500mg/kg b.w. and 4500mg/kg b.w. groups may be indicators of the drug effect on the PCV values of sheep. This may also suggest that the two doses whose PCVs dropped may have had a negative stimulatory effect on the hemopoietic system or the animals may have increased water intake (Githiori, 2004).

As no abnormal adverse effects were observed during this study after administration of the herbal drug, water intake may not have contributed to this effect as no abnormal water intake was observed in all the groups under the same experimental conditions. Each worm is responsible for a daily loss of blood of about 0.05ml through ingestion and seepage from lesions (Eguale *et al.*, 2007). PCV values are directly related to anaemia, correlated with high FEC and parasite burdens (Dawo and Tibbo, 2005; Worku *et al.*, 2009). The drop in PCVs in the animals treated with the herbal extract at 1500mg/kg b.w. and 4500mg/kg b.w. correlated with the lower anthelmintic activities (FECR) of the two groups. On the other hand, the improved PCV values meant that there

was reduction of blood loss from the animals due to parasite inhibition or clearance, which can signify recovery from helminthosis and improvement in health status (Agaie and Onyeyili, 2007) as observed with the extract mixture dose of 500mg/kg b.w.. Negative stimulatory effects were enhanced in the herbal extract mixture groups compared to the control groups with notable increase in anaemia in 1500mg/kg b.w. and 4500mg/kg b.w. groups. Notable contradiction is the PCV increment in the untreated group despite the fact that the egg count over the period indicated a general increase. However, a higher egg count may not necessarily indicate the level of worm infection (Tarpoff, 2010).

Other similar *in vivo* work has been reported. A maximum EPG reduction of 50.3% on Arsi Bale goats was achieved by Dawo and Tibbo (2005) using crude powder of *Halothamnus somalensis.* Aqueous extract of *Daniellia oliveri* reduced feacal egg count in naturally infected sheep by 12.8% by day 14 PT at a dose of 1200mg/kg b.w. (Adama *et al.*, 2012). Crude powder and crude methanolic extract of *Ferula costata* showed significantly higher reduction in EPG compared to untreated control at all stages PT with a maxima on day 14 (47.9%) for 3g/kg b.w. but which was significantly lower than the positive control (Levamisole) at 7.5mg/kg b.w. (99.39%) (Kakar *et al.*, 2013). The average PCV values were not significantly different for goats treated with aqueous leaf extract of *C. pyramidalis* compared to Doromectin-treated groups and the untreated groups. The LBWs of the animals treated with the extract did not change significantly except those treated at the highest dose of 5mg/kg b.w. (Robson *et al.*, 2012). In evaluation of *in vivo* anthelmintic activity using Boer goats, Worku *et al.* (2009) observed that groups treated with wormwood and tobacco with added copper sulphate resulted in dramatic decreases in PCV values and related this to toxic effects of these plant extracts.

3.5 Results of chemical and spectroscopic profiling of the individual plants and formulated herbal anthelmintic drug of *E. leptostachya* and *P. juliflora*

3.5.1 Extraction yields of crude saponins from *E. leptostachya* and *P. juliflora* extracts and extract mixture 1:7 (*E. leptostachya:P. juliflora*)

The crude saponin extract yields of *P. juliflora*, *E. leptostachya* and their mixture 1:7 are presented below in **Figure 3.7**. *E. leptostachya* gave the highest percentage yield of 8.82±0.03%, followed by 1:7 mixture (5.11±0.02%) while *P. juliflora* gave the least yield (4.41±0.02%).

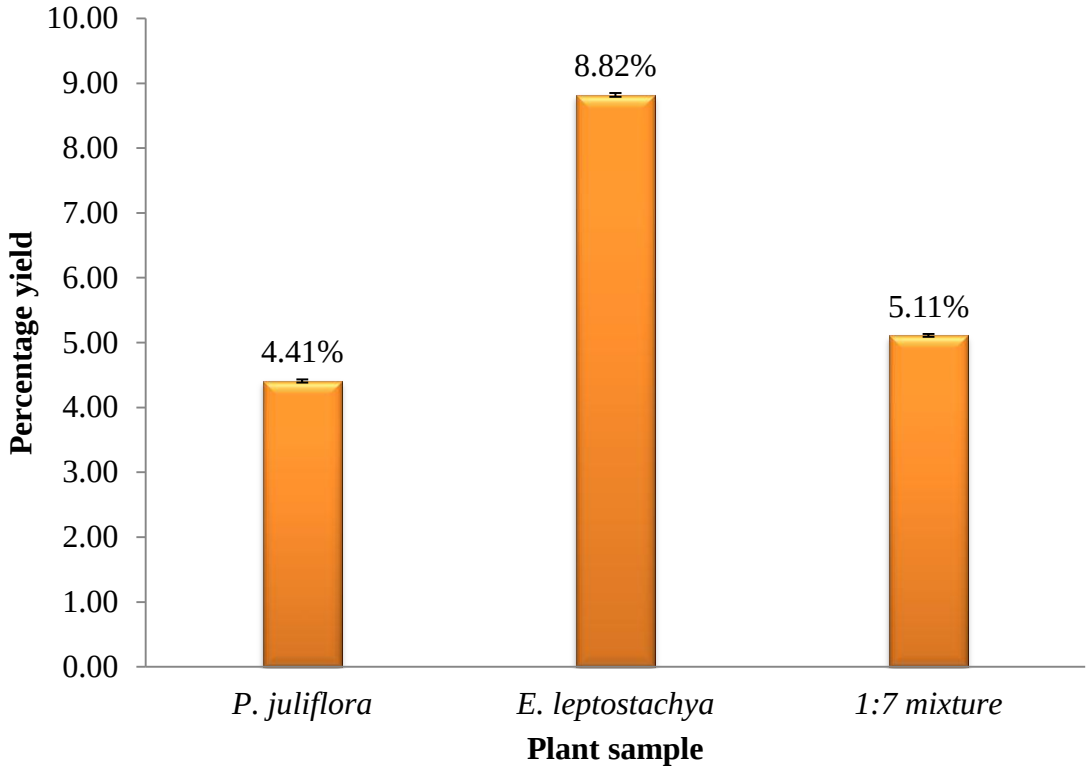


Figure 3.7: Gravimetric mean±S.D. (n=3) of crude saponin extract yields (% w/w) from *P. juliflora, E. leptostachya* and extract mixture 1:7 (*E. leptostachya:P. juliflora*)

From these yields, mathematical computations reveal that the two individual plants contributed near equivalent amounts of crude saponins as per their ratio 1:7. A theoretical percentage of 4.96% is achieved from mixing the crude saponins from the two plants at the 1:7 ratio and this figure is comparable to the experimental percentage

figure of 5.11±0.02% achieved. An assumption can then be made to the effect that the two plants, going by the experimental protocol of mixing that was followed, contributed equivalent crude saponin contents into the 1:7 mixture. Practically, this means that the method of mixing the two plant crude aqueous extracts can be validated and can be replicated in commercial production of the drug.

Kareru (2008) got a crude saponin yield of 1.23±0.02% from *E. leptostachya*. This yield differs from the yield achieved in this study of 8.82% despite using a similar protocol (Obadoni and Ochuko, 2001) of extraction. This variation could be attributed to the differences in the geographical source of the plant sample used (Suleiman *et al.*, 2005;Worku *et al.*, 2009), the season and environmental variability at the time of collection of the two plant samples (Suleiman *et al.*, 2005; Monglo *et al.*, 2006; Eguale and Giday, 2009; Zafar *et al.*, 2009; Saraf *et al.*, 2009; Worku *et al.*, 2009), edaphic factors (soil pH, soil composition, macro and micro nutrient) (Dawo and Tibbo, 2005; Saraf *et al.*,2009), the age (Lateef *et al.*, 2003;Suleiman *et al.*, 2005; Eguale and Giday, 2009; Saraf *et al.*, 2009) or growing stage of the plant (Suleiman *et al.*, 2005; Zafar *et al.*, 2009), whether the plant was freshly harvested or preserved (Suleiman *et al.*, 2005; Monglo *et al.*, 2006; Eguale and Giday, 2009) and the drying process and storage technique (Zafar *et al.*, 2009;Monglo *et al.*, 2006; Eguale and Giday, 2009). For instance, Quinoa grown in low soil water deficit gave saponin contents of 0.456% while the high water deficit gave 0.386%. Among the growing stages, highly significant differences were observed where the branching stage gave 0.309% while the blooming stage gave 0.608% (Solíz-Guerrero *et al.*, 2002). Due to these uncontrollable variations in phytochemical profiles as a result of above factors, standardization of herbal products becomes very important in order to ensure their batch to batch consistency (Saraf *et al.*, 2009). Microwave irradiation or microwave assisted process (MAP) has been developed and used in the extraction of saponins from some medicinal plants (Lahlou, 2004).

3.5.2 Results of infrared (FT-IR) and ultraviolet (UV) fingerprinting

3.5.2.1 Infrared spectra of *P. juliflora, E. leptostachya* and extract mixture 1:7

Presence of saponins in *Entada leptostachya* and *Prosopis juliflora* has been confirmed by phytochemical analyses and by spectrophotometric (FT-IR) method and documented (Kareru *et al*, 2008; Wamburu *et al.*, 2013). The FT-IR fingerprints of crude saponin extracts of the two plants and their formulated mixture 1:7 (*E. leptostachya:P. juliflora*) were recorded (**Appendix I**). A typical FT-IR spectrum recorded from methanolic extract of *Cassia spectabilis* (Sasidharan *et al.*, 2010b) is also shown on **Table I(d)** in **Appendix I**. The crude saponins showed characteristic infrared absorbance (**Table 3.11**) of the hydroxyl group stretch (-OH) ranging from 3381cm^{-1} (*E. leptostachya*) to 3384 cm^{-1} (*P. juliflora*); C-H asym/sym stretch from 2929 cm^{-1} (*E. leptostachya, P. juliflora*) to 2858cm^{-1} (*P. juliflora*) respectively; alkenyl C=C stretch ranging from 1631cm^{-1} (*E. leptostachya*) to 1645cm^{-1} (*P. juliflora*); carboxylic acid/ketone C=O stretch at 1712cm^{-1} (*E. leptostachya*); C-O-C (due to C-O stretch of the glycosidic linkage or cyclic ether of the glycone) ranging from 1045cm^{-1} (*E. leptostachya*) to 1074cm^{-1} (*P. julifora*); C=C-C aromatic ring stretch from 1452cm^{-1} (*E. leptostachya*) to 1454cm^{-1} (*P. juliflora*); aryl-O stretch (aromatic ethers) from 1242cm^{-1} (*E. leptostachya*) to 1220cm^{-1} (*P. juliflora*); carboxylate (R-COO^{-}) absorptions from 1377cm^{-1}, 1317cm^{-1} (*E. leptostachya*) to 1367cm^{-1} (*P. juliflora*). The crude saponin extracts of the extract mixture 1:7 also showed characteristic infrared absorbances (**Table 3.11**) of the hydroxyl group stretch (-OH) at 3417cm^{-1}; C-H asym/sym stretch from 2931cm^{-1} to 2860cm^{-1}; alkenyl C=C stretch at 1641cm^{-1}; C-O-C stretch (due to C-O stretch of the glycosidic linkage or cyclic ether of the glycone) at 1074cm^{-1}; C=C-C aromatic ring stretch at 1452cm^{-1}; aryl-O stretch (aromatic ethers) at 1224cm^{-1}and carboxylate (R-COO^{-}) absorptions at 1373cm^{-1},1434 cm^{-1}.

Table 3.11: FT-IR spectra of crude saponins of *E. leptostachya, P. juliflora* and their mixture 1:7 (*E. leptostachya:P. juliflora*) as KBr pellets

	Sample /wave number (cm^{-1})		
Functional group	***E. leptostachya***	***P. juliflora***	**Mixture 1:7**
OH_{str}	3381	3384	3417
C-$H_{str(asym)}$	2929	2929	2931
C-$H_{str(sym)}$		2858	2860
C=$O_{str(carboxylic\ acid)}$	1712		
C=C_{str}	1631	1645	1641
	1556	1512	1512
	1537		
	1523		
C=C-$C_{str(aromatic\ ring)}$	1452	1454	1452
$RCOO^-_{str}$	1377,1317	1367	1373,1434
Aryl-O_{str}	1242	1220	1224
	1159	1180	1170
C-O-C_{str}	1045	1074	1074

Most of the infrared functional group absorptions characteristic of saponins recorded in this study have been cited in some literature (Coates, 2000; Evangelista *et al.*, 2002; Solíz-Guerrero *et al.*, 2002; Raimundo *et al.*, 2004; Kareru *et al.*, 2008; Kannabiran *et al.*, 2009; Sasidharan *et al.*, 2010a,b) and compare well with the findings of this study. The -OH, C-H, C=C, C=O/carboxylate and C-O-C/Aryl-O determined in the two plants are suggestive of triterpenoid (C_{30}) saponins (Evangelista *et al.*, 2002; Kannabiran *et al.*, 2009; Kareru *et al.*, 2008; Raimundo *et al.*, 2004). Though further analytical techniques like nuclear magnetic resonance and mass spectroscopy need to be employed to ascertain this, the crude saponins in *E. leptostachya* and *P. juliflora* could be bidesmosidic due to detection of the carboxylic acid C=O stretch which could mean a

further attachment of glycone molecule via an ester linkage as confirmed by carboxylate absorptions from both plants. Presence of the C-O-C ether linkage (link between the saccharide and triterpenoid) is confirmed by aryl-O absorptions by both plants. The aglycone may contain one or more unsaturated C-C bonds (Becker *et al.*, 2002) and absorptions at C=C for both plants confirms that fact.

Absorptions as contributions by the two plants are confirmed by similar characteristic absorptions displayed by their extract mixture 1:7, confirming that all the functional groups present in the two individual plants gave characteristic absorptions in their mixture. Absence of the characteristic absorption of C=O at 1712 cm^{-1} (observed in *E. leptostachya* alone) in the mixture could have been due to the presence of *E. leptostachya* in the mixture in a smaller ratio compared to *P. juliflora* and hence, falling below detectable limit.

Infrared fingerprints from the crude saponin extracts of the two plants and their mixture could be used in standardization of the drug formula mixture 1:7 in quality control procedures as direct FT-IR determination of saponins is possible from their crude plant powders or dry crude extracts (Kareru *et al.*, 2008). Confirmation of the contributory evidence of individual absorptions into the mixture then means that long and exhausting procedures of standardizing individual plants are not needed in this case and direct determination of infrared fingerprints of the drug mixture is representative of individual plants.

Similar characteristic IR absorption spectra from saponins have been reported. Two saponins, dasyscyphin C and gymnemagenol, isolated from *E. prostrata* and *G. sylvestre*, showed IR spectrum at 3435.8, 2921.82, 1635.05, 1245.75, 1050.66 for dasyscyphin C and at 3445.41, 2924.10, 1635.38, 1457.48 cm^{-1} for gymnemagenol (Kannabiran *et al.*, 2009). Sirma (2009) did a FTIR spectrum of *P. juliflora* leaves toluene/ethanol and acetone extractives. The FTIR analyses indicated absorption bands at 3400cm^{-1} which were attributed to OH group, 1119 and 1071cm^{-1} to sugars, strong

bands at 2917cm^{-1} and 2849cm^{-1} were characteristic of C-H vibrations and 1728cm^{-1} characteristic of C=O vibrations, were attributed to fatty acids in their free or esterified form while bands at 1510 and 1450cm^{-1} were linked to aromatic structures.

3.5.2.2 Ultraviolet spectra of *P. juliflora, E. leptostachya* and extract mixture 1:7

The UV spectra for the individual plants and their mixture are presented (**Appendix II**). **Table 3.12** shows UV absorbances (λ_{max}) ranging between 198.5 and 330.5 nm for *E. leptostachya* and *P. juliflora* and their mixture ratio 1:7.

Table 3.12: Ultraviolet (UV) spectra of crude saponin extracts of *Entada leptostachya, Prosopis juliflora* and their extract mixture 1:7

Sample	λ_{max} (nm)
Entada leptostachya	277.5, 199.5
Prosopis juliflora	329.5, 269.5, 198.5
Mixture 1:7 (*E. leptostachya:P. juliflora*)	330.5, 269.5

The data in **Table 3.12** represent the UV fingerprints of the marker compounds from *E. leptostachya, P. juliflora* and their mixture. The chromophores within the crude saponins responsible for UV absorption in the individual plants could be traced in their extract mixture apart from those absorbing at around 198-199 nm. Given that the *P. juliflora* proportion in the mixture was much higher than *E. leptostachya*, the absorptions due to chromophores emanating from saponins in *P. juliflora* led to fingerprints in the extract mixture being very similar to those in *P. juliflora*. UV absorbance data achieved in this investigation was nearly identical to those reported in literature. Kareru (2008) has reported UV absorption of crude saponin extract mixture from *E. leptostachya* at 204.8 and 277.4 nm. He was able to further prove that the same chromophores responsible for the UV absorption could be traced in the organic extracts (hexane, dichloromethane, ethylacetate and methanol) and methanol column eluates of *E. leptostachya* which had similar UV absorbances ranging between 202.5-283.1 nm and 201.7-280.4 nm

respectively. UV absorbances at 199, 205, 268 and 315 nm for two new triterpenoid saponins from *Zygophyllum aptriplicoides* were reported (Viqar *et al.*, 2005). Similarities of these absorbances recorded by Viqar *et al.* (2005) to absorbances recorded in this study may indicate a high probability of crude saponins present in *E. leptostachya* and *P. juliflora* being triterpenoid in nature. Radosevich and Delfel (1986) report a UV maximum absorption value of 254 nm for underivatized glycyrrhizic acid. Two saponins, dasyscyphin C and gymnemagenol, isolated from *E. prostrata* and *G. sylvestre*, showed maximum absorption peaks at 234, 238, 302 nm for dasyscyphin C and at 223, 237, 274 nm for gymnemagenol (Kannabiran *et al.*, 2009).

Absence of chromophore moieties in most saponin molecular structures has presented a huge challenge in analyzing this class of compounds using UV detection due to their poor UV absorption (Bassani *et al.*, 2005; Kim *et al.*, 2006; Negi *et al.*, 2011; Oleszek, 2002). However, UV absorptions by saponins from other plants at wavelength of 210nm (Bassani *et al.*, 2005) and longer wavelengths have been reported.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

From the ethnoveterinary information obtained from the traditional healers in Embu and Mbeere areas, the *in vitro* studies done on *E. leptostachya* and *A. anthelmintica* validated their use as anthelmintics in traditional folklore in communities within these areas. The anthelmintic activities of these plants were also comparable to Albendazole at the test concentrations in inhibiting egg hatch of mixed nematode eggs. *P. juliflora* and *E. leptostachya* aqueous extracts of their leaf and root extracts respectively, exhibited the best *in vitro* anthelmintic activity and this is attributed to presence of saponins, tannins, alkaloids and flavonoids in leaf extracts of *P. juliflora* and saponins, tannins, triterpenes and glycosides in root extracts of *E. leptostachya*. Crude aqueous extract from *P. juliflora* exhibited better *in vitro* anthelmintic activity than Albendazole.

Mixture 1:7 generally showed a dose-dependent *in vitro* anthelmintic activity. The mixture ratio 1:7 (*E. leptostachya:P. juliflora*) showed the highest *in vitro* anthelmintic activity in inhibiting egg-hatching of mixed nematode eggs, which was comparable to Albendazole. The anthelmintic activity of this mixture is attributed to the secondary metabolites existing in the two plants which worked together to produce the total anthelmintic effect.

The results of toxicity studies indicated that the extract mixture 1:7 had an LD_{50} greater than 5000 mg/kg b.w. and is therefore, safe when consumed acutely as a single dose orally. However, the AST/ALT ratio from the two rats in upper-bound (5000 mg/kg b.w.) was indicative of extract-induced liver cirrhosis as the cause of elevated AST levels. Use of much fewer test animals in the toxicity studies justified the use of the OECD Up-and-Down procedure.

The extract mixture is safe as it did not produce any physical toxicity signs when a single dose was administered to sheep orally. However, the extract mixture showed time-dependent and not dose dependent *in vivo* anthelmintic activity in experimental

sheep. The extract mixture 1:7 produced biologically significant *in vivo* anthelmintic activity in feacal egg count reduction in sheep. There could be evidence of highlighted problem of adulteration of synthetic drugs as observed from the Albendazole brand used in this study, which produced lower FECR values lower than most Albendazole brands used in literature.

The crude saponin yields obtained from *E. leptostachya* and *P. juliflora* revealed that the individual plants contributed near equivalent amounts of crude saponins into the mixture ratio using the protocol of mixing and this protocol can be replicated in commercial production of this drug mixture.

The FT-IR fingerprints confirmed that the extracted saponins in the individual plants and their mixture 1:7 are most likely triterpenoid saponins. The individual plants contributed their individual FT-IR and UV absorption bands into their mixture 1:7. The FT-IR and UV fingerprints obtained from the crude saponins of the extract mixture can, therefore, be directly used in standardization of the herbal mixture drug, avoiding the long and exhausting procedures of standardizing the individual plants present in the mixture. FT-IR and UV proved to be simple and fast standardization methods.

The project was faced by a number of limitations. Insufficient population size for the sheep during the *in vivo* studies without which, more accurate results could have been achieved. With more time and financial resources more studies and study parameters like biochemical parameters and qualitative and quantitative phytochemical studies could have been done.

In general, the formulated herbal mixture drug showed comparable *in vitro* anthelmintic activity while producing better *in vivo* anthelmintic activity compared to albendazole at the label-prescribed dose. The formulation is also non-toxic and hence, safer than Albendazole. Upon further work in formulating it into the most stable presentation (tablet, liquid drench, powder, extruded feed pellets, e.t.c.), this drug could prove to be cheaper than most available synthetic drugs as the method of extraction medium utilized

is water and plants used to make this drug mixture are locally available, both of which may positively contribute to a cheaper product. This extract mixture preparation is therefore, recommended for use by SHFs and pastoralists.

RECOMMENDATIONS

1. Comparative *in vitro* anthelmintic studies using similar range of aqueous concentrations of *E. leptostachya* and *P. juliflora* should be done for individual plants and for the extract mixture 1:7 to conclusively ascertain whether there exists synergistic, antagonistic or additive mechanism in phytochemical interactions.
2. Other stages of nematode life-cycle like the larval and adult stages could be used in the *in vitro* studies using the extract mixture 1:7 to compare its anthelmintic effects at these stages.
3. Further toxicity studies should be done to ascertain long-term effects based on repeated doses of the extract mixture 1:7 on internal organs using sub-chronic and chronic protocols. Microscopic organ examinations should be done to determine cellular integrity. Other routes of administration like intraperitoneal should also be considered to validate absence of toxicity signs and even eradicate any doubt that there could exist poor absorption of the extract mixture or poor bioavailability of the active constituents in the extract mixture.
4. Further confirmatory *in vivo* work should be done to validate and explain the observed anthelmintic trend where the extract mixture showed lower anthelmintic activity with rising dose levels.
5. Preliminary work on pharmacokinetics using FT-IR and other techniques should be done to monitor peak plasma concentration of the drug by tracking the intensity and presence of one or more phytochemical fingerprints. This will help in determining the dosing regime for maximum worm eradication in the GIT of the host animal and to determine the best dosing frequency to avoid drug resistance. This could also be helpful in initiating clinical trials after conclusive sub-chronic and chronic toxicity studies have been carried out.

6. Concentrations and doses based on dry, crude aqueous extracts from the individual plants could be used to do the *in vitro* and *in vivo* tests and results compared with those in this study.

7. Total worm count (TWC) determination is essential to determine efficacy of the herbal mixture as EPG count is not reliable to make conclusions on worm loads.

8. Fingerprints of other phytochemicals of anthelmintic importance could also be determined using the same methods while additionally, using chromatographic and hyphenated methods and a standardization protocol established with all factors that affect the phytochemical variations in plants taken to mind.

REFERENCES

Abebe, W. and Esayas, G. (2001). Survey of ovine and caprine gastro-intestinal helminthosis in eastern part of Ethiopia during the dry season of the year, *Revue Méd. Vét.*,152(5): 379-384.

Adama, K., Amadou, T., Man, N., Hamidou, T.H. and Gaston, B.A.M. (2012). Anthelmintic activity of *Daniellia oliveri* (Rolfe) Hucht. and Dalz (Caesalpiniaceae) in sheep in Burkina Faso, *Journal of Research in Antimicrobials*, 1(1), 049-055.

Ademola, I.O., Ajayi, A., and Okotie, S.V. (2008). Larvicidal effects of *Aframomum danieli* seed extracts against gastrointestinal nematode of sheep: *in vitro* studies, *African Ethnomedicines Network*, 5(3), 173-179.

Ademola, I.O. and Eloff, J.N. (2010). *In vitro* anthelmintic activity of *Combretum molle* (R. Br. ex G. Don) (Combretaceae) against *Haemonchus contortus* ova and larvae, *Veterinary Parasitology*, 169(1-2), 198-203.

Adeyemi, O.O. and Adeneye, A.A. (2009). Hypoglycaemic effects of the aqueous seed extract of *Hunteria umbellate* in normoglycaemic and glucose- and nicotine-induced hyperglycaemic rats, *International Journal of Applied Research in Natural Products*,2 (1), 9-18.

Afolayan, A.J., Ashafa, A.O.T., Yakubu, M.T. and Grierson, D.S. (2009). Toxicological evaluation of the aqueous extract of *Felicia muricata* Thunb. leaves in wistar rats, *African Journal of Biotechnology*, 8(6): 949-954.

Agaie, B.M. and Onyeyili, P.A. (2007). Anthelmintic activity of the crude aqueous leaf extracts of *Anogeissus leiocarpus* in sheep, *African Journal of Biotechnology:* 6(13): 1511-1515.

Agbaje, E.O., Adeneye, A.A. and Daramola, A.O. (2009). Biochemical and toxicological studies of aqueous extract of *Syzigium aromaticum* (L.) Merr. & Perry (Myrtaceae) in rodents, *African Journal of Traditional, Complementary and Alternative Medicines:* 6(3): 241-254.

Akanmu, M.A., Alade, G.O., Obuotor, E.M., Osasan, S.A. and Omobuwajo, O.R. (2009). Acute and oral sub-acute toxicity of methanolic extract of *Bauhinia monandra* leaf in rats, *African Journal of Pharmacy and Pharmacology*, 3(7), 354-358.

American Society for Testing and Materials (1987). Standard test method for estimating acute oral toxicity in rats. American Society for Testing and Materials, Philadelphia, U.S.A..

Andersson, S. (2005). Spread of the introduced tree species *Prosopis juliflora* (Sw.) DC in the Lake Baringo area, Kenya, pp. 7. ©Stefan Andersson 2005, Institutionen för skoglig vegetationsekologi, Sveriges lantbruksuniversitet, 901 83, UMEÅ.

Andola, H.C., Giri, L., Purohit, V.K., Rawat, M.S.M., Rawal, R.S. and Bhatt, I.D. (2010a). Chromatographic and spectral fingerprinting standardization of traditional medicines: An overview as modern tools, *Research Journal of Phytochemistry*, 4(4), 234-241.

Andola, H.C., Rawal, R.S., Rawat, M.S.M., Bhatt, I.D. and Purohit, V.K. (2010b). Analysis of berberine content using HPTLC fingerprinting of root and bark of three Himalayan *Berberis* species, *Asian Journal of Biotechnology*, 2, 239-245.

Andrews, J.R.H. (1969). A guide to the identification of helminthes parasites recorded from wild animals in New Zealand, *Tuatara: Journal of the Biological Society,* 17(2), 68-81.

Aniagu, S.O., Nwinyi, F.C., Akumka, D.D., Ajoku, G.A., Dzarma, S., Izebe, K.S., Ditse, M., Nwaneri, P.E.C., Wambebe, C. and Gamaniel, K. (2005). Toxicity studies in rats fed nature cure bitters, *African Journal of Biotechnology*, 4(1), 71-78.

Athanasiadou, S., Kyriazakis, I., Jackson, F. and Coop, R.L. (2000a). Effects of short-term exposure to condensed tannins on adult *Trichostrongylus colubriformis*, *Veterinary Research,* 146,728-732.

Athanasiadou, S., Kyriazakis, I., Jackson, F. and Coop, R.L. (2000b). Consequences oflong-term feeding with condensed tannins on sheep parasitized with

Trichostrongylus colubriformis, *International Journal of Parasitology*, 30, 1025-1033.

Badmanaban, R. and Patel, C.N. (2010). Studies on anthelmintic and antimicrobial activity of the leaf extracts of *Lagenaria siceraria Mol, Journal of Global Pharma Technology*, 2, 66-70.

Barry, T. N., McNeill, D. M. and McNabb, W. C. (2001). Plant secondary compounds; their impact on nutritive value and upon animal production, pp. 445–452. Proc. XIX Int. Grass. Conf., Sao Paulo, Brazil.

Bassani, V.L., Gnoatto, S.C.B. and Schenkel, E.P. (2005). HPLC method to assay total saponins in *Ilex paraguariensis* aqueous extract, *Journal of Brazilian Chemical Society*, 16(4), 723-726.

Batista, A.M., Mustafa, A.F., McKinnon, J.J. and Kermasha, S. (2002). In situ ruminal and intestinal nutrient digestibilities of mesquite (*Prosopis juliflora*) pods, *Animal Feed Science and Technology*, 100(1-2), 107-112.

Becker, K., Francis, G., Kerem, Z. and Makkar, H.P.S. (2002). The biological action of saponins in animal systems: a review, *British Journal of Nutrition*, 88, 587-605.

Behnke, J.M., Buttle, D.J., Stepek, G., Lowe, A. and Duce, R.I. (2008). Parasites and vectors, *Creative Commons Attribution*, 1(29), 1756-3305.

Burke, J.M., Wells, A., Casey, P. and Kaplan, R.M. (2009). Herbal dewormer fails to control gastrointestinal nematodes in goats, *Veterinary Parasitology, 160* (1-2), 168-70.

Burnouf-Radosevich, M. and Delfel, N.E. (1986). High-performance liquid chromatography of triterpene saponins, *Journal of Chromatography*, 368, 433-438.

Chitwood, D.J. (2002). Phytochemical based strategies for nematode control, *Annual Review of Phytopathology*, 40, 221-249.

Clarke, E.G.C. and Clarke, M.L. (1977). Veterinary toxicology, pp. 268-277. Casell and Collier Macmillan Publishers, London.

Coates, J. (2000). Interpretation of infrared spectra, a practical approach, in *Encyclopaedia of Analytical Chemistry*, R.A. Meyers (Ed.), pp. 10815-10837. © John Wiley and Sons Ltd, Chichester, United Kingdom.

Dawo, F. and Tibbo, M. (2005). Anthelmintic effect of *Halothamnus somalensis* in Arsi-Bale goats, *Livestock Research for Rural Development*, 17(6), 1-8.

Dave, S., Patil, U.K., Bhaiji, A., Baghel, U.S., Yadav, S.K. and Sharma, V.K. (2009). *In vitro* anthelmintic activity of leaves of *Spinacia oleracea* Linn., *International Journal of Toxicological and Pharmacological Research*, 1(1), 21-23.

Demirezer, L.O., Karahan, N., Ucakturk, E., Kuruuzum-Uz, A., Guvenalp, Z. and Kazaz, C. (2011). HPLC fingerprinting of sennosides in laxative drugs with isolation of standard substances from some senna leaves, *Records of Natural Products*, 5(4), 261-270.

Deore, S.L. and Khadabadi, S.S. (2010). *In vitro* anthelmintic studies of *Chlorophytum borivilianum Sant.* and *Fernandez* tubers, *Indian Journal of Natural Products and Resources*, 1(1), 53-56.

Ebenshade, H.W. and Grainger, A. (1980). The Bamburi reclamation project, *International Tree Crops Journal*, 1, 199-202.

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

Eguale, T., Tilahun, G., Debella, A., Feleke, A. and Makonnen, E. (2007). *In vitro* and *in vivo* anthelmintic activity of crude extracts of *Coriandrum sativum* against *Haemonchus contortus*, *Journal of Ethnopharmacology*, 110, 428-433.

Eguale, T., Tilahun, G., Gidey, M. and Mekonnen, Y. (2006). *In vitro* anthelmintic activities of four Ethiopian medicinal plants against *Haemonchu contortus, Pharmacology Online*, 3, 153-165.

Evangelista, L., Teixeira de Sousa P.F., Bastida, P.J. and Schmeda-Hirschmann G.G. (2002). Saponins from *Cariniana rubra* (Lecythidaceae), *Boletín de la Sociedad Chilena de Química*, 47(4), 441-447.

Falodun, A., Okunrobo, L.O. and Agbo, L.O. (2007). Evaluation of the anti-edematogenic activity of the aqueous extract of *Leea guineensis*, *African Journal of Biotechnology*, 6(9), 1151-1153.

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

Food and Agriculture Organization of the United Nations (FAO). Naturally occurring plant tannins as a means of controlling intestinal nematode infections in ruminants, http//:www.fao.org/teca/content/naturally-occurring-plant-tannins-means-controlling-intestinal-nematode-infections-ruminants. Accessed on March 11, 2011.

Gathuma, J.M., Mbaria, J.M., Wanyama, J., Kaburia, H.F., Mpoke, L., Mwangi, J.N. and Samburu and Turkana healers (2004). Efficacy of *Myrsine Africana*, *Albizia anthelmintica* and *Hilderbrantia sepalosa* herbal remedies against mixed natural sheep helminthosis in Samburu district, Kenya, *Journal of Ethnopharmacology*, 91(1), 7-12.

Geidam, Y.A., Ambali, A.G. and Onyeyili, P.A. (2007). Preliminary phytochemical and antibacterial evaluation of crude aqueous extract of *Psidium guajava* leaf, *Journal of Applied Sciences*, 7(4), 511-514.

Giknis, M.L.A. and Clifford, C.B. (2008). Clinical laboratory parameters for Crl:WI (Han), pp. 9. Charles Rivers Laboratories Preclinical Services Montreal Inc., Senneville, Quebec, Canada.

Giri, L., Andola, H.C., Purohit, V.K., Rawat, M.S.M., Rawal, R.S. and Bhatt, I.D. (2010). Chromatographic and Spectral Fingerprinting Standardization of Traditional Medicines: An Overview as Modern Tools, *Research Journal of Phytochemistry*, 4(4), 234-241.

Githiori, J.B. (2004). *Evaluation of anthelmintic properties of ethnoveterinary plant preparations used as livestock dewormers by pastoralists and smallholder*

farmers in Kenya, Ph.D. Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Githiori, J.B., Höglund, J., Waller, P.J. and Baker, R.L. (2003). Evaluation of anthelmintic properties of extracts from some plants used as livestock dewormers by pastoralist and smallholder farmers in Kenya against *Heligmosomoides polygyrus* infections in mice, *Veterinary Parasitology*, 118, 215-226.

Hansen, J. and Perry, B. (1994). The epidemiology, diagnosis and control of helminthes parasites of ruminants, pp. 62-63. International Laboratory for research on Animal Diseases (ILRAD), Nairobi, Kenya.

Hussain, A (2008). *Evaluation of anthelmintic activity of some ethnobotanicals,* PhD Thesis, University of Agriculture, Faisalabad, Pakistan.

Jesupillai, M. and Palanivelu, M. (2009). Anthelmintic activity of leaves of *Erythrina indica ,Lam, The Internet Journal of Alternative Medicine,* 7(1), 1.

Joint FAO/IAEA division of nuclear techniques in food and agriculture (Animal production and health sub-programme) (2000). *Quantification of tannins in tree foliage*, A laboratory manual for the FAO/IAEA co-ordinated research project on 'Use of nuclear and related techniques to develop simple tannin assays for predicting and improving the safety and efficiency of feeding ruminants on tanniferous tree foliage', IAEA, Vienna.

Kagira, J.M., Waruiru, R.M., Munyua, W.K. and Kanyari, P.W.N. (2003). Resistance to anthelmintics in commercial pig herds in Thika district, Kenya, *Israel Journal of Veterinary Medicine*, 58(1), 31-36.

Kakar, S.A., Tareen, R.B., Sandhu, Z.U., Azam, M.K., Saeed, U.K., Zafar, I. and Jabeen, H. (2013). *In vitro* and *in vivo* anthelmintic activity of *Ferula costata* (Kor.) against gastrointestinal nematodes of sheep, *Pak. J. Bot.*, 45(SI), 263-268.

Kannabiran, K., Khanna, V.G. and Getti, G. (2009). Leishmanicidal activity of saponins isolated for the leaves of *Eclipta prostrata* and *Gymnema sylvestre*, *Indian Journal of Pharmacology*, 41(1), 32-35.

Kaplan, R.M., Mortensen, L.L., Williamson, L.H., Terrill, T.H., Kircher, R.A. and Larsen, M. (2003). Evaluation of prevalence and clinical implications of anthelmintic resistance in gastrointestinal nematodes in goats, *Journal of American Veterinary Medicine Association*, 223(4), 495-500.

Kareru, P.G. (2008). *Ethnomedicine practices, analysis and standardization of a herbal anthelmintic drug used by the Embu and Mbeere peoples of Kenya,* PhD thesis, Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya.

Kareru, P.G., Keriko, J.M., Gachanja, A.N., and Kenji, G.M. (2008). Direct detection of triterpenoid saponins in medicinal plants, *African Journal of Traditional, Complementary and Alternative Medicines*, 5(1), 56-60.

Kareru, P.G., Ombasa, O., Rukunga, G., Mbaria, J., Keriko, J.M., Njonge, F.K. and Owuor, B.O. (2012). *In vitro* anthelmintic effects of two Kenyan plant extracts against *Haemonchus contortus* adult worms, *International Journal of Pharmacological Research*, 2(3), 113-115.

Keiser, J., Tritten, L., Adelfio, R. and Vargas, M. (2012). Effect of combinations of marketed human anthelmintic drugs against *Trichuris muris in vitro* and *in vivo*, *Parasites and Vectors*, 5, 292.

Khan, R.A., Riaz, A. Ahmed, S. and Afroz, S. (2010).Assessment of acute toxicity and reproductive capability of a herbal combination, *Pakistan Journal of Pharmaceutical Sciences*, 23(3), 291-294.

Kim, Y.S., Ha, Y.W., Na, Y.C., Seo, J.J., Kim, S.N. and Linhardt, R.J. (2006). Qualitative and quantitative determination of ten major saponins in *Platycodi radix* by high performance liquid chromatography with evaporative light scattering detection and mass spectrometry, *Journal of Chromatography A*, 1135, 27-35.

Komen, C., Kiprono, P.C. and Wanjala, F.M. (2005). Efficacy of *Jasminum abyssinicum* treatment against *Haemonchus contortus* in sheep, *African Journal of Traditional, Complementary and Alternative Medicines*, 2(3), 264-268.

Krimpen, V.M.M., Binnendijk, G.P., Borgsteede, F. and Gaasenbeek, C. (2008). Effect of some herbs as alternative for conventional treatment of *Ascaris Suum* in pigs, *Report 169 for Animal Sciences Group van Wageningen UR.*

Kumar, S.V.S. and Mishra, S.H. (2009). Hepatoprotective activity of *Baliospermum montanum* (willd) Muell.-Arg. in rats treated with carbon tetrachloride; *in vivo* and *in vitro* studies, *Pharmacognosy Magazine,* 5(19), 196-202.

Kumsa, B., Tolera, A. and Nurfeta, A. (2010). Comparative efficacy of seven brands of Albendazole against naturally acquired gastrointestinal nematodes in sheep in Hawassa, Southern Ethiopia, *Turkish Journal of Veterinary Animal Science,* 34(5), 417-425.

Lahlou, M. (2004). Methods to study the phytochemistry and bioactivity of essential oils, *Phytotherapy Research,* 18, 435-448.

Lalchhandama, K. (2009). Cestocidal activity of *Acacia caesia* stem bark on *Raillietina echinobothrida, Pharmacognosy Research,* 1(4), 179-184.

Lanusse, C.E., Prichard, R.K. (1993). Relationship between pharmacological properties and clinical efficacy of ruminant anthelmintics, *Veterinary Parasitology*, 49, 123-158.

Lateef, M., Iqbal, M., Rauf, U. and Jabbar, A. (2006). Anthelmintic activity of *Carum capticum* seeds against gastro-intestinal nematodes of sheep, *J. Anim. Pl. Sci.*, 16, 35-36.

Lateef, M., Zafar, I., Khan, M.N., Akhtar, M.S. and Jabbar, A. (2003). Anthelmintic activity of *Adhatoda vesica* roots, *International Journal of Agriculture and Biology,* 5(1), 86-89.

Levecke, B., Dobson, R.J., Speybroeck, N., Vercruysee, J. and Charlier, J. (2012). Novel insights in the feacal egg count reduction test for monitoring drug efficacy against gastrointestinal nematodes of veterinary importance, *Veterinary Parasitology,* 188(3-4), 391-396.

Liang, Y., Xie, P. and Chan, K. (2004). Quality control of herbal medicines, *Journal of Chromatography B,* 812, 53-70.

Lorke, D. (1983). A new approach to practical acute toxicity testing, *Archives of Toxicology*, 54(4), 275-287.

Maghembe, J.A., Kariuki, E.M. and Haller, R.D. (1983). Biomass and nutrient accumulation in young *Prosopis juliflora* at Mombasa, Kenya, *Agroforestry Systems*, 1, 313-321.

Masilamani, P. and Vadivelu, K.K. (1997). Seed Development and Maturation in Honey Mesquite (*Prosopis juliflora* Swartz. DC.), *Bangladesh Journal of Forest Science*, 26(1), 68-73.

Matthews, S. and Brand, K. (2004). Africa invaded. The growing danger of invasive alien species, *The Global Invasive Species Programme*, pp. 28-29.

Matthee, S. (2003). Anthelmintic treatment in horses: the extra-label use of products and the danger of under-dosing, *Journal of the South African Veterinary Association*, 74(3), 53-56.

Maurer, V., Cabaret, J., Hertzberg, H., Langhans, W. and Hördegen, P. (2006). In vitro screening of six anthelmintic plant products against larval *Haemonchus contortus* with a modified methyl-thiazolyl-tetrazolium reduction assay, *Journal of Ethnopharmacology*, 108, 85-89.

Mbaka, G.O., Adeyemi, O.O. and Oremosu, A.A. (2010). Acute and sub-chronic toxicity studies of the ethanol extract of the leaves of *Sphenocentrum jollyanum* (Menispermaceae), *Agriculture and Biology Journal of North America*, 1 (3), 265-272.

Min, B.R. and Hart, S.P. (2003). Tannins for suppression of internal parasites, *Journal of Animal Science*, 81(E. Suppl. 2), E102-E109.

Mohammed, M.S., Mohammed, M., Yusuf, O.A. and Ologunja, J.A. (2005). Anthelmintic activity of the crude methanol extract of *Xylopia aethiopica* against *Nippostrongylus braziliensis* in rats, *Veterinarski Arhiv*, 75 (6), 487-495.

Monglo, D., Njongmeta, L.M., Musongong, G., Ngassoum, M. and Nukenine, E.N. (2006). Evaluation of anthelmintic potential of ethanolic plant extracts from

Northern Cameroon against eggs and infective larvae of *Haemonchus contortus*, *Journal of Biological Sciences,* 6, 426-433.

Monteiro, A.M., Wanyangu, S., Kariuki, D.P., Bain, R., Jackson, F. and McKellar, Q.A. (1998). Pharmaceutical quality of anthelmintics sold in Kenya, *Veterinary Research,* 142, 396–398.

Mounnissamy, V.M., Kavimani, S., Sankari, G., Quine, S.D. and Subramani, K. (2010). Evaluation of acute and sub-acute toxicity of ethanol extracts of *Cansjera rheedii* J. Gmelin (Opiliaceae), *Journal of Brewing and Distilling,* 1 (1), 011-014.

Mukinda, J.T. (2005). *Acute and chronic toxicity of the flavonoid-containing plant, Artemisia afra in rodents,* MSc Thesis, School of Pharmacy, University of the Western Cape, Bellville, South Africa.

Nalule, A.S., Karue, C.N. and Katunguka-Rwakishaya, E. (2011). Anthelmintic activity of *Phytolacca dodecandra* and *Vernonia amygdalina* leaf extracts in naturally infected small East African goats, *Livestock Research for Rural Development*, 23(12).

Negi, J.S., Singh, P., Pant, G.J.N. and Rawat, M.S.M. (2011). High performance liquid chromatography analysis of plant saponins: An update 2005-2010, *Pharmacognosy Review,* 5(10), 155-158.

Netpana, N., Wanapat, M., Poungchompu, O. and Toruban, W. (2001). *Effect of condensed tannins in cassava hay on fecal parasitic egg counts in swamp buffaloes and cattle,* In proceedings of International workshop current research and development on use of cassava as animal feed held in July 23-25, 2001 in Khon Kaen University, Thailand.

Niezen, J.H., Charleston, W.A.G., Robertson, H.A., Shelton D., Waghorn, G.C. and Green, R. (2002). The effect of feeding sulla (*Hedysarum coronarium*) or lucerne (*Medicago sativa*) on lamb parasite burdens and immunity to gastrointestinal nematodes, *Veterinary Parasitology,* 105, 229-245.

Njoroge, G.N. and Bussmann, R.W. (2006). Herbal usage and informant consensus in ethnoveterinary management of cattle diseases among the Kikuyu (Central Kenya), *Journal of Ethnopharmacology*, 108(3), 332-339.

Nonaka, G. (1989). Isolation and structure elucidation of tannins, *Pure and Applied Chemistry*, 61(3), 357-380.

Obadoni, B. and Ochuko, P. (2001). Phytochemical studies and comparative efficacy of the crude extracts of some homeostatic plants in Edo and Delta States of Nigeria, Global, *Journal of Pure and Applied Science,* 8, 203-208.

Organization for Economic Co-operation and Development, OECD/OCDE (2000). Guidance document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation, pp. 21. Environment Directorate, OECD, Paris, France.

Organization for Economic Co-operation and Development, OECD/OCDE (2001). OECD guideline for testing of chemicals (Acute oral toxicity-fixed dose procedure), *Revised guideline 420 as adopted by the council on 17th December 2001.*

Organization for Economic Co-operation and Development, OECD/OCDE (2001). OECD guideline for testing of chemicals (OECD 425. Acute Oral Toxicity-Revised Up and Down procedure), Organization for Economic Co-operation and Development, Paris.

Oleszek, W.A. (2002). Chromatographic determination of plant saponins, *Journal of Chromatography A,* 967, 147-162.

Olorunfemi, C.J., Ologunja, J.A., Adaudi, A.O. and Agbede, R.I.S. (2006). Anthelmintic efficacy of extracts of *Spigelia anthelmia linn* on experimental *Nippostrongylus braziliensis* in rats, *Journal of Veterinary Science,* 7(3), 229-232.

Orisakwe, O.E., Afonne, O.J., Chude, M.A., Obi, E. and Dioka, C.E. (2003). Sub-chronic toxicity studies of the aqueous extract of *Boerhavia diffusa* leaves, *Journal of Health Science,* 49(6), 444-447.

Padgham, J.. Internal parasites and ruminants: Organic answers, retrieved from www.mosesorganic.org.

Pasiecznik, N.M., Felker, P., Harris, P.C., Harsh, L.N., Cruz, G., Tewari, J.C., Cadoret, K. and Maldonado, L.J. (2001). The *Prosopis juliflora-Prosopis pallida* complex: A monograph, *HDRA, Coventry, UK*, pp. 172.

Pasiecznik, N.M., Harris, P.C. and Smith, S.J. (2004). *Identifying Tropical Prosopis Species: A Field Guide, London HDRA.*

Patil, P.S. and Shettigar, R. (2010). An advancement of analytical techniques in herbal research, *Journal of Advanced Scientific Research,* 1(1), 08-14.

Patra, K.C., Pareta, S.K., Harwansh, R.K. and Kumar, K.J. (2010). Traditional approaches towards standardization of herbal medicines-A review, *Journal of Pharmaceutical Science and Technology*, 2(11), 372-379.

Penlap, V.B., Assam Assam, J.P., Dzoyem, J.P. and Pieme, C.A. (2010). *In vitro* antibacterial activity and acute toxicity studies of aqueous-methanol extract of *Sida rhombifolia* Linn. (Malvaceae), *BMC Complementary and Alternative Medicine*, 10, 40 doi: 10.1186/1472-6882-10-40.

Prichard, R. (1994). Anthelmintic resistance, *Veterinary Parasitology*, 54, 259-268.

Radosevich, M.B. and Delfel, N.E. (1986). High-performance liquid chromatography of triterpene saponins, *Journal of Chromatography A,* 368, 433-438.

Raimundo, B., Viana, F.A., Pouliquen, Y.B.N., Neto, M.A., Santiago, G.M.P., and Rodrigues-Filho, E. (2004). Triterpenoid saponins from the stem bark of *Pentaclethra macroloba*, *Journal of the Brazilian Chemical Society*, 15(4), 595-602.

Ramalingam, R., Bindu, K.H., Madhavi, B.B., Nath, A.R. and Banji, D. (2010). Phytochemical and anthelmintic evaluation of corm of *Amorphophallus campanulatus*, *International Journal of Pharma and Bio Sciences,* 1(2), 1-9.

Research Animal Resources, University of Minnesota. Reference values for laboratory animals, www.ahc.umn.edu/rar/refvalues.html. Accessed on July 13, 2013.

Robson, R.B., López, J.A., Santos, L.C., Zacharias, F., David, J.M., David, J.P. and Lima, F.W.M. (2012). Biological effect of leaf aqueous extract of *Caesalpinia pyramidalis* in goats infected with gastrointestinal nematodes, *Evidence-Based Complementary and Alternative medicine,* 2012(2012), http://dx.doi.org/10.1155/2012/510391.

Salawu, O.A., Aliyu, M. and Tijani, A.Y. (2008). Hematological studies on the ethanolic stem bark extract of *Pterocarpus erinaceus* Poir (Fabaceae), *African Journal of Biotechnology*, 7(9), 1212-1215.

Saraf, S., Saraf, S. and Shukla, S.S. (2009). Approaches towards standardization and quality assessment of herbals, *J. Res. Educ. Indian Med.,* XV (1), 25-32.

Sasidharan, S., Rajoo, A., Ramanathan, S. and Mansor, S.M. (2010a). Standardization of *Elaeis guineensis* with respect to authencity, assay and chemical constituent analysis, *African Journal of Biotechnology*, 9 (44), 7544-7549.

Sasidharan, S., Torey, A., Yeng, C. and Latha, L.Y. (2010b). Standardization of *Cassia spectabilis* with Respect to Authenticity, Assay and Chemical Constituent Analysis, *Molecules*, 15, 3411-3420.

Satrija, F., Retnani, E.B., Ridwan, Y. and Tiuria, R. (2001). *Potential use of herbal anthelmintics as alternative antiparasitic drugs for small holder farms in developing countries,* In proceedings of the 10th conference of the association of institutions for tropical veterinary medicine held in 2001 in Copenhagen, Denmark.

Scheuerle, M. (2009). *Anthelmintic resistance of Haemonchus contortus and the FAMACHA®-method as a tool to delay the development of anthelmintic resistance,* PhD thesis, Universität München, Germany.

Sheng, Y.X., Zhang, J.L., Sun, S.Q. and Xu, J.T. (2006). Quality analysis and evaluation of *Rhizoma coptidis* under different cultivation conditions, Acta. *Pharm. Sin.* 41, 1010-1014.

Sirma, P.K. (2009). *Towards valorization of Prosopis juliflora as an alternative to the declining wood resources in Kenya*, PhD Thesis, Université Henri Poincaré, Nancy I, France.

Solíz-Guerrero, J.B., Rodriguez, D.J., Rodriguez-García, R., Angulo-Sánchez, J.L. and Méndez-Padilla, G. (2002). Quinoa saponins: Concentration and composition analysis, pp110-114. J. Janick and A. Whipskey (eds.), Trends in new crops and new uses, ASHS Press, Alexandria, VA.

Sujon, M.A., Mostofa, M., Jahan, M.S., Das, A.R. and Rob, S. (2008). Studies on medicinal plants against gastrointestinal nematodes of goats, *Bangladesh Journal of Veterinary Medicine*,6 (2), 179-183.

Suleiman, M.M., Mamman, M., Aliu, Y.O. and Ajanusi, J.O. (2005). Anthelmintic activity of the crude methanol extract of *Xylopia aethiopica* against *Nippostrongylus brasiliensis* in rats, *Veterinarski Arhiv*, 75(6), 487-495.

Talpada, P.M. and Shukla, P.C. (1988). Study on Sugar and Amino Acids Composition of *Prosopis juliflora* Pods, *Gujarat Agricultural University Research Journal*, 14(1), 32-35.

Tandon, V., Yadav, A.K., Roy, B., and Das, B. (2011). Phytochemicals as cure of worm infections in traditional medicine systems, *Emerging Trends in Zoology*, 351-378.

Tardiff, R.G. (1978). *In vitro* methods of toxicity evaluation, *Annual Review of Pharmacology and Toxicology*, 18, 357-369.

Tarpoff, A.J. (2010). *Comparative efficacy of two ivermectin pour-on anthelmintics in beef steers in a commercial feed yard*, MSc. Thesis, Kansas State University, Manhattan, Kansas, United States of America.

Thoithi, G.N., Maingi, N., Karume, D., Gathumbi, P.K., Mwangi, J.W. and Kibwage, I.O. (2002). Anthelmintic and other pharmacological activities of the root bak extracts of *Albizia anthelmintica* Brongn, *East and Central African Journal of Pharmaceutical Sciences*, 5, 60-66.

Tong, L., Wang, Y., Xiong, J., Cui, Y., Zhou, Y.G. and Yi, L. (2008). Selection and fingerprints of the control substances for plant drug *Eucommiaulmodies* Oliver by HPLC and LC-MS, *Talanta*, 76, 80-84.

Umezawa, T. (2001).Chemistry of extractives. Wood and cellulosic chemistry. Ed New York Marcel Dekker, Inc., 2, 213-241.

Váraday, M., Königová, A., Čerňanská, D. and Čorba, J. (2005). Effect of combined therapy of an anthelmintic and an immunomodulator on the elimination of gastrointestinal nematodes in sheep, *Helminthologia,* 42(3), 133-136.

Viqar, U.A., Iqbal, S., Kousar, R., Bader, S., Arshad, S and Tareen, R.B. (2005). Two new saponins from *Zygophyllum atriplicoides*, *Chemical and Pharmaceutical Bulletin* 53 (9), 1126–1130.

Waghorn, G.C., Charleston, W.A.G., Niezen, J.H. and Robertson, H. (1995). Effects of condensed tannins on parasitism in lambs. In: Novel approaches to the control of helminthes parasites, of livestock, pp. 56. University of New England, Armidale, NSW, Australia.

Wamburu, R.W., Kareru, P.G., Mbaria, J.M., Njonge F.K., Nyaga, G. and Rechab, S.O. (2013). Acute and sub-acute toxicological evaluation of ethanolic leaves extract of *Prosopis juliflora* (Fabaceae), *Journal of Natural Sciences Research*, 3(1), 8-11.

Wedge, D.E., Klun, J.A., Tabanca, N., Demirci, B., Ozek, T., Baser, K.H.C., Liu, Z., Zhang, S., Cantrell, C.L. and Zhang, J. (2009). Bioactivity-guided fractionation and GC-MS fingerprinting of *Angelica sinensis* and *Angelica archangelica* root components for antifungal and mosquito deterrent activity, *Journal of Agricultural and Food Chemistry*, 57, 464-470.

Wood, I. B., Amaral, N. K., Bairden, K., Duncan, J. L., Kassai, T., Malone, J. B., Jr., Pankavich, J. A., Reinecke, R. K., Slocombe, O. and Taylor, S. M. (1995). World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine), *Veterinary Parasitology,* 58, 181-213.

Worku, M., Franco, R. and Miller, J.H. (2009). Evaluation of the activity of plant extracts in Boer goats, *American Journal of Animal and Veterinary Sciences*, 4(4), 72-79.

Xiao-he, X., Tian-tian, Z. and Hai-long, Y. (2011). Quality control approaches for Chinese herbal medicines, *Chinese Herbal Medicines*, 3(1), 17-22.

Zafar, I., Abdul, J., Muhammad, S.A., Ghulam, M. and Muhammad, L. (2005). Possible role of ethnoveterinary medicine in poverty reduction in Pakistan: use of botanical anthelmintics as an example, *Journal of Agriculture and Social Science*, 1 (2), 189.

Zafar, I., Bachaya, H.A., Nisar, M.K., Jabbar, A., Gilani, A.H., and Islam, U.D. (2009). *In vitro* and *in vivo* anthelmintic activity of *Terminalia arjuna* bark, *International Journal of Agriculture and Biology*, 11(3), 273-278.

Zafar, I., Mufti, K.A. and Nisar, M.K. (2002). Anthelmintic effects of condensed tannins, *International Journal of Agriculture and Biology*, 4(3), 438-440.

Zafar, I., Muhammad, S., Abdul, J., Shahbaz, A., Nisa, M., Muhammad, S., Muhammad, N.K., Kamran, A.M. and Muhammad, Y. (2007). Direct and indirect anthelmintic effects of condensed tannins in sheep, *Veterinary Parasitology*, 144(1-2), 125-131.

Zang, X (2000). General guidelines for methodologies on research and evaluation of traditional medicine, World Health Organization (WHO), pp. 1-71.

APPENDICES

Appendix I: Infrared spectra profiles of crude saponin extracts of *E. leptostachya*, *P. juliflora* and crude saponin extract mixture 1:7 (*E. leptosctachya:P. julifora*)

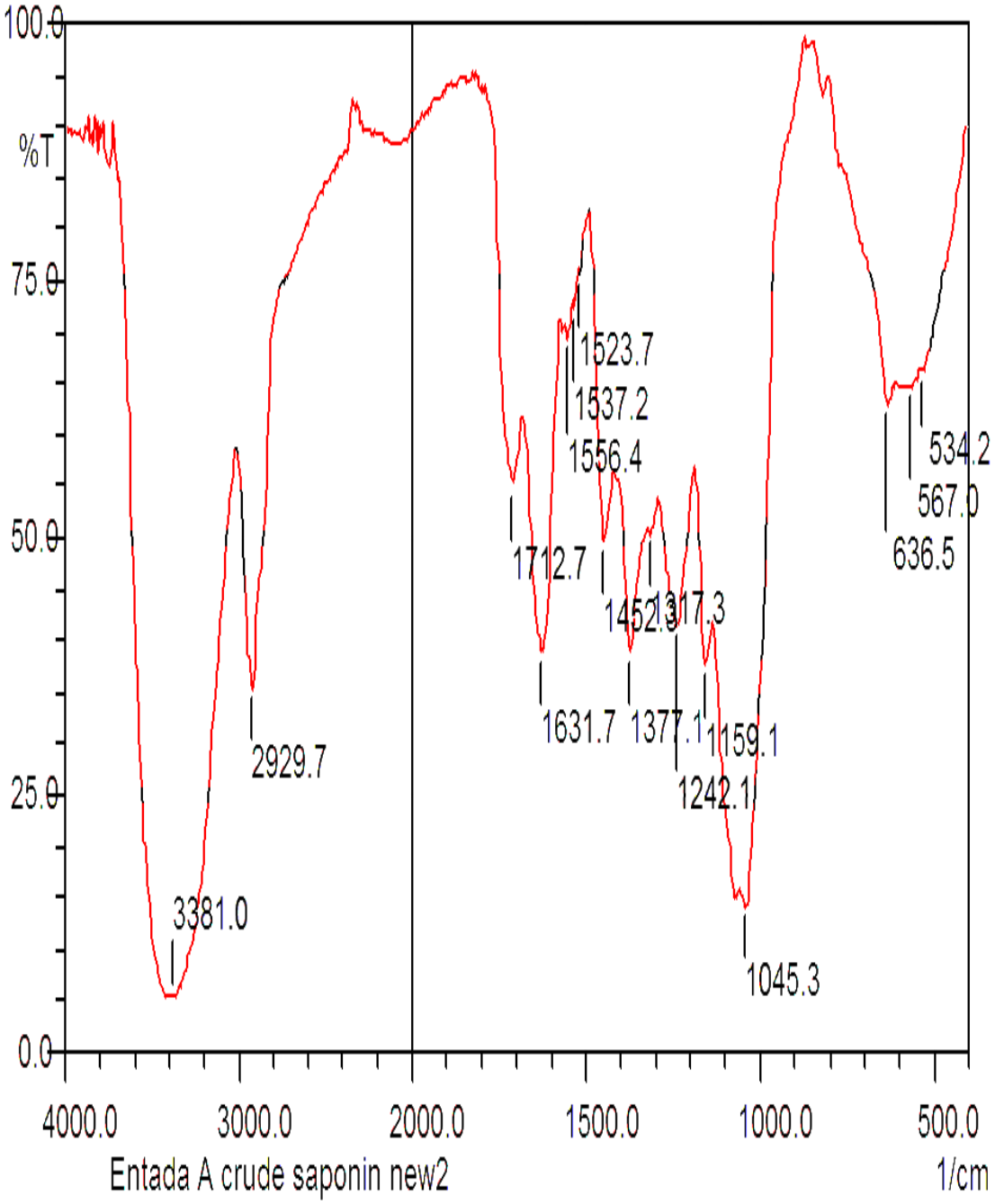


Figure I(a): Infrared spectrum of crude saponin extract of *E. leptostachya*

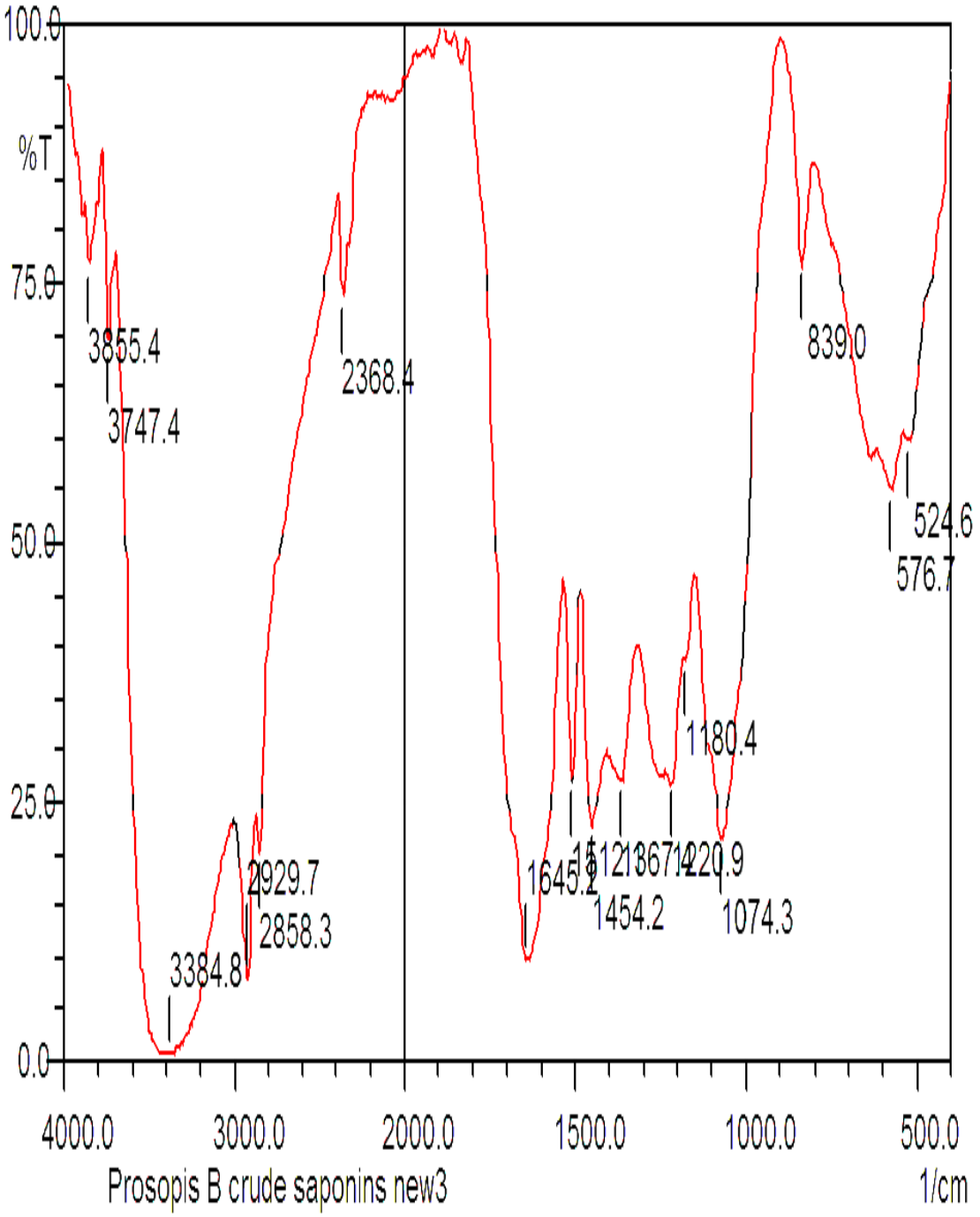


Figure I(b): Infrared spectrum of crude saponin extract of *P. juliflora*

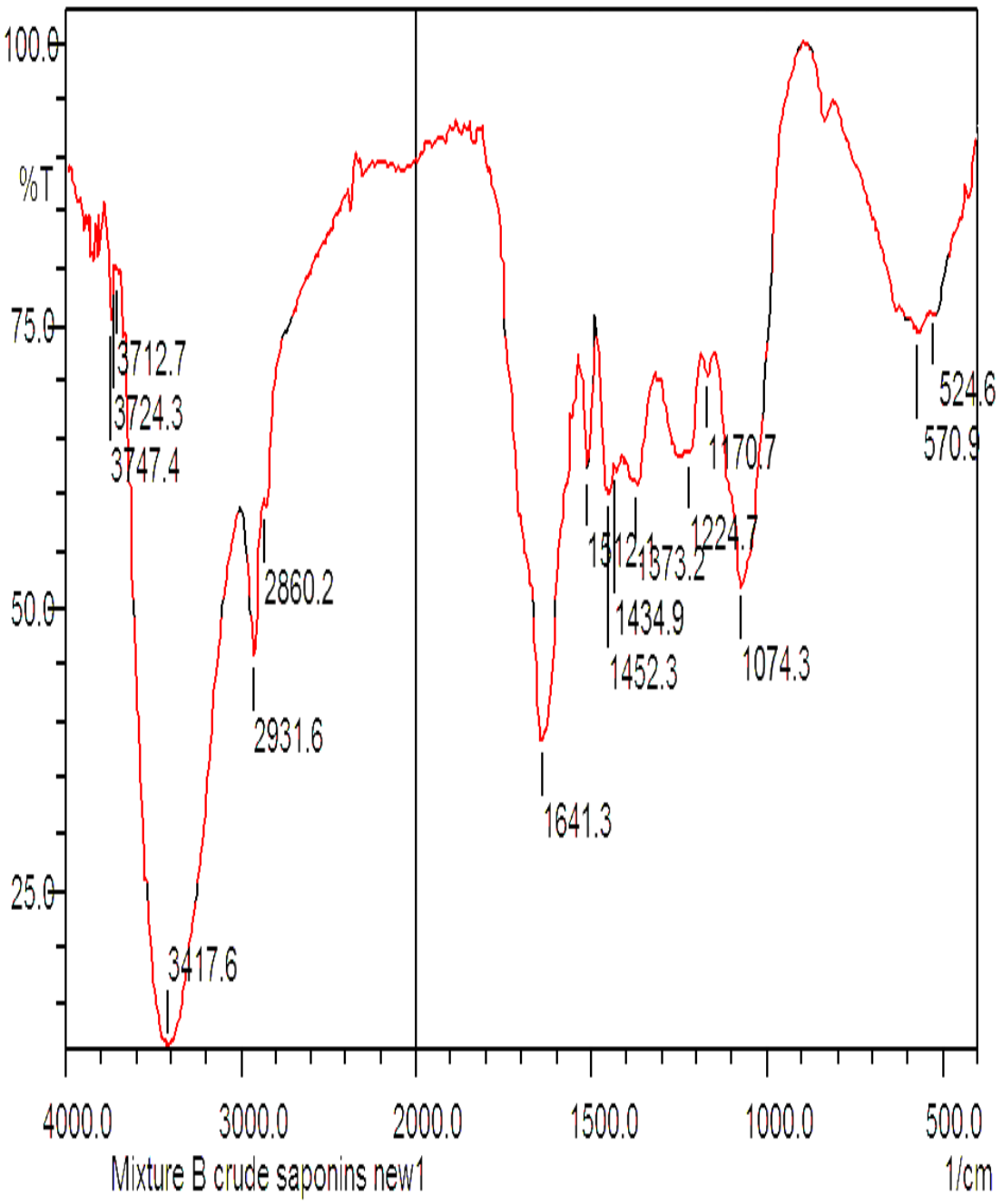


Figure I(c): Infrared spectrum of crude saponin extract of mixture 1:7 (*E. leptostachya:P. juliflora*)

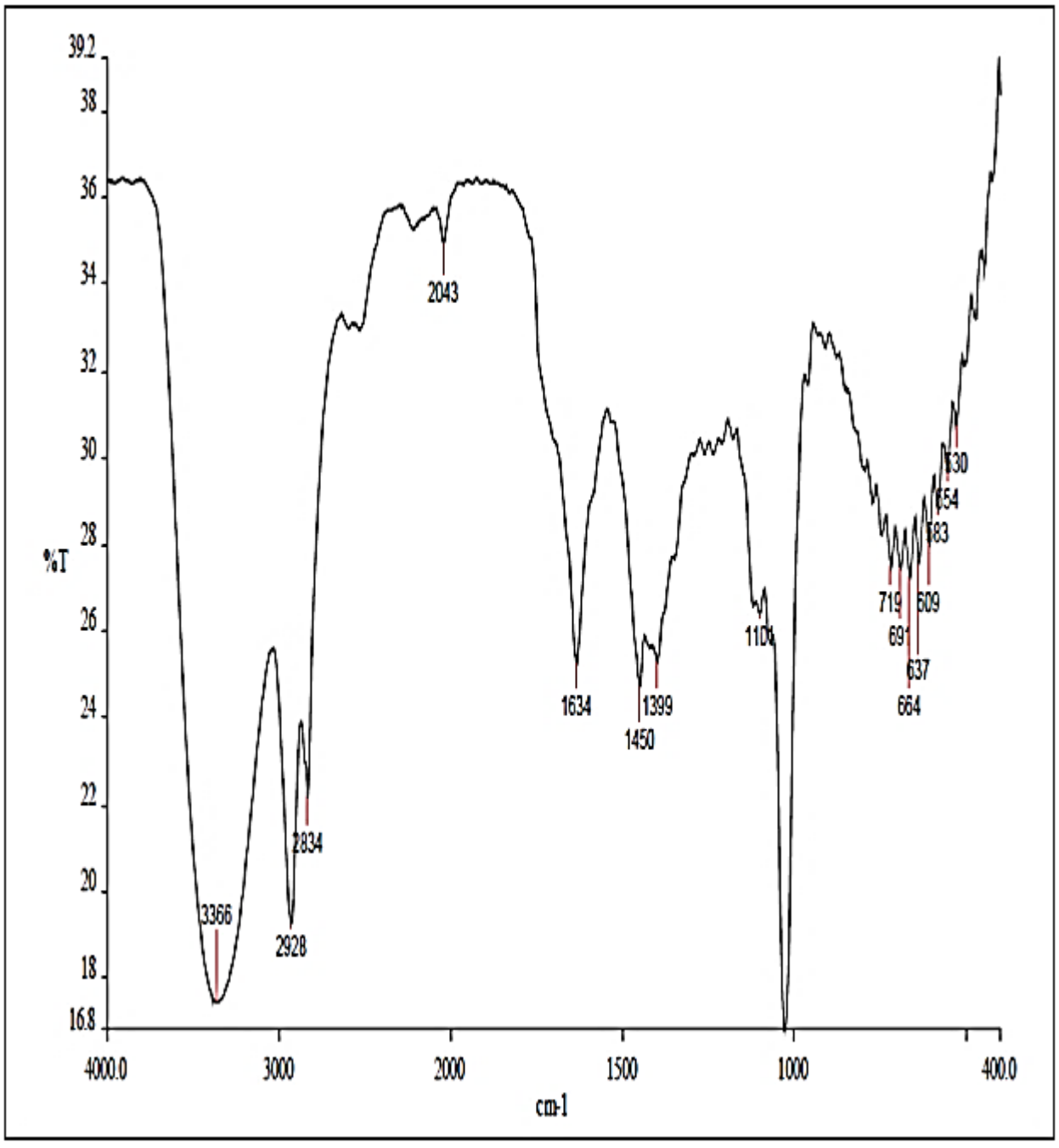
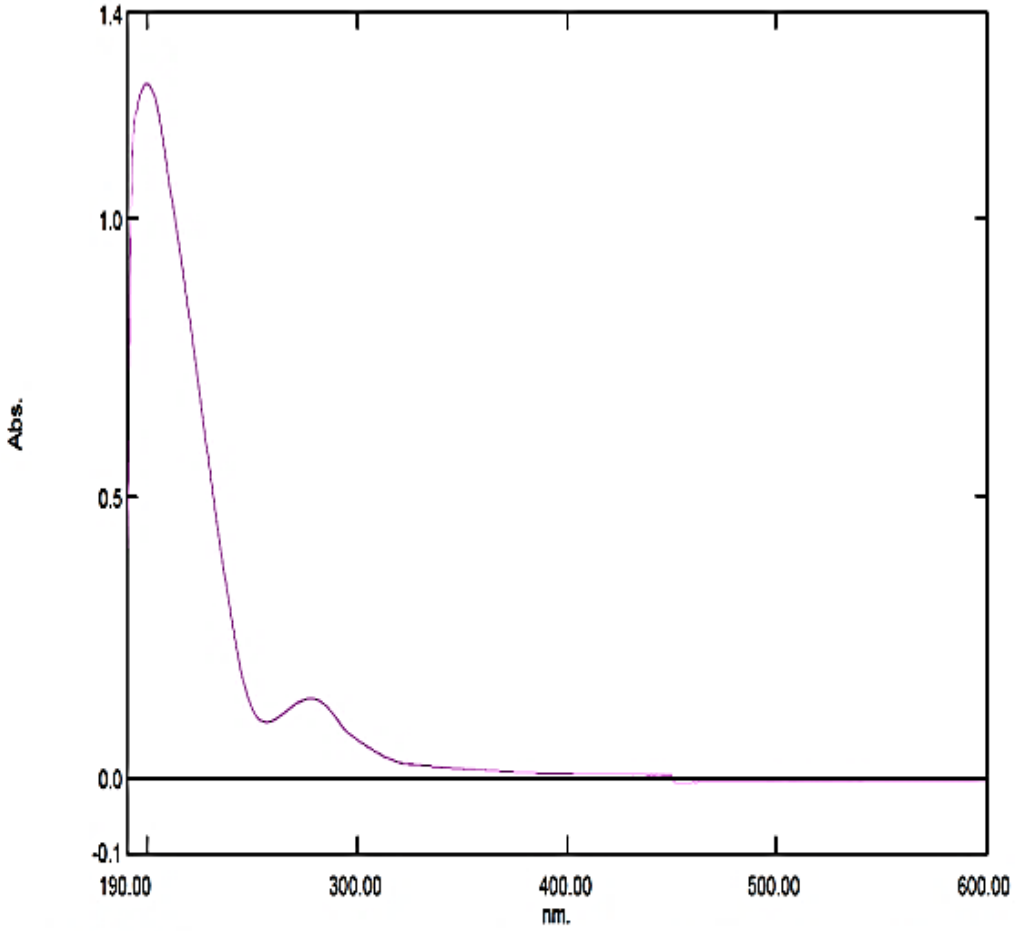


Figure I(d): Infrared spectrum of methanolic extract of *Cassia spectabilis* (KBr disc), cm^{-1} (Source: Sasidharan *et al.*, 2010b)

Appendix II: Ultraviolet spectra profiles of crude saponin extracts of *E. leptostachya, P. juliflora* and crude saponin extract mixture 1:7 (*E. leptostachya:P. juliflora*)

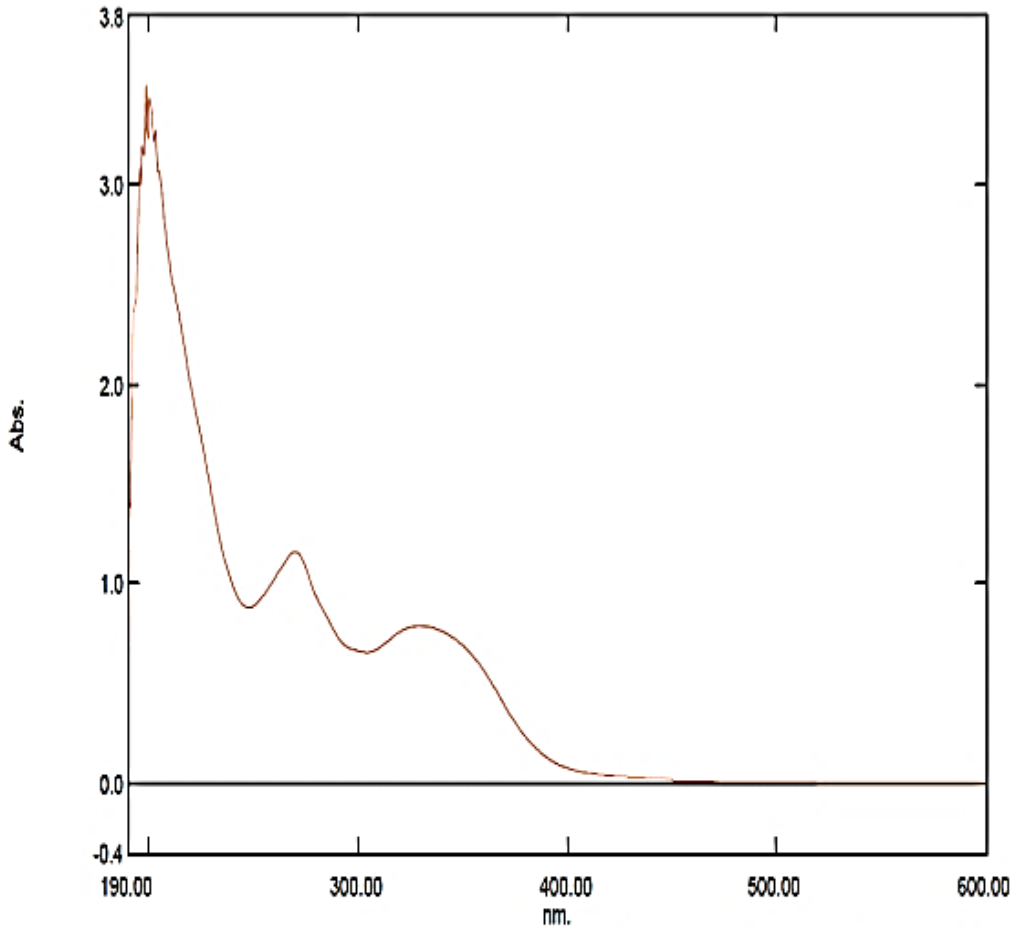


Measurement Properties	
Wavelength Range (nm.):	190.00 to 600.00
Scan Speed:	Fast
Sampling Interval:	0.5
Auto Sampling Interval:	Enabled
Scan Mode:	Auto

Instrument Properties	
Instrument Type:	UV-1800 Series
Measuring Mode:	Absorbance
Slit Width:	1.0 nm
Light Source Change Wavelength:	340.0 nm
S/R Exchange:	Normal

No.	P/V	Wavelength	Abs.	Description
1	↑	277.50	0.1	
2	↑	199.50	1.2	
3	↓	451.50	-0.0	
4	↓	257.00	0.1	

Figure II(a): UV spectrum of crude saponins from *Entada leptostachya*

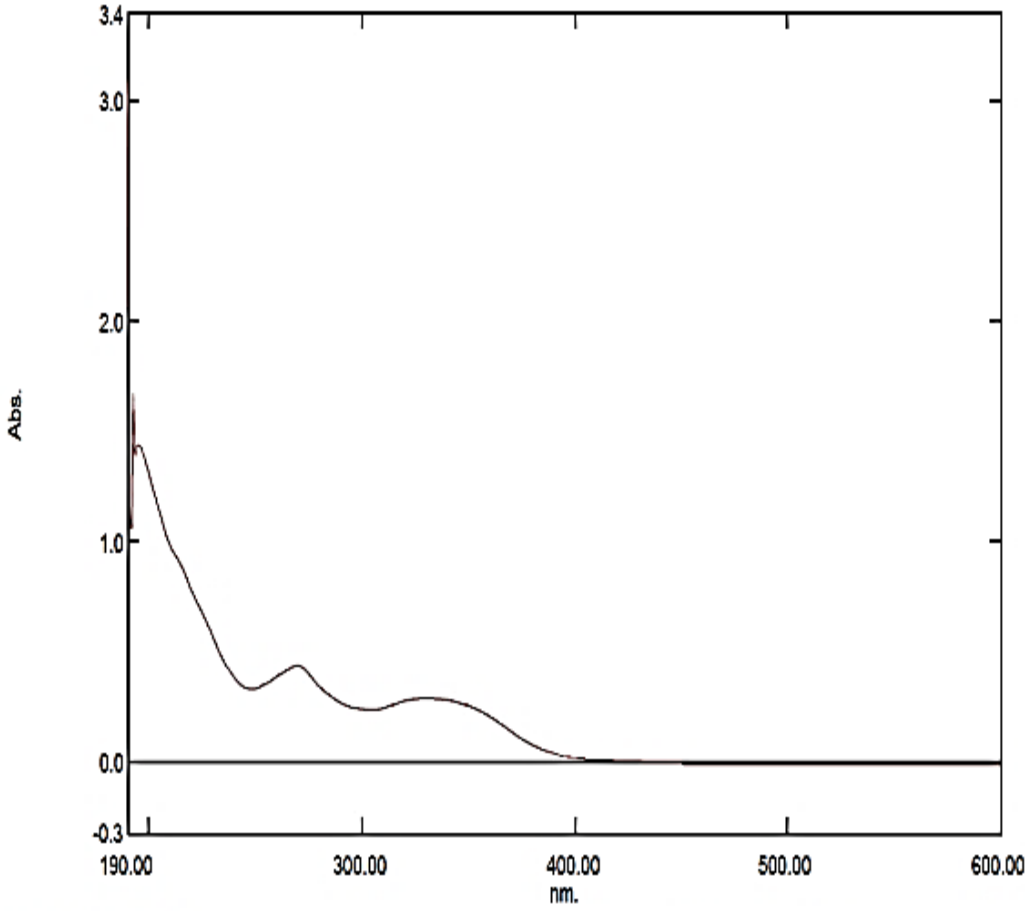


Measurement Properties	
Wavelength Range (nm.):	190.00 to 600.00
Scan Speed:	Fast
Sampling Interval:	0.5
Auto Sampling Interval:	Enabled
Scan Mode:	Auto

Instrument Properties	
Instrument Type:	UV-1800 Series
Measuring Mode:	Absorbance
Slit Width:	1.0 nm
Light Source Change Wavelength:	340.0 nm
S/R Exchange:	Normal

No.	P/V	Wavelength	Abs.	Description
1	↑	329.50	0.8	
2	↑	269.50	1.2	
3	↑	198.50	3.5	
4	↓	304.50	0.7	
5	↓	247.50	0.9	

Figure II(b): UV spectrum of crude saponins of *Prosopis juliflora*



Measurement Properties	
Wavelength Range (nm.):	190.00 to 600.00
Scan Speed:	Fast
Sampling Interval:	0.5
Auto Sampling Interval:	Enabled
Scan Mode:	Auto

Instrument Properties	
Instrument Type:	UV-1800 Series
Measuring Mode:	Absorbance
Slit Width:	1.0 nm
Light Source Change Wavelength:	340.0 nm
S/R Exchange:	Normal

No.	P/V	Wavelength	Abs.	Description
1	↑	330.50	0.3	
2	↑	269.50	0.4	
3	↓	451.50	-0.0	
4	↓	304.50	0.2	
5	↓	248.00	0.3	

Figure II(c): UV spectrum of crude saponins of mixture ratio 1:7 (*E. leptostachya:P. juliflora*)

Appendix III: Percentage (%) mean egg inhibition of aqueous herbal extract mixture ratios (*E. leptostachya, En*:*P. juliflora, Pro*) at concentrations of 0.4, 0.8, 1.6 and 3.2mg/ml

	Concentration of herbal extract mixtures (*En:Pro*) in mg/ml			
Sample	**0.4**	**0.8**	**1.6**	**3.2**
1:2	50.59	78.83	97.67	95.27
1:3	83.56	81.16	85.89	95.27
1:4	76.50	74.10	92.94	90.61
1:5	71.77	88.21	97.67	95.27
1:6	52.93	69.44	90.61	90.61
1:7	59.99	95.27	95.27	90.61
1:8	76.50	69.44	83.56	95.27
1:9	76.50	83.56	95.27	97.67
2:1	22.37	48.27	41.21	50.60
3:1	64.71	36.49	81.16	92.94
4:1	48.27	55.33	69.44	95.27
5:1	69.44	67.04	92.94	88.21
6:1	48.27	45.87	95.27	100
7:1	59.99	50.60	90.61	97.67
8:1	64.71	59.99	69.44	95.27
9:1	59.99	83.56	92.94	97.67
1:1	22.37	43.54	55.33	64.71
Albendazole	83.56	83.56	90.61	92.94

Appendix IV: Effect of herbal extract mixture ratio 1:7 (*E. leptostachya:P. juliflora)* on live body weights (in grams) of rats

Dose (mg/kg b.w)	DAY 0	DAY 7	DAY14
Control	196.89	219.19(11.3%)	217.86(10.7%)
175	169.95	189.65(11.6%)	192.93(13.5%)
550	174.95	194.84(11.4%)	212.86(21.7%)
1750	176.85	183.89(4.0%)	201.44(13.9%)
5000	165.18	203.45(23.2%)	189.12(14.5%)
5000	167.05	189.95(13.7%)	211.59(26.7%)
5000	167.92	195.02(16.1%)	209.87(25.0%)

The % values in parentheses indicate the corresponding percentage weight gains from day 0.

Appendix V: Ethics committee approval letter

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY
OFFICE OF THE DIRECTOR, PRODUCTION SERVICES
(RESEARCH, PRODUCTION AND EXTENSION DIVISION)

INTERNAL MEMO

DATE: 22nd JULY 2014

TO: Prof. Patrick G. Kareru – Chemistry Department

FROM: Director, Production Services **REF:** JKU/2/4/IPC/IP/013

RE: APPROVAL OF FUNDING FOR PROJECT PROPOSAL: 'DEVELOPMENT AND EVALUATION OF HERBAL ANTHELMINTIC DRUGS FOR RUMINANT ANIMALS'
Ref: JKU/2/4/IPC/IP/013

This is to confirm that prior to funding by the University, the above innovation proposal was subjected to peer-review and approval by the Ethics Sub-committee of the Innovations and Production Committee of Senate, Jomo Kenyatta University of Agriculture & Technology during the year 2009.

Prof. Martin Obanda
Director- Production Services

Copy to: DVC, RPE

Appendix VI: Published paper on *in vivo* (FECR) studies

IOSR Journal of Applied Chemistry (IOSR-JAC)
e-ISSN: 2278-5736.Volume 7, Issue 8 Ver. II. (Aug. 2014), PP 21-28
www.iosrjournals.org

In vivo activity of two herbal plant mixtures against gastrointestinal nematodes in ruminants

Kimani, D[1*], Kareru, P G[1], Karanja, J M[1], Njonge, F K [2], Githira P N[1], Kutima, H L[3], Mercy, G[1] and Nyagah, G C[1]

[1]Chem*istry Department, College of Pure and Applied Sciences, Jomo Kenyatta University of Agriculture and Technology, Kenya*

[2]*Land Resource and Animal Sciences Department, Jomo Kenyatta University of Agriculture and Technology, Kenya*

[3]*Zoology Department, Jomo Kenyatta University of Agriculture and Technology, Kenya*

Abstract: *Helminthes infestation is a major constraint to livestock production. Increasing anthelmintic resistance and the impact of conventional anthelmintics on the environment has led to increased interest on new novel plant-based compounds. In this study, the in vivo activity of a herbal extract mixture containing Entada leptostachya and Prosopis juliflora was determined using faecal egg count (FEC) reduction tests on sheep. There were no signs of toxicity in all the groups throughout the study period apart from reduced feed intake in the initiation stage of the experiment. The herbal formula exhibited a time-dependent but not dose-dependent in- vivo anthelmintic activity. The 500mg/kg b.w.dose produced the maximum faecal egg reduction of 84% while 4500mg/kg b.w.dose gave the least reduction of -59% on day 19 post-treatment (PT). The results of the FEC reduction tests indicated that the herbal formulation tested passed the threshold FEC reduction of 80%. All the groups had an increase in their mean live body weights (LBWs) by day 19 PT except the untreated group. However, none of the increase was significant (P>0.05). All the animals recorded pre- and post-treatment packed cell volume (PCV) values that were within the permissible range of between 24 -45% for experimental sheep. All the groups recorded improved PCV values except the doses at 1500mg/kg b.w. and 4500mg/kg b.w. In conclusion, the herbal mixture was, therefore, safe and sufficiently active and has potential as a novel anthelmintic drug for the treatment of gastro-intestinal nematodes in ruminants.*

Key words: *Gastro-intestinal nematode, anthelmintic, faecal egg count, Prosopis juliflora*

I. Introduction

Helminthiasis is one of the most common setbacks in production and reproductive performance of livestock (Agaie and Onyeyili, 2007; Dawo and Tibo, 2005). Most of the effects caused by helminth parasitoses go unnoticed because of sub-clinical or chronic nature of the diseases they cause unless the parasites cause death of the animal (Dawo and Tibo, 2005). Control of gastro-intestinal nematode (GIN) parasitism is usually based on the use of chemical anthelmintics, whose effectiveness and consistent use has been limited by high levels of anthelmintic resistance and high cost.

In this study, *in vivo* tests were conducted on sheep naturally infected with a mixture of gastrointestinal nematodes. *In vivo* tests using FEC involve feeding the ruminant animal with herbal extract followed by monitoring helminthes eggs in the animal feaces over time after administration. Reduction of feacal egg counts with time is an indication of *in vivo* anthelmintic activity (Githiori, 2004; Agaie and Onyeyili, 2007; Dawo and Tibbo, 2005; Krimpen *et al.*, 2008; Burke *et al.*, 2009; Deore and Khadabadi, 2010).

Entada leptostachya is found in several parts of Kenya (Machakos, Embu and Mbeere districts) and other parts of Africa such as Somalia, Ethiopia and Tanzania. The communities in Embu and Mbeere districts of Eastern Province, Kenya use the root bark decoction to treat worms in humans and animals (Kareru, 2008). *Prosopis juliflora* (locally known as 'Mathenge') was introduced in Kenya in the early 1970s (Ebenshade and Grainger, 1980, Maghembe *et al.*, 1983) and is generally considered a noxious weed locally. This study, therefore, sought to determine the safety and *in vivo* activity using live animal model (sheep) of a formulated herbal extract mixture containing the two plants. This is in order to determine its safety and activity so as to confirm possible application as an anthelmintic for ruminants as a way of mitigating the negative attributes of *P.juliflora* while providing a cheaper alternative for control of helminthes for small holder farmers and pastoralists.

II. Materials And Methods

2.1 Collection and preparation of medicinal plant material

Entada leptostachya root barks were collected from Embu and Mbeere areas of Kenya. *Prosopis juliflora* leaves were collected from Marigat, Baringo County, Kenya. The plant specimen were identified in the field and authenticated by a plant taxonomist from the Botany Department at Jomo Kenyatta University of Technology (J.K.U.A.T.) where voucher specimens were also deposited. The samples were sorted, cleaned and air dried on the laboratory benches away from direct sunlight before being ground into fine powder and separately stored in air-tight plastic bags for further use to avoid contact with moisture.

Standard procedures (Lateef *et al.* (2003; 2006); Sujon *et al.*, 2008; Krimpen *et al.*, 2008) were used during the *in vivo* studies with a few modifications.

2.2 Sourcing, housing and grouping of animals

Sheep (male and female stock of 6-12 months) weighing between 13-26.5 kg were bought from Kariobangi livestock market in Nairobi, Kenya. They were transported to

J.K.U.A.T. and left to graze and acclimatize for a month. The animals were housed in pre-designed animal shed in the university's animal farm consisting of five cubicles and a feed storage cubicle with each cubicle having a meshed window for ventilation and raised wooden floor, a wooden feeding trough and watering bucket. The animals were then grouped according to the various test concentrations of 500mg/kg, 1,500mg/kg and 4,500mg/kg for the extract treatment groups, 10mg/kg for the treated control group (Albendazole) and untreated control group. Each group consisted of two males and two females and the females were confirmed to be non-pregnant. The animals were randomly selected and assigned to the various groups so that the individual weights in each group were as close as possible. There was no physical contact between animals in different cubicles.

2.3 Pre-treatment procedure

The animals were left to acclimatize in the cubicles for about 2 days. The animals were fed on commercial feed supplemented with grass and they had *ad libitum* access to tap water. Feacal samples from each group were collected and egg count per gram (EPG) of their fresh feacal samples determined to confirm that the animals were naturally infected with mixed species of worms. The infected sheep were then regrouped again so that each group had low, medium and highly infected sheep. The sheep were then given unique individual identifications on laminated paper wound on their necks.

The fresh crude aqueous extract mixture and positive control (albendazole) were diluted in distilled water in graduated doses of 500mg/kg, 1,500mg/kg and 4,500mg/kg for the aqueous extract mixture and 10mg/kg for the positive control. The animals were fasted overnight with *ad libitum* access to water. On day 0, the sheep were weighed using an overhead spring balance (0-50kg scale), fresh feacal samples collected directly via rectum into clean, capped, air-tight plastic sample containers ready for EPG counting. Fresh blood sampling was done directly from the sheep's ear capillary into heparinized microhaematocrit tubes ready for packed cell volume (PCV) determination (Hansen and Perry, 1994). The sheep were then treated with single doses of the crude extract mixture according to the animals' live body weights (LBWs) by varying the volume using the following equation:

$$Volume\ (ml)\ = \frac{Dose\ rate\ (mg/kg\ b.w.) \times Body\ weight\ (kg)}{Concentration\ (mg/ml)}$$

The sheep dosing was followed by further withdrawal of food for about 3 hours with *ad libitum* access to water and then fed with commercial feed supplemented with grass with *ad libitum* access to water. Fresh feacal samples were obtained from the sheep each morning via rectum on day 3, 7, 11, 15 and 19 post– treatment and screened for presence of nematode eggs by salt floatation technique and the EPG determined. The eggs were observed and counted using a modified McMaster egg counter with a sensitivity of 50 eggs per gram of feaces and feacal egg count percent reduction (FECR) calculated using the following formula:

$$\%FECR = \frac{\text{Pre}-treatment\ egg\ count\ per\ gram - Post-treatment\ egg\ count\ per\ gram}{\text{Pre}-treatment\ egg\ count\ per\ gram}$$

Individual live body weights of the animals were taken on day 3, 7, 11, 15 and 19 using an overhead spring balance (0-50kg scale) and PCV also determined on day 0 and day 19.

III. Results And Discussion

3.1 Feacal egg count results

Feacal egg counts (FEC) and their percentage reduction/increase are recorded in **Table 1**. These *in vivo* anthelmintic results for extract mixture are being reported for the first time by the time of conducting this study.

Table 1: Mean ± S.D. (n=4) EPG results after single oral administration of the herbal extract mixture to experimental sheep

Day PT	Controls		Herbal extract mixture dose (mg/kg b.w.)		
	Untreated	Albendazole (10mg/kg b.w.)	500	1500	4500
0	1325±1024.3 (0%)	1800±2079.7 (0%)	7963±6713.7 (0%)	6038±5861.9 (0%)	1513±904.9 (0%)
3	12250±15033.4 (-825%)	3188±2131.7 (-77%)	18513±18896.7 (-132%)	8750±6559.1 (-45%)	6338±8877.3 (-319%)
7	3050±2949.3 (-130%)	2225±1274.4 (-24%)	12263±8479.0 (-54%)	10537±7243.8 (-75%)	3825±1756.2 (-153%)
11	6413±5906.3 (-384%)	2350±1931.3 (-31%)	6250±3946.1 (22%)	10587±10575.6 (-75%)	3750±1239.6 (-148%)
15	1613±1438.4 (-22%)	883±800.5^{e} (51%)	1775±885.5^{a} (78%)	4938±4659.5^{b} (18%)	2663±832.0^{c} (-76%)
19	7288±7530.1 (-450%)	1333±548.5 (-26%)	1313±438.5^{a} (84%)	4863±3336.8^{b} (19%)	2413±2148.8 (-59%)

PT=Post treatment; **Untreated**=Naturally infected but untreated control group; **b.w.**=body weight; values with same lettered superscripts are significantly different ($P<0.05$) from day 0 FEC in the same column; Negative percentage values indicate the extent of increase in FEC value compared to day 0 PT

There were no signs of toxicity such as salivation, diarrhea and skin reaction in all the groups throughout the study period apart from reduced feed intake in the initiation stage of the experiment. The herbal extract mixture showed a time-dependent but not dose-

dependent *in vivo* anthelmintic activity. Albendazole and the herbal extract mixture 1:7 showed a general positive *in vivo* anthelmintic activity compared to untreated control. The high faecal egg counts could be attributed to the high presence of adult parasites in reproductive stages in the host (Worku *et al.*, 2009). There was a general increase in FEC in all the animal groups on day 3, with a general drop from day 7 except for treatment group at 1500mg/kg b.w. This increase in FEC could have been due to reduced faecal output hence, a virtually higher faecal nematode egg concentration (Githiori, 2004). This was caused by general reduction in the animals' feed intake by day 0 post treatment. This may be attributed to a shorter period of acclimatization to the commercial feed leading to poor palatability despite supplementing the commercial feed with grass. However, the feed intake had improved by day 5 post-treatment after mineral supplementation was introduced on day 4 post-treatment.

The peak anthelmintic effects of the herbal extract mixture 1:7 doses show a decreasing anthelmintic activity with increasing dosage with a similar trend having been observed by Lateef *et al.* (2006) with crude methanolic extract of *Carum copticum*. The peak FEC reductions for Albendazole and extract mixtures at 500mg/kg b.w. and 1500mg/kg b.w. were significant ($P<0.05$). The 500mg/kg b.w. group produced a maximum faecal egg count reduction (FECR) of 84% on day 19 post-treatment (PT) followed by 1500mg/kg b.w. group which gave 19% on day 19 PT while 4500mg/kg b.w. group gave the least reduction of -59% on day 19 PT while Albendazole gave 51% on day 15 PT. This, however, should not be construed to mean that the 1500mg/kg and 4500mg/kg doses were ineffective as there was a consistent FEC reduction from day 7 post-treatment for both dose levels though the herbal drug at 4500mg/kg b.w. was not able to reduce the FEC below the pre- treatment EPG. Probably, drug metabolism/breakdown could have taken longer with increase in dosage. The other reason for this observation could have been due to early saturation of the aqueous solutions of the individual plants during preparation of the extracts. With a peak faecal egg reduction of 84%, the 500mg/kg b.w. dose passed the threshold FEC reduction.

Wood *et al.* (1995) reports that any anthelmintic product that reduces FEC by less than 80% during FECR test trial should be considered insufficiently active as a curative agent. Githiori (2004) considered FEC and total worm count (TWC) reductions greater than or equal to 70% biologically significant based on the same guideline. The 500mg/kg b.w. dose may not have been affected by slow drug metabolism and this could provide direction on the maximum dose necessary to produce better *in vivo* anthelmintic effects.

The herbal extract mixture formula exhibited *in vivo* anthelmintic activity against mixed gastrointestinal nematodes. This could be attributed to the mixture of polar phytochemicals present in *E. leptostachya* and *P. juliflora* which are soluble in aqueous medium. Phytochemical studies done on the two plants confirm the presence of alkaloids, flavonoids, saponins, tannins and sterols/triterpenes from the leaf aqueous extracts of P. juliflora (Wamburu *et al.*, 2013) while root aqueous extracts of *E. leptostachya* have tested positive for sterols/triterpenes, glycosides, saponins and tannins (Kareru, 2008; Kareru *et al.*, 2012). Condensed tannins can impair vital processes such as feeding and reproduction of the parasite or may bind and disrupt the integrity of the

parasite's cuticle (Dave *et al.*, 2009; Zafar *et al.*, 2009). It is reported that monodesmoside saponins have shown to destabilize membranes and increase cell permeability by combining with membrane-associated sterols (Geidam *et al.*, 2007; Ademola *et al.*, 2008; Ademola and Eloff, 2010) and producing changes in cell morphology leading to cytolysis (Geidam *et al.*, 2007). Alkaloids may improve tonicity of the gastrointestinal tract and thus expel the worms or may have a direct effect on the nervous system of the nematodes (Ademola *et al.*, 2008; Lateef *et al.*, 2003). Albendazole works by interference with polymerization of microtubule, where the drug binds to the protein tubulin of the parasite leading to death by starvation (Kareru *et al.*, 2012; Lalchhandama, 2009). The other phytochemicals in the two plants like flavonoids and oleanane type triterpenes may have had their independent or synergistic anthelmintic effects (Zafar *et al.*, 2009).

3.2 Results of live body weights of the experimental sheep

Changes in the live body weights of the sheep are recorded in **Table 2**. There were no significant changes ($P>0.05$) in the LBWs of the sheep treated with the herbal extract mixture and Albendazole on day 19 PT compared with day 0. All the groups had an increase in their mean LBWs by day 19 PT except the untreated group. However, none of the increase was significant ($P>0.05$).

Table 2: Effect of herbal extract mixture (*E. Leptostachya: P. juliflora*) on mean (± S.D., n=4) live body weights of sheep

	LIVE BODY WEIGHTS (KG)				
DAY PT	**UNTREATED (10mg/kg)**	**ALBENDAZOLE**	**500mg/kg**	**1500mg/kg**	**4500mg/kg**
0	19.4±4.2 (0%)	21.0±3.7 (0%)	19.6±5.1 (0%)	22.3±4.4 (0%)	21.0±5.5 (0%)
3	18.5±3.2[a] (-4.6%)	20.3±4.4[b] (-3.3%)	19.6±5.4 (0%)	21.0±3.0[d] (-5.8%)	21.0±6.5 (0%)
7	18.5±3.5[a] (-4.6%)	22.2±2.3 (5.7%)	19.6±5.2 (0%)	20.6±3.0[d] (-7.6%)	19.6±6.3[e] (-6.7%)
11	18.4±3.2[a] (-5.2%)	20.8±2.4 (-1%)	19.0±4.7[c] (-3.1%)	20.6±4.1[d] (-7.6%)	20.4±6.4[e] (-2.6%)
15	18.9±3.6 (-2.6%)	21.7±2.5 (3.3%)	20.1±4.5 (2.6%)	21.0±4.2[d] (-5.8%)	21.4±6.1 (1.9%)
19	19.0±2.5 (-2.1%)	25.0±2.6 (19.0%)	20.5±4.6 (4.6%)	22.8±3.4 (2.2%)	24.5±6.2 (16.7%)

PT=Post treatment; Mean LBWs with same lettered superscripts are significantly different (P<0.05) from day 0 LBW in the same column; **Untreated**=Naturally infected but untreated control group; Negative percentage values (in parentheses) indicate percentage mean LBW decrease from value on day 0 PT

Nematodes have been reported to cause severe damages to the gastrointestinal tract (GIT) and the host therefore, expends energy and protein repairing these damages caused by these parasites other than for growth (Agaie and Onyeyili, 2007). This could have been responsible for the insignificant changes (P>0.05) in LBWs of the sheep despite anthelmintic treatment.

Presence of tannins in the leaf and root aqueous extracts of *P. juliflora* and *E. leptostachya* respectively is reported (Kareru *et al.*, 2012; Wamburu *et al.*, 2013). Because of their reactivity with plant proteins as they are being chewed by ruminant animals, condensed tannins partially protect animals against rumen degradation of proteins, and so increase the flow of amino acids to the small intestines hence, increasing their absorption (FAO, 2011; Min and Hart, 2003; Zafar *et al.*, 2002). This increase may help to counteract the losses of protein attributed to gastrointestinal

nematode infection and for immune response to nematode parasites (Barry *et al.*, 2001; Niezen *et al.*, 2002; Zafar *et al.*, 2002; Min and Hart, 2003).
The herbal extract mixture treated groups could have benefitted from better utilization of proteins in the presence of tannins or the weight gains could have been as a result of improved feeding or both or due to better nutrient utilization due to lower worm load. Weight gains in the Albendazole-treated group could have been due to maximization of nutrient utilization as a result of lower worm load. However, determination of total worm count will be essential in future work with this extract mixture to validate weight gains as a result of lower worm loads as EPG counts cannot reliably be used to make conclusions on worm loads.

3.3 Results of packed cell volumes (PCV) of the experimental sheep

From **Table 3**, there were no significant changes (P>0.05) in the packed cell volumes (PCVs) by day19 PT compared to day 0 PT except for the extract mixture dose of 1500mg/kg b.w. whose PCV significantly dropped (P<0.05). All the animals recorded pre- and post-treatment PCV values that were within the permissible range of between 24-45% (Research Animal Resources, University of Minnesota, 2013; Worku *et al.*, 2009) for experimental sheep.

Table 3: Effect of the herbal extract mixture on the mean±S.D. (n=4) percentage packed cell volume (PCV) of the sheep

	%PCV				
DAYS PT	**UNTREATED**	**ALBENDAZOLE (10mg/kg)**	**500mg/kg**	**1500mg/kg***	**4500mg/kg**
0	28.71±2.61	25.97±4.52	30.29±2.55	25.17±2.09	26.33±2.30
19	31.74±4.34 (10.55%)	30.60±3.39 (17.83%)	30.63±3.95 (1.12%)	24.21±3.67 (-3.81%)	25.51±3.00 (-3.11%)

*Significant difference (P<0.05) between day 0 and day19; Negative percentage values (in parentheses) indicate percentage decrease in PCV from value on day 0 PT

All the groups recorded improved PCV values (thus, reduced degree of anaemia) except the doses at 1500mg/kg b.w. and 4500mg/kg b.w. Anaemia, following administration of an agent, could be as a result of lysis of blood cells and/or inhibition of blood cell synthesis by the active constituents of the extract (Orisakwe *et al.*, 2003). The higher doses of the extract mixture (1500 and 4500mg/kg b.w.) could have had this effect on the experimental animals thus, recording decreased PCV values. Besides the negative effects worms have on increasing anaemia, the lower percentage increase on mean PCV

of the treated animals at 500mg/kg b.w. compared to the controls and percentage PCV drops at 1500mg/kg b.w. and 4500mg/kg b.w. groups may be indicators of the drug effect on the PCV values of sheep. This may also suggest that the two doses whose PCVs dropped may have had a negative stimulatory effect on the hemopoietic system or the animals may have increased water intake (Githiori, 2004). As no abnormal adverse effects were observed during this study after administration of the herbal drug, water intake may not have contributed to this effect as no abnormal water intake was observed in all the groups under the same experimental conditions. Each worm is responsible for a daily loss of blood of about 0.05ml through ingestion and seepage from lesions (Eguale *et al.*, 2007). PCV values are directly related to anaemia, correlated with high FEC and parasite burdens (Dawo and Tibbo, 2005; Worku *et al.*, 2009).

The drop in PCVs in the animals treated with the herbal extract at 1500mg/kg b.w. and 4500mg/kg b.w. correlated with the lower anthelmintic activities (FECR) of the two groups. On the other hand, the improved PCV values meant that there was reduction of blood loss from the animals due to parasite inhibition or clearance, which can signify recovery from helminthosis and improvement in health status (Agaie and Onyeyili, 2007) as observed with the extract mixture dose of 500mg/kg b.w. Negative stimulatory effects were enhanced in the herbal extract mixture groups compared to the control groups with notable increase in anaemia in 1500mg/kg b.w. and 4500mg/kg b.w. groups. Notable contradiction is the PCV increment in the untreated group despite the fact that the egg count over the period indicated a general increase. However, a higher egg count may not necessarily indicate the level of worm infection (Tarpoff, 2010).

Other similar *in vivo* work has been reported. A maximum EPG reduction of 50.3% on Arsi Bale goats was achieved by Dawo and Tibbo (2005) using crude powder of *Halothamnus somalensis*. Aqueous extract of *Daniellia oliveri* reduced feacal egg count in naturally infected sheep by 12.8% by day 14 PT at a dose of 1200mg/kg b.w. (Adama *et al.*, 2012). Crude powder and crude methanolic extract of *Ferula costata* showed significantly higher reduction in EPG compared to untreated control at all stages PT with a maxima on day 14 (47.9%) for 3g/kg b.w. but which was significantly lower than the positive control (Levamisole) at 7.5mg/kg b.w. (99.39%) (Kakar *et al.*, 2013). The average PCV values were not significantly different for goats treated with aqueous leaf extract of *C. pyramidalis* compared to Doromectin -treated groups and the untreated groups. The LBWs of the animals treated with the extract did not change significantly except those treated at the highest dose of 5mg/kg b.w. (Robson *et al.*, 2012). In evaluation of *in vivo* anthelmintic activity using Boer goats, Worku *et al.* (2009) observed that groups treated with wormwood and tobacco with added copper sulphate resulted in dramatic decreases in PCV values and related this to toxic effects of these plant extracts.

Acknowledgements

The authors thank Research, Production and Extension Division of Jomo Kenyatta University of Agriculture and Technology for funding this project. Mr. Kimanthi (Animal house caretaker), for dedicated care of the experimental animals and a helping hand during faecal sampling of the animals.

References

[1]. Adama, K., Amadou, T., Man, N., Hamidou, T.H. and Gaston, B.A.M. (2012). Anthelmintic activity of *Daniellia oliveri* (Rolfe) Hucht. and Dalz (Caesalpiniaceae) in sheep in Burkina Faso, Journal of Research in Antimicrobials: **1**(1): 049-055.

[2]. Ademola, I.O., Ajayi, A., and Okotie, S.V. (2008). Larvicidal effects of *Aframomum danieli* seed extracts against gastrointestinal nematode of sheep: *in vitro* studies, African Ethnomedicines Network: **5**(3): 173-179.

[3]. Ademola, I.O. and Eloff, J.N. (2010). In vitro anthelmintic activity of *Combretum molle* (R. Br. ex G. Don) (Combretaceae) against *Haemonchus contortus* ova and larvae, Veterinary Parasitology: **169**(1-2): 198-203.

[4]. Agaie, B.M. and Onyeyili, P.A. (2007). Anthelmintic activity of the crude aqueous leaf extracts of *Anogeissusleio carpus* in sheep, African Journal of Biotechnology: **6**: 1511-1515.

[5]. Barry, T. N., McNeill, D. M. and McNabb, W. C. (2001). Plant secondary compounds; their impact on nutritive value and upon animal production, pp. 445–452. Proc. XIX Int. Grass. Conf., Sao Paulo, Brazil.

[6]. Burke, J.M., Wells, A., Casey, P. and Kaplan**.** (2009). Herbal dewormer fails to control gastrointestinal nematodes in goats, VeterinaryParasitology: 160 (1-2): 168-70.

[7]. Dave, S., Patil, U.K., Bhaiji, A., Baghel, U.S., Yadav, S.K. and Sharma, V. K. (2009). *In vitro* anthelmintic activity of leaves of *Spinacia oleracea Linn.*, International Journal of Toxicological and Pharmacological Research: **1**(1): 21-23.

[8]. Dawo, F. and Tibbo, M. (2005). Anthelmintic effect of *Halothamus somalensis* in Arsi-Bale goats,Livestock Research for Rural Development: **17**.

[9]. Deore, S.L. and Khadabadi, S.S. (2010). *In vitro* anthelmintic studies of *Chlorophytum borivilianum* Sant. and Fernandez tubers, Indian Journal of Natural Products and Resources: **1**(1): 53-56.

[10]. Ebenshade, H.W. and Grainger, A. (1980). The Bamburi reclamation project, International Tree Crops Journal: **1**: 199-202.

[11]. Eguale, T. and Giday, M. (2009). *In vitro* anthelmintic activity of three medicinal plants against *Haemonchus contortus*, International Journal of Green Pharmacy: **3**(1): 29-34.

[12]. Food and Agriculture Organization of the United Nations (FAO). Naturally occurring plant tannins as a means of controlling intestinal nematode infections in ruminants, http//:www.fao.org/teca/content/naturally-occurring-plant-tannins-means-controlling-intestinal-nematode-infections-ruminants. Accessed on March 11, 2011.

[13]. Geidam, Y.A., Ambali, A.G. and Onyeyili, P.A. (2007). Preliminary phytochemical and antibacterial evaluation of crude aqueous extract of *Psidium guajava* leaf, Journal of Applied Sciences: **7**(4): 511-514.

[14]. Githiori, J.B. (2004). Evaluation of anthelmintic properties of ethnoveterinary plant preparations used as livestock dewormers by pastoralists and smallholder farmers in Kenya, Ph.D. Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

[15]. Hansen, J. and Perry, B. (1994). The epidemiology, diagnosis and control of helminthes parasites of ruminants, pp. 62 -63. International Laboratory for research on Animal Diseases (ILRAD), Nairobi, Kenya.
[16]. Kareru, P.G. (2008). Ethnomedicine practices, analysis and standardization of a herbal anthelmintic drug used by the Embu and Mbeere peoples of Kenya, PhD thesis, Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya.
[17]. Kareru, P.G., Ombasa, O., Rukunga, G., Mbaria, J., Keriko, J.M., Njonge, F.K. and Owuor, B.O. (2012). *In vitro* anthelmintic effects of two Kenyan plant extracts against *Haemonchus contortus* adult worms, International Journal of Pharmacological Research: **2**(3): 113-115.
[18]. Kakar, S.A., Tareen, R.B., Sandhu, Z.U., Azam, M.K., Saeed, U.K., Zafar, I. and Jabeen, H. (2013). *In vitro* and *in vivo* anthelmintic activity of *Ferula costata* (Kor.) against gastrointestinal nematodes of sheep, Pak. J. Bot.: **45**(SI): 263-268.
[19]. Lalchhandama, K. (2009). Cestocidal activity of *Acacia caesia* stem bark on *Raillietina echinobothrida*, Pharmacognosy Research: **1**(4): 179-184.
[20]. Lateef, M., Iqbal, M., Rauf, U. and Jabbar, A. (2006). Anthelmintic activity of *Carum capticum* seeds against gastro -intestinal nematodes of sheep, J. Anim. Pl. Sci.: **16**: 35-36.
[21]. Lateef, M., Zafar, I., Khan, M.N., Akhtar, M.S. and Jabbar, A. (2003). Anthelmintic activity of *Adhatoda vesica* roots, International Journal of Agriculture and Biology: **5**(1): 86-89.
[22]. Maghembe, J.A., Kariuki, E.M. and Haller, R.D. (1983). Biomass and nutrient accumulation in young *Prosopis juliflora* at Mombasa, Kenya, Agroforestry Systems: **1**: 313-321.
[23]. Min, B.R. and Hart, S.P. (2003). Tannins for suppression of internal parasites, Journal of Animal Science: **81**(E. Suppl. 2): E102- E109.
[24]. Niezen, J.H., Charleston, W.A.G., Robertson, H.A., Shelton D., Waghorn, G.C. and Green, R. (2002). The effect of feeding sulla (*Hedysarum coronarium*) or lucerne (*Medicago sativa*) on lamb parasite burdens and immunity to gastrointestinal nematodes, Veterinary Parasitology: **105**: 229-245.
[25]. Orisakwe, O.E., Afonne, O.J., Chude, M.A., Obi, E. and Dioka, C.E. (2003). Sub-chronic toxicity studies of the aqueous extract of *Boerhavia diffusa* leaves, Journal of Health Science: **49**(6): 444-447.
[26]. Research AnimalResources, University of Minnesota. Reference values for laboratory animals, www.ahc.umn.edu/rar/refvalues.html. Accessed on July 13, 2013.
[27]. Robson, R.B., López, J.A., Santos, L.C., Zacharias, F., David, J.M., David, J.P. and Lima, F.W.M. (2012). Biological effect of leaf aqueous extract of *Caesalpinia pyramidalis* in goats infected with gastrointestinal nematodes, Evidence -Based Complementary and Alternative medicine: **2012**(2012): http://dx.doi.org/10.1155/2012/510391.
[28]. Sujon, M.A., Mostofa, M., Jahan, M.S., Das, A.R. and Rob, S. (2008). Studies on medicinal plants against gastrointestinal nematodes of goats, Bangladesh Journal of Veterinary Medicine:**6** (2): 179-183.

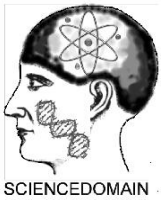
[29]. Tarpoff, A.J. (2010). Comparative efficacy of two ivermectin pour-on anthelmintics in beef steers in a commercial feed yard, MSc. Thesis, Kansas State University, Manhattan, Kansas, United States of America.
[30]. Wamburu, R.W., Kareru, P.G., Mbaria, J.M., Njonge F.K., Nyaga, G. and Rechab, S.O. (2013). Acute and sub -acute toxicological evaluation of ethanolic leaves extract of *Prosopis juliflora* (Fabaceae), Journal of Natural Sciences Research: **3**(1): 8-11.
[31]. Wood, I. B., Amaral, N. K., Bairden, K., Duncan, J. L., Kassai, T., Malone, J. B., Jr., Pankavich, J. A., Reinecke, R. K., Slocombe, O. and Taylor, S. M. (1995). World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine), Veterinary Parasitology: **58**: 181-213.
[32]. Worku, M., Franco, R. and Miller, J.H. (2009). Evaluation of the activity of plant extracts in Boer goats, American Journal of Animal and Veterinary Sciences: **4**(4): 72-79.
[33]. Zafar, I., Bachaya, H.A., Nisar, M.K., Jabbar, A., Gilani, A.H., and Islam, U.D. (2009). *In vitro* and *in vivo* anthelmintic activity of *Terminalia arjuna* bark, International Journal of Agriculture and Biology: **11**(3): 273-278.
[34]. Zafar, I., Mufti, K.A. and Nisar, M.K. (2002). Anthelmintic effects of condensed tannins, International Journal of Agriculture and Biology: **4**(3): 438-440.

Appendix VII: Published paper on toxicity studies

British Journal of Pharmaceutical Research
4(21): 2475-2483, 2014
ISSN: 2231-2919

SCIENCEDOMAIN *international*

www.sciencedomain.org



Safety of *Prosopis juliflora* (Sw.) DC. (*Fabaceae*) and *Entada leptostachya* Harms (*Leguminosae*) Extract Mixtures Using Wistar Albino Rats

D. Kimani[1*], P. G. Kareru[1], H. L. Kutima[2], F. K. Njonge[3], G. C. Nyagah[1], J. M. Karanja[1], K. Mutembei[1], P. Githira[1] and G. Mercy[1]

[1]Department of Chemistry, Jomo Kenyatta University of Agriculture and Technology (JKUAT), P.O. Box 62000, Nairobi, Kenya.
[2]Department of Zoology, Jomo Kenyatta University of Agriculture and Technology (JKUAT), P.O. Box 62000, Nairobi, Kenya.
[3]Department of Land Resource and Animal Sciences, Jomo Kenyatta University of Agriculture and Technology (JKUAT), P.O. Box 62000, Nairobi, Kenya.

Authors' contributions

This work was carried out in collaboration between all authors. Author DK designed the study, wrote the protocol, performed the statistical analysis and wrote the first draft of the manuscript. Author PGK supervised and managed all aspects of the study and review of the draft. Authors HLK and FKN managed the animal welfare aspect including pathology. Author GCN managed the analytical and biochemical analyses. Authors PG and GM managed all information on natural products chemistry. Authors JMK and KM helped in the practical aspect of the study and also contributed in review of the manuscript from time to time. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJPR/2014/10993
Editor(s):
(1) Dongdong Wang, Dept. of Pharmacogonosy, West China College of Pharmacy, Sichuan University, P. R. China.
Reviewers:
(1) Cristiane Bezerra da Silva, Federal University of Paraná, Brazil. (2) Anonymous, Herbal Medicine Research Centre, Institute for Medical Research, Malaysia.
(3) Anonymous, National Institute for Pharmaceutical Research & Development (NIPRD), Nigeria. (4) Anonymous, University of Abuja, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=788&id=14&aid=6684

Original Research Article

Received 21st April 2014
Accepted 21st August 2014
Published 24th October 2014

**Corresponding author: Email: davykym@gmail.com;*

ABSTRACT

Aim: Many medicinal plants have been used traditionally in treating ailments in humans and animals. However, for most of herbal remedies, no scientific toxicity profiles exist in literature. In this study, the safety profile of an herbal extract mixture containing *Entada leptostachya* (EL) and *Prosopis juliflora* (PJ) was determined using acute oral toxicity tests using adult female Wistar albino rats.
Place and Duration of Study: Laboratories in the departments of Chemistry, Zoology, Botany and Biochemistry of Jomo Kenyatta University of Agriculture and Technology (J.K.U.A.T.) between March 2012 and April 2012.
Methodology: The OECD 425 guidelines (Up-and-Down procedure) were followed. Different dosages (control, 175, 550, 1750 and 5000 mg/kg body weight) were used in the experiment. Selective observations and analysis were made and recorded on mortality, signs of pain or distress and moribund animals, biochemical and macroscopic (pathological, organ and live body weights) analyses.
Results: During the entire period of the study, no signs of pain or enduring distress were observed neither was there any mortality. Alanine aminotransferase (ALT) values were within range (for experimental rats) apart from the rat in control while Aspartate aminotransferase (AST) values were within range (for experimental rat) apart from two rats in the upper limit. Macroscopic organ observations did not show colour or texture consistent with drug-induced inflammation or lesions. The toxicity studies of the extract mixture showed that the median lethal dose (LD_{50}) was above the upper limit of 5000mg/kg body weight.
Conclusion: In conclusion, the LD_{50} of the extract mixture was found to be greater than 5000 mg/kg body weight and was, therefore, considered safe and has potential as a novel herbal preparation.

Keywords: Toxicity; Prosopis juliflora; alanine aminotransferase; LD_{50}; upper limit.

1. INTRODUCTION

Herbs and herbal formulations as medicinal alternatives have continued to receive increased attention because of strong belief that they are safe. It is this assumption, to a larger extent, that has influenced the indiscriminate use of herbal formulations leading to incidences of adverse effects and sometimes life-threatening conditions [1,2]. The traditional uses of any plant for medicinal purposes do not necessarily guarantee their safety, especially with regard to mutagenicity, carcinogenicity, embryotoxicity, nephrotoxicity and hepatotoxicity, especially if such effects are complex and not easily recognized by the local populations [3].

Although phytochemicals or plant secondary metabolites (PSMs) with pharmacological properties continue to play a vital role in the health management of people and animals, in most cases, not much information is known about their possible toxic effects. For this reason, alongside confirming their activities, it becomes important to investigate and confirm their toxicity profiles in order to appraise adequately their suitability for use by humans or target host animals [4]. These experiments fall into four categories: a) microorganism systems, b) mammalian cell culture systems, c) tissue preparations, and d) organ cultures [5]. The animal tests investigate the effect of a test substance on the various body systems such as respiratory, nervous, and cardiovascular systems [6]. In the context of this study, OECD guideline Up-and-Down procedure (UDP) provides a more humanely acceptable animal use protocol. OECD's UDP uses a maximum of six animals in dose administration of test substances in a sequential manner after survival of

previously dosed animal has been assured. Animal found in moribund state or showing signs of severe pain or enduring signs of severe distress are used as end-points rather than mortality [7].

Entada leptostachya is found in several parts of Kenya (Machakos, Meru, Embu and Mbeere districts) and other parts of Africa such as Somalia, Ethiopia and Tanzania. The communities in Embu and Mbeere districts of Eastern Province, Kenya use the root bark decoction to treat worms in humans and animals [8]. *Prosopis juliflora* (locally known as 'Mathenge') was introduced from Latin America into Kenya in the early 1970s [9,10] and is generally considered a noxious weed locally. This study, therefore, sought to determine the toxicity of a formulated herbal extract mixture containing the two plants in order to determine its safety as well as confirm possible application as an anthelmintic for ruminants as a way of mitigating the negative attributes of *P. Juliflora* while providing a cheaper alternative for controlling worm-infestations, especially among flocks of small holder farmers and pastoralists. For animal welfare reasons, OECD 425 guideline was used as it uses fewer animals and considers moribund conditions or severe signs of pain and distress as end- points.

2. MATERIALS AND METHODS

Standard procedures using OECD 425 guidelines (Up-and-Down procedure) were followed [7] during the toxicity studies with minor, selective additional data on biochemical analysis and relative organ weights. The main test was performed at a starting dose of 175 mg/kg b.w. as no toxicity data for the herbal mixture existed in literature or was documented. Approval letter from the ethics sub-committee (ref: JKU/2/4/IPC/IP/013) was obtained from Jomo Kenyatta University of Agriculture and Technology. The herbal preparation is at the pre-patent stage hence, the ratio of the mixture could not be divulged at this stage.

2.1 Collection and Preparation of Medicinal Plant Material

Entada leptostachya root barks were collected from Embu and Mbeere areas of Kenya. *Prosopis juliflora* leaves were collected from Marigat, Baringo County, Kenya. The plant specimen were identified in the field and authenticated by a plant taxonomist from the Botany department at Jomo Kenyatta University of Technology (J.K.U.A.T.) where voucher specimens were also deposited (voucher numbers: **En-jkuat/092010** and **Pro-jkuat/092010** for *E. Leptostachya* and *P. Juliflora* respectively). The *E. Leptostachya* root and *P. Juliflora* leaf samples were sorted, cleaned using tap water to remove adhering soil and other foreign matter and air dried at room temperature with intermittent turning for about three weeks on the laboratory benches away from direct sunlight before being ground into fine powder using an electric grinder (manufactured by J.K.U.A.T. mechanical engineering department) and separately stored in air-tight plastic bags at room temperature for further use to avoid contact with moisture.

2.2 Care and Preparation of Experimental Animals

Seven (1 control) adult female Wistar albino rats, 10-12 weeks old and weighing 165-196g, were used in the study. The animals were procured from Zoology Department animal house of Kenyatta University at the age of between 7-9 weeks. The animals were left to acclimatize in their cages (bedded with wood shavings) for three weeks with *ad libitum* access to food (standard commercial rat pellets from Unga Feeds Limited, Kenya) and tap water. Twelve hours artificial lighting and 12 hours darkness sequence was

followed. The rats were uniquely marked on their tails for easy individual identification using permanent ink.

2.3 Preparation and Administration of Doses

The plant powders were weighed and separately soaked for one hour in hot, distilled water and filtered through cotton swabs using a funnel. Separate aqueous stock solutions for the two plants at various concentrations were prepared using the equation below:

$$Concentration\ (mg/ml) = \frac{Dose\ rate\ (mg/kg\ b.w.) \times Body\ weight\ (kg)}{Volume\ (ml)}$$

The rats were fasted overnight with *ad libitum* access to water and weighed prior to dosing with the extract mixture. A constant volume of 2 ml per rat based on a single dose was given by gavage using a stomach tube. A dose progression factor of half log (equivalent to 3.2) was followed with a starting dose of 175 mg/ml. Food, but not water, was withheld for a further 3 hours after dosing and any toxicity signs noted during the first 30 minutes and then hourly for the next six hours. Individual observations were made daily for the next 48 hours for any signs of pain or enduring distress, being moribund or mortality before dosing the next animal. Each animal was then observed on a daily basis for 14 days and each rat weighed once weekly and at the end of the study. The dose progression sequence and LD_{50} estimate in toxicity testing was done using AOT425statpgm (version 1) program software.

2.4 Biochemical and Macroscopic Analyses

Each animal was humanely sacrificed at the end of the study by euthanizing it using carbon dioxide gas in a closed dessicator. Fresh blood samples were obtained by cardiac puncture using a needle and syringe into blood sample tubes containing EDTA (anticoagulant) and the biochemical parameters recorded using an auto analyzer using assay kits from Roche diagnostics, GmbH, Mannheim, Germany. The lungs, spleen, kidneys and liver were immediately weighed on an electronic balance and their weights recorded.

3. RESULTS AND DISCUSSION

3.1 Clinical Toxicological Signs

Table 1 shows the effects of the drug mixture on mortality after single drug administration.

Table 1. Toxicity test data for the extract mixture

Test sequence	Animal ID	Dose (mg/kg b.w.)	Short-term result (48hrs)	Long-term result
1	A	175	O	O
2	B	550	O	O
3	C	1750	O	O
4	D	5000	O	O
5	E	5000	O	O
6	F	5000	O	O

Key: (X = Died, O = Survived)

During the entire study, no signs of toxicity were observed after administration of a single oral dose of aqueous extract mixture. No signs of pain or enduring distress were evident during the 14 days (long-term) of observation and no mortalities were observed as seen in Table 1. The study had to be stopped when no deaths were observed after three consecutive rats were dosed at the upper bound (5000 mg/kg b.w.). The median lethal dose (LD_{50}) was, therefore, estimated at >5000 mg/kg b.w. following the stopping rule procedure. No delayed behavioural toxicity signs were observed.

3.2 Biochemical Data

Table 2 shows the drug effect on the various blood chemistry parameters. From Table 2, the creatinine values obtained from all the animals treated and the control were similar at <44.2 µmol/L which was within the normal range of between 15-61 µmol/L [6] for experimental rats. This implies that none of the rats suffered from impaired renal functioning. Creatinine blood levels rise if the kidney filtering process is deficient and this is a reliable indicator of kidney malfunction/impaired function. The alanine aminotransferase (ALT) values for all the animals apart from the control were within range of 35-80 U/L [11] for experimental rats. Low ALT levels in the control may indicate a normal healthy liver or a low/non-functioning liver which may fail to release a lot of ALT into the blood. A high level of ALT in the blood usually signifies liver cell damage [1]. ALT is found mainly in the liver, but also in smaller amounts in the kidneys, heart, muscles and pancreas [12]. Aspartate aminotransferase (AST) values were within range of 65-203 U/L for experimental rats [13] for all the rats apart from two rats in the 5000mg/kg b.w. category. AST is also a hepatic health/function indicator but may also be used to assess damage in the heart and cell necrosis of many tissues [1]. A mild elevation of AST level has been associated with liver injury or myocardial infarction. The amount of AST in the blood is directly related to the extent of the tissue damage. An AST/ALT ratio is sometimes useful in differentiating causes of tissue damage. A typical myocardial infarction gives an AST/ALT ratio greater than 1 while an AST/ALT ratio less than 1 is due to liver injury and AST/ALT of more than 2 indicates alcoholic hepatitis or cirrhosis [14]. The AST/ALT ratio (>2) for the two rats in upper-bound doses indicate possible plant extract-induced liver cirrhosis as the cause of elevated AST levels. However, macroscopic observation of the organs from the two animals did not show any form of inflammation or colour consistent with cirrhosis or abnormal texture. The AST levels were consistently higher than the ALT levels which was expected since the body cells contain more AST than ALT. Since ALT is localized mainly in cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocellular damage than AST [12,14,15]. Toxin-induced hepatocellular damage is caused by leakage of cytosolic enzymes out of the cells due to increase in cell permeability, membrane damage and cell necrosis [16]. Cell permeability, and hence the cellular leakage, could be caused by saponins which combine with membrane-associated sterols reducing the membrane integrity [17].

Table 2. Effect of treatment of rats with the aqueous extract mixture on biochemical parameters (n=1)

		Dose (mg/kg b.w.)					
Parameter	**Control**	**175**	**550**	**1750**	**5000**	**5000**	**5000**
Creatinine (µmol/L)	<44	<44	<44	<44	<44	<44	<44
ALT (U/L)	18.5	66.6	38.1	63.3	44.4	73.5	36.6
AST (U/L)	110	67	152	82.1	423	336	148

3.3 Changes in Organ and Live Body Weights

Figs. 1 to 2 show the drug effects on the organ weights and live body weights, respectively, of the rats during and after the study.

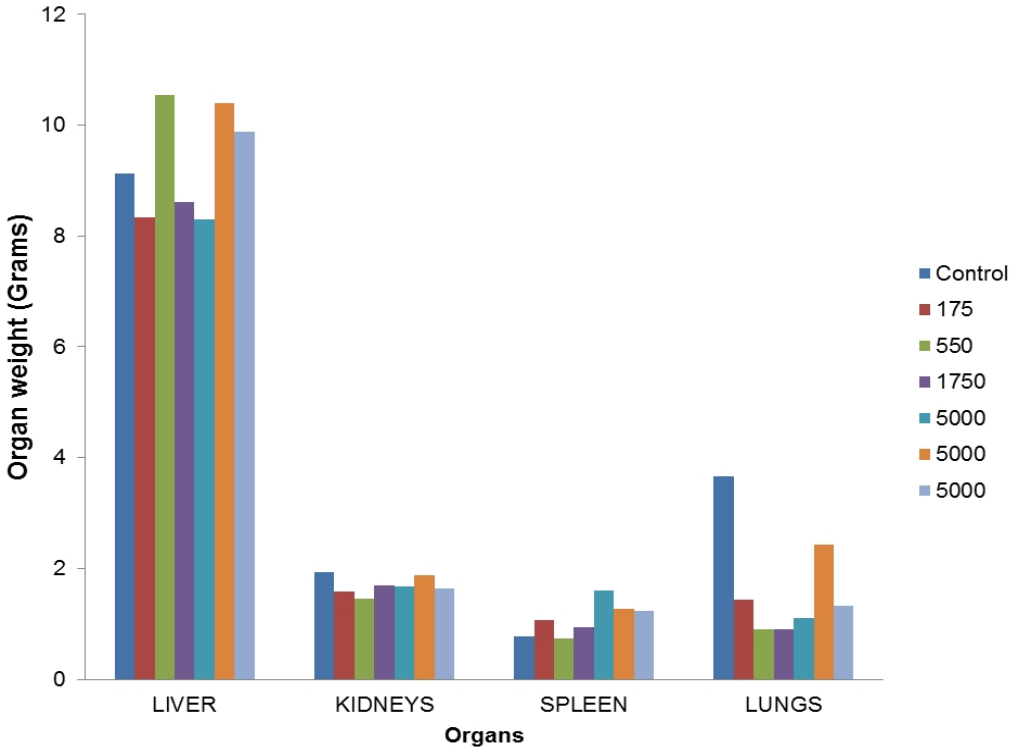


Fig. 1. Internal organ weights of rats after acute oral administration of the aqueous extract mixture, n=1

There were no major differences in the gross pathological parameters in the study. In Fig. 1, the lung weights of the rat in control and one rat in upper bound were the only conspicuously visible outliers. There were no major deviations in the other organ weights compared to control. The slight differences in liver and spleen weights could not be directly attributed to any internal pathological processes. Macroscopic examination of the organs showed no changes in colour and texture or any visible inflammation compared to the control.

Generally, the animals had progressive weight gains (Fig. 2). The progressive weight gains indicate positive growth response.

Similar studies using similar and other guidelines to establish toxicity of plant extracts in rat or mice models are documented. Acute oral toxicity testing of *Hunteria umbellate* was done using the Up-and–Down procedure (OECD guideline 425). The study showed that the plant extract had an LD_{50} of 1020mg/kg and was therefore slightly toxic [18]. Akanmu *et al.* [4] investigated acute and sub-acute oral toxicity of methanolic extract of *Bauhinia monandra* (used for treatment of diabetes). Acute administration of the extract up to a dose of 8000 mg/kg b.w. did not cause any deaths nor any toxicity signs. The study concluded that *B. Monandra* may possess relatively low toxicity. Acute toxicity study of the leaves of *Sphenocentrum jollyanum* showed no toxicity when administered up to 11000 mg/kg b.w. orally while intra-peritoneal (IP) administration produced dose dependent mortality with an LD_{50} of 1445.4 mg/kg b.w. The results suggested that the leaves extract was potentially safe for oral consumption [2]. Acute and sub-acute toxicity of 95% ethanolic extract of aerial parts of *Cansjera rheedii* J. Gmelin (*Opiliaceae*) was evaluated in Swiss mice and Wistar albino rats. The acute toxicity study was conducted following the OECD 420 guideline where a limit test dose of 2000 mg/kg b.w. was used. No significant changes in the organ weights between the control and treatment groups were observed nor were there any gross pathological and histopathological changes observed after 28 days. There were no mortalities during the entire treatment period. In conclusion, the study presented strong evidence of non-toxic effects of the ethanol extract of C. *rheedii* and the extract was considered safe and could be extensively used [14].

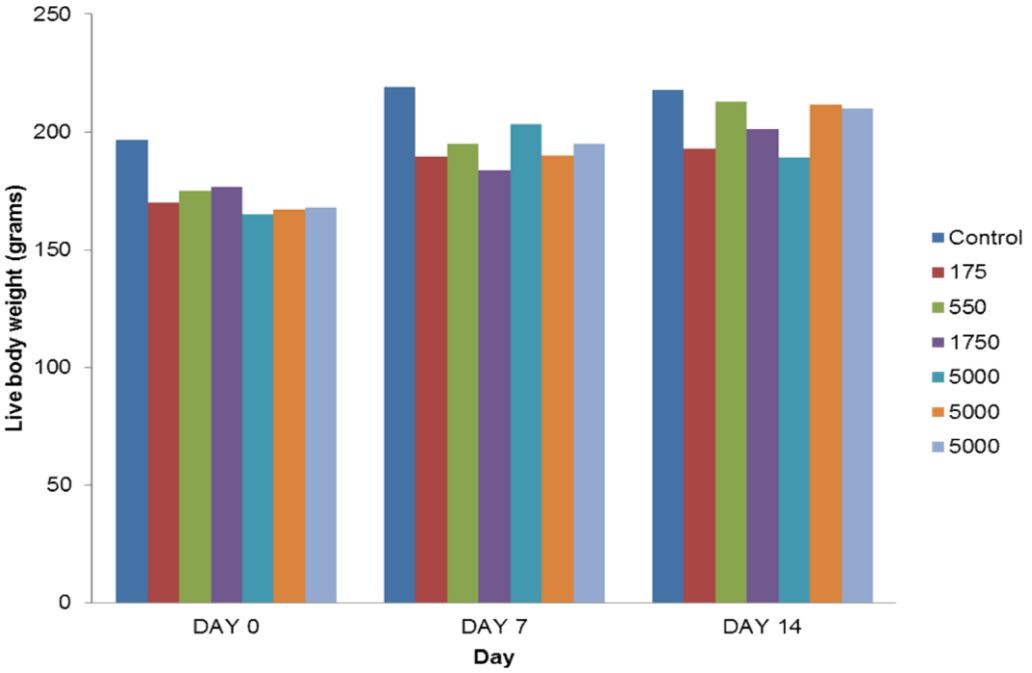


Fig. 2. Effect of the aqueous extract mixture on live body weights of rats (n=1)

In this study, the constituent phytochemicals in the extract mixture may not have had any or may have had very minimal negative effects on the internal vital organs to produce any noticeable physical toxicity signs. This could have been due to poor absorption and

bioavailability of the herbal extract mixture from the gastrointestinal tract [19]. According to the American Society for Testing and Materials (ASTM) [20], any chemical substance with an LD_{50} estimate less than 2000 mg/kg/oral route but greater than 1000 mg/kg/oral route could be considered to be slightly toxic although Clarke and Clarke [21] consider any compound with an estimated LD_{50} greater than 1000 mg/kg/oral route to be safe. A scale proposed by Lorke [22], roughly classifies substances according to their LD_{50} as follows: very toxic (LD_{50}<1.0 mg/kg), toxic (LD_{50} up to 10.0 mg/kg), less toxic (LD_{50} up to 100.0 mg/kg) and only slightly toxic (up to 1000.0 mg/kg). Substances with LD_{50} values greater than 5000 mg/kg are practically non-toxic [23]. The extract mixture formula qualified as a safe substance (non-toxic) using the proposed toxicological scales [7,20,21,22].

4. CONCLUSION

The study confirmed that *Entada leptostachya* (EL) and *Prosopis juliflora* (PJ) extract mixture is safe at the prescribed acute exposure, hence it is recommended for use by farmers and pastoralists.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee".

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Agbaje EO, Adeneye AA, Daramola AO. Biochemical and toxicological studies of aqueous extract of *Syzigium aromaticum* (L.) Merr. & Perry (*Myrtaceae*) in rodents. African Journal of Traditional, Complementary and Alternative Medicines. 2009;6(3):241-254.
2. Mbaka GO, Adeyemi OO, Oremosu AA. Acute and sub-chronic toxicity studies of the ethanol extract of the leaves of *Sphenocentrum jollyanum* (*Menispermaceae*). Agriculture and Biology Journal of North America. 2010;1(3):265-272.
3. Afolayan AJ, Ashafa AOT, Yakubu MT, Grierson DS. Toxicological evaluation of the aqueous extract of *Felicia muricata* Thunb., leaves in wistar rats. African Journal of Biotechnology. 2009;8(6):949-954.
4. Akanmu MA, Alade GO, Obuotor EM, Osasan SA, Omobuwajo OR. Acute and oral sub-acute toxicity of methanolic extract of *Bauhinia monandra*leaf in rats. African Journal of Pharmacy and Pharmacology. 2009;3(7):354-358.
5. Tardiff RG. *In vitro* methods of toxicity evaluation. Ann Rev Pharmacol Toxicol. 1978;18:357-369.

6. Wamburu RW, Kareru PG, Mbaria JM, Njonge FK, Nyaga G, Rechab SO. Acute and sub-acute toxicological evaluation of ethanolic leaves extract of *Prosopis juliflora*(*Fabaceae*). Journal of Natural Sciences Research. 2013;3(1):8-11.
7. OECD/OCDE Guidelines for Testing of Chemicals. OECD 425. Acute Oral Toxicity- Revised Up and Down procedure, Organization for Economic Co-operation and Development, Paris; 2001.
8. Kareru PG. Ethnomedicine practices, analysis and standardization of a herbal anthelmintic drug used by the Embu and Mbeere peoples of Kenya, PhD thesis, Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya; 2008.
9. Ebenshade HW, Grainger A. The Bamburi reclamation project. International Tree Crops Journal. 1980;1:199-202.
10. Maghembe JA, Kariuki EM, Haller RD. Biomass and nutrient accumulation in young *Prosopis juliflora* at Mombasa, Kenya. Agroforestry Systems. 1983;1:313-321.
11. Research Animal Resources, University of Minnesota. Reference values for laboratory animals. Accessed on July 13, 2013. Available: www.ahc.umn.edu/rar/refvalues.html.
12. Penlap VB, Assam Assam JP, Dzoyem JP, Pieme CA. *In vitro* antibacterial activity and acute toxicity studies of aqueous-methanol extract of *Sida rhombifolia* Linn. (Malvaceae). BMC Complementary and Alternative Medicine. 2010;10:40. DOI: 10.1186/1472-6882-10-40.
13. Giknis MLA, Clifford CB. Clinical laboratory parameters for Crl: WI (Han). Charles Rivers Laboratories Preclinical Services Montreal Inc., Senneville, Quebec, Canada; 2008;9.
14. Mounnissamy VM, Kavimani S, Sankari G, Quine SD, Subramani K. Evaluation of acute and sub-acute toxicity of ethanol extracts of *Cansjera rheedii* J Gmelin (*Opiliaceae*). Journal of Brewing and Distilling. 2010;1(1):011-014.
15. Aniagu SO, Nwinyi FC, Akumka DD, Ajoku GA, Dzarma S, Izebe KS, et al. Toxicity studies in rats fed nature cure bitters. African Journal of Biotechnology. 2005;4(1):71-78.
16. Kumar SVS, Mishra SH. Hepatoprotective activity of *Baliospermum montanum* (willd) Muell. Arg. in rats treated with carbon tetrachloride; *in vivo* and *in vitro* studies. Pharmacognosy Magazine. 2009;5(19):196-202.
17. Geidam YA, Ambali AG, Onyeyili PA. Preliminary phytochemical and antibacterial evaluation of crude aqueous extract of *Psidium guajava* leaf. Journal of Applied Sciences. 2007;7(4):511-514.
18. Adeyemi OO, Adeneye AA. Hypoglycaemic effects of the aqueous seed extract of *Hunteria umbellate* in normoglycaemic and glucose-and nicotine-induced hyperglycaemic rats. International Journal of Applied Research in Natural Products. 2009;2(1):9-18.
19. Mukinda JT. Acute and chronic toxicity of the flavonoid-containing plant*, Artemisia afra* in rodents*.* MSc Thesis, School of Pharmacy, University of the Western Cape, Bellville, South Africa; 2005.
20. American Society for Testing and Materials. Standard test method for estimating acute oral toxicity in rats. American Society for Testing and Materials, Philadelphia, U.S.A.; 1987.
21. Clarke E G C, Clarke ML. Veterinary toxicology. Casell and Collier Macmillan Publishers, London. 1977;268-277.
22. Lorke D. A new approach to practical acute toxicity testing. Arch Toxicol. 1983;54(4):275-287.

23. Salawu OA, Aliyu M, Tijani AY. Hematological studies on the ethanolic stem bark extract of *Pterocarpus erinaceus* Poir (*Fabaceae*). African Journal of Biotechnology. 2008;7(9):1212-1215.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.