Anthelmintic activity of microencapsulated *Prosopis juliflora* (sw.) extracts against *Haemonchus contortus* of sheep

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A Thesis Submitted in Partial Fulfillment for the Degree of Master of Science in Zoology (Animal Parasitology) of Jomo Kenyatta University of Agriculture and Technology

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DECLARATION

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

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DEDICATION

I dedicate this work to my parents and friends who continually supported me in prayers during the course of my project.

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LIST OF ABBREVIATIONS AND ACRONYMS

EHA	Egg Hatch Assay
LMA	Larval Mortality Assay
AMA	Adult Mortality Assay
DMSO	Dimethyl Sulfoxide
GIN	Gastrointestinal Nematode
L1	First stage larvae
L3	Third stage infective larvae
JKUAT	Jomo Kenyatta University of Agriculture and Technology
RPM	Revolutions Per Minute
FAEP	Fuel wood Afforestation Extension Project
WAAVP	World Association for Advancement Veterinary Parasitology
PBS	Phosphate Buffered Saline
ANOVA	Analysis of Variance
KVB	Kenya Veterinary Board
TLC	Thin Layer Chromatography

ABSTRACT

Haemonchus contortus is one of the most prevalent nematode parasite in small ruminants worldwide. Development of anthelmintic resistance and high cost of synthetic drugs prompted evaluation of microencapsulated medicinal plant extracts as alternative drugs. The aim of this study was to evaluate the anthelmintic activity of microencapsulated *Prosopis juliflora* extracts against *H. contortus* isolated from naturally infected sheep. Roots and leaves of *P. juliflora* were collected from Marigat, Baringo County, air-dried, ground and extracted with ethanol. The extracts were subjected to phytochemical screening and evaluated for anthelmintic activity according to standard procedures. The mean percentage extraction yields for roots and leaves ethanolic extract were 11.2% and 5.6%, respectively. The phytochemical screening on both ethanolic extracts tested positive for tannins, saponins, alkaloids, flavonoids and sterols. The amounts of saponins, alkaloids and tannins on roots were 5.9, 1.7, 1.8 and leaves 2.4, 3.7, 0.17 percent respectively. The results showed that microencapsulated leaves ethanolic extract at drug to polymer ratio of 2:1, leaf ethanolic extract and albendazole at 2.0 mg/ml concentration exhibited 100% on inhibition of egg hatchability. However, there was no statistically significant difference ($p= 0.272$) in mean percentage egg hatch inhibition on both leaf and root ethanolic extracts in comparison with albendazole which showed a significant difference ($p= 0.000$) in activity. In larval mortality assay, all microencapsulated extracts of *P. juliflora* (leaves and roots) induced over 50% mortality of larvae at the highest concentration used (2mg/ml). Albendazole required a maximum concentration of 0.25 mg/ml to induce 100% larval mortality. There was a significant difference ($p= 0.000$) in larval mortality compared to that of egg hatchability. The results on adult mortality assay indicate that there was a significant difference ($p< 0.05$) in mean percentage adult mortality of *H. contortus* at different concentrations and ratios. All the assays showed extract concentration dependent response. The EC_{50} values from microencapsulated leaves and roots extracts on adult mortality assay, ranged from 1.950 to a maximum of 26.87 mg/ml. These values were significantly different ($p< 0.05$) compared with albendazole (0.05 mg/ml) and leaves

ethanolic extracts (0.71 mg/ml). In conclusion, this study has shown that microencapsulated *P. juliflora* extracts had anthelmintic activity on eggs, larvae and adults of *H. contortus* parasite. The activity could be related to the presence of phytochemicals such as saponins, alkaloids and tannins. Therefore, microencapsulated herbal drug may be evaluated further as novel anthelmintic drug for control of *H. contortus* in small ruminants and thus *in vivo* studies are recommended to ascertain its efficacy.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Background information

Livestock production covers up to 40 percent gross value of agricultural production worldwide (Bachaya, Iqbal, Jabbar, & Ali, 2006), and livestock, especially small ruminants represent a major asset among resource-poor farmers (Perry, Randolph, McDermott, Sones, & Thornton, 2002). Gastro-intestinal nematode parasites pose a major constraint in sheep industry and it causes production loss, increased cost of management, treatment and mortality in severe cases (Barger & Cox, 1984; Larsen, Vizard, & Anderson, 1995). In Kenya, the annual economic loss due to helminthosis on livestock estimates to about 11.8% of the total slaughtered cattle and 46.0% for both sheep and goats (Olorunfemi, Ologunja, Adaudi, & Agbede, 2006).

Gastrointestinal nematodes (GINs) infection in sheep and goats is usually controlled with synthetic anthelmintics in combination with grazing management (Alawa *et al.*, 2010; Badar, Iqbal, Khan, & Akhtar, 2011). Synthetic anthelmintics are currently being used to control GIN (Pomroy, Hart, & Min, 2002), however, the evolution of resistance in GIN species to anthelmintic drugs has been reported in all sheep producing countries (VanWyk, Stenson, Vander Merwe, Vorster, & Viljoen, 1999). Keeping in view the problem of resistance, medicinal plants are potential candidates for their use as anthelmintics. Plant extracts with anthelmintic properties are considered one of the most promising alternatives for control of GINs (Waller, Knox, & Faedo, 2001; Gillian, Behnke, Buttle, & Duce, 2004).

1.2 Previous studies on use of medicinal plants as alternative anthelmintics

A number of medicinal plants have been used to treat parasitic infections in man and animals (Akhtar, Kabal, Khan, & Lateef, 2000). *Dregea volubilis* (family Asclepediaceae) is widely used as anthelmintic in traditional system of medicine in India (Hossain, Chandra, Nandy, Manda, & Gupta, 2012). In Kenya, plants that have been used as anthelmintics in livestock include *Vernonia lasiopus* (Njonge *et al.*,

2013) and *Entanda leptostachya* (Kareru, Kenji, Gachanja, Keriko, & Mungai, 2007; Njonge *et al*., 2013).

A study done by Kareru (2008) reported the presence of triterpenes, tannins, saponins and glycosides in *E. leptostachya*, while triterpenes, saponins, tannins and anthraquinones were found in *Albizia anthelmintica*. Phytochemical studies have also revealed the presence of saponins, tannins, flavonoids and alkaloids in *Prosopis juliflora* (Sathiya & Muthuchelian, 2008).

P. juliflora (Sw.) DC is an evergreen tree with a large crown and an open canopy, growing to a height of 5-10 m. A study by Wamburu *et al.* (2013) investigating the toxicity and safety levels of *P. juliflora* using *Swiss albino* rats revealed no toxicity symptoms and post mortem did not show any major gross effects on the internal organs. Saponins from its bark have also been reported in South West Africa to be effective against intestinal worms (Githiori, 2004).

The main problem of using plant derived natural compound is their degradation in gastrointestinal system before reaching the circulation system which limits the area of usage of these compounds (Luo *et al.*, 2013). Thus, there is need for microencapsulation system to link this gap so that its potential therapeutic benefits of natural compounds are maximized for effective control of gastrointestinal nematodes in small ruminants.

1.3 Literature review

1.3.1 Losses associated with GINs

Gastrointestinal nematodes (GINs) cause serious economic losses and is the most important factor limiting sheep production worldwide (Kaplan, 2004; Menkir, Uggla, & Waller, 2006; Abebe, Gebreyohannes, Mekuria, Abunna, & Regassa, 2010). In small ruminants production systems its consequences can be extensive, ranging from reduced animal performance (Fox, 1993; Gatongi, Munyua, Cheruiyot, & Prichard, 1997) and blood loss resulting in decrease in erythrocytes, lymphocytes, hemoglobin, packed cell volume, body weight and wool growth (Hayat, Hussain, Iqbal, Hayat, & Akhtar, 1996).

1.3.2 Gastrointestinal nematodes

Wide variety of GIN parasites are highly distributed in different geographical regions of the world, *Haemonchus contortus, Ostertagia ostertagi, Trichostrongylus* species, *Oesophagostomum* species. Among these GINs, *H. contortus* is the most important blood sucking parasitic nematode causing haemonchosis in small ruminants' throughout the world (Gibbs, 1977; Tritschler *et al.*, 1989; MacGlaflin, Zajac, Rego, & Petersson, 2011). In Kenya, *H. contortus* causes annual loss of US$26 million in sheep and goats as described by Kareru (2008).

1.3.2.1 *Haemonchus contortus* (Abomasal nematode)

The abomasal wireworm, *H. contortus* was first time described in 1803 by Karl Rodolphi (Soulsby, 1982). Twelve species of *Haemonchus* were described of which *H. contortus*, *H. placei* and *H. similis* are found globally in the abomasum of domesticated ungulates within the super-family Ovine, Caprine and Bovine (Hoberg, Linhtenfels, & Gibbons, 2004).

H. contortus is placed in phylum Nemathelminthes; class Nematoda, Order Strongylida, Family Trichostronglylidae, and subfamily Haemoncinae, Genus *Haemonchus,* and Species *H. contortus*.

1.3.2.2 Life cycle of *Haemonchus contortus*

The parasite has a direct life cycle, meaning no intermediate host is required for its development (Kaplan, 2003). Figure 1.1 shows the direct life cycle of *H. contortus* parasite in sheep and goats.

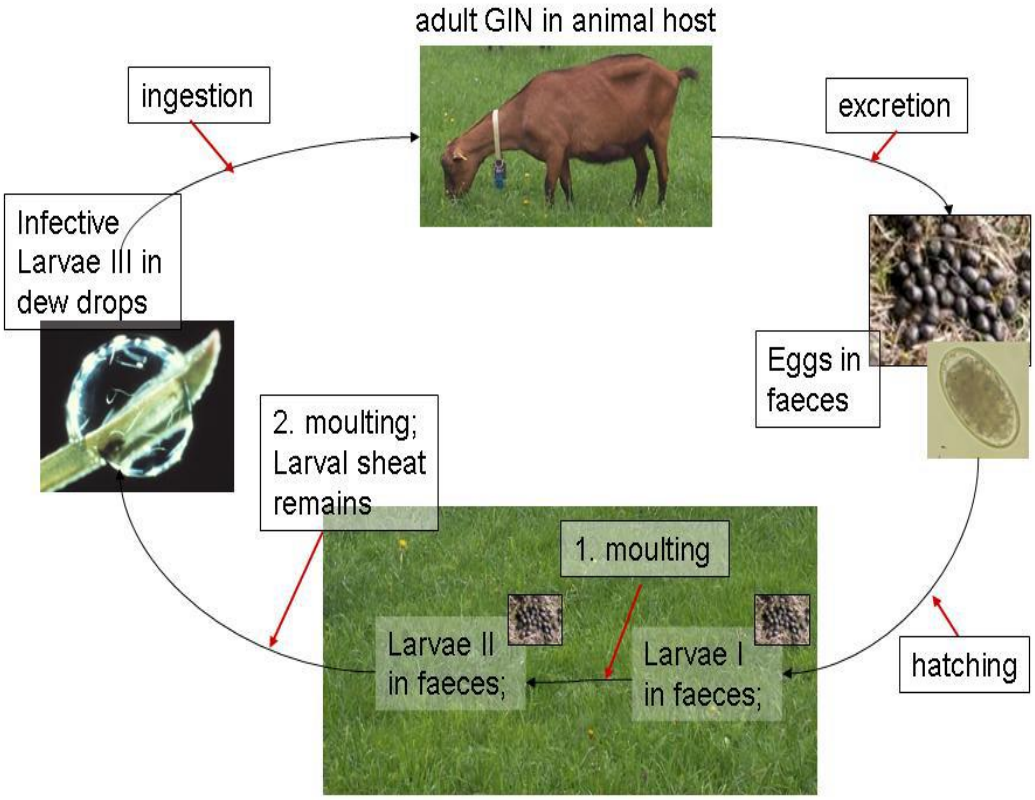

Figure 1.1: Life cycle of *H. contortus* nematode (Scheuerle, 2009)

It mainly depends on small ruminant host for its development and completes life cycle in 21 days (Miller & Horohov, 2006). The adult male and female worms live and mate in the abomasum of ruminant animals. Eggs in the early morula stage pass out of the host with the feces. First stage larvae hatch from the eggs within 24 to 48 hours and feed on bacteria present in the feces. These free-living larvae molt and the 2nd stage larvae continue to feed on bacteria as they develop into non-feeding infective 3rd stage larvae. The 3rd stage larvae retain the 2nd stage cuticle as a protective sheath to yield a double cuticle. Infective larval stage leaves the fecal pellet and climb up blades of moist grass on pasture where it is consumed by grazing animals (Besier, 2009). After ingestion, the larvae shed their outer protective layer in

the rumen and then invade the mucosa of the host abomasum, molting into the L_4 stage larvae (Miller & Horohov, 2006).

The L_4 late larval stage develops a piercing lancet before the final molting which enables them to obtain blood from the mucosal vessels. Adults move freely on the surface of the mucosa (Urquhart, Armour, Duncan, Dunn, & Jennings, 1996). The final molt to the 5^{th} stage occurs in the abomasal lumen and the worms mature into adults. Egg production commences when the sexually mature worms mate (Osen, 1986) and the eggs are passed out of the host in the feces. The prepatent period is 2-3 weeks in sheep (Urquhart *et al.,* 1996).

1.3.2.3 Signs and symptoms of haemonchosis

The larval and adult stages of *H. contortus* feed on the blood of the host causing severe anemia and death in heavily infected animals (Bethony, 2006). Anemia is characterized by pale mucous membranes, which is mainly visible in the lower eyelid. Sudden deaths in heavily parasitized animals occur as a result of hemorrhagic anemia caused by severe blood loss from the gut. Although animals with low levels of infection do not generally exhibit clinical signs, sub-clinical infections are also of great concern to producers due to weakness and significant decreases in weight, fertility and milk yield within a herd or flock (Mir, 2007). When feeding on the host, *H. contortus* exposes an oral lancet used to slit capillaries of the abomasal mucosa open and feed on the released blood, simultaneously secreting an anticoagulant causing prolonged bleeding at the site (Johnstone, 2000). At peak levels of infection, *H. contortus* accounts for the loss of up to one fifth of the circulating erythrocyte volume per day from lambs, and one tenth of the circulating erythrocyte volume per day from adult sheep (Bowman, Lynn, & Eberhard, 2003). The disease is acute in animals with heavy worm burdens. The parasite is extremely prolific and females can produce up to 10,000 eggs each day at a rate of one egg in every ten seconds (Lee, 2002). Edema under the jaw commonly called “bottle jaw” is another frequent sign of *H. contortus* infection caused by significant blood protein and plasma loss from the host (Barriga, 1997).

1.3.3 Anthelmintics drugs and their use

Anthelmintics are the most widely used means of controlling helminth infections (Roos, 1997). Anthelmintics are drugs that kill parasitic helminthes (Howell & Burke, 2008).

Since 1960s there have been three major classes of anthelmintics used in veterinary medicine, these include benzimidazoles, imidazothiazoles and macrocyclic lactone. Benzimidazole includes albendazole, thiabendazoles, mebendazole and fenbendazole which all have broad spectrum action against gastrointestinal nematodes (Martin, Robertson, & Bjorn, 1997).

Control of nematode parasites is essential for maximizing livestock productivity and feed efficiency. However, control of nematode parasites is becoming more difficult with increasing resistance of the parasites to common anthelmintics (Ketzis, 2003).

1.3.4 Anthelmintic resistance and cost of conventional de-wormers

Anthelmintic resistance is defined as a decline in the efficacy of an anthelmintic against a population of parasites that is generally susceptible to that drug (Wolstenholme, Fairweather, Prichard, Von Samson-Himmelstjerna, & Sangster, 2004).

The earliest reports of anthelmintic resistance involved nematode parasites of sheep and horses (Kaplan, 2004). Currently resistance has appeared in nematodes that affect many animal as well as humans. These include several phyla of helminths and cover all of the major chemical groups of anthelmintics (Sangster & Gill, 1999). Parasite resistance to benzimidazoles (i.e. albendazole, thiabendazole, and fenbendazole), imidazothiazoles (levamisole), and macrolides (ivermectin) has been reported in Australia, Africa, Europe, North America and South America; wherever animals are regularly treated with anthelmintics and investigations have been made (Prichard, 1994).

Helminth control in domestic animals is mainly based on chemical method in combination with grazing management. The efficacy of drugs currently has been

reduced because of development of resistant nematode strains. The main methods for delaying drug resistance are: infrequent use of anthelmintics, utilization of the most active anthelmintic compounds at the highest practical dose, yearly alternation of anthelmintics from different groups, management of pastures to avoid the buildup of resistant populations and surveillance of newly acquired stock (Barriga, 1997).

High cost of modern anthelmintics and unavailability has limited the effective control of helminthes by rural farmers. In addition, widespread and intensive use of sometimes low quality anthelmintics (Githiori, 2004; Mohammed, Mohammed, Yusuf, & Ologunja, 2005) has led to resistance, leading to reduction in effectiveness of available anthelmintics (Mohammed *et al.,* 2005). Chemical residues from anthelmintics are also reported to be toxic and can pose side effects to the administrator and the animal (Kareru, 2008; Nalule, Karue, & Katunguka-Rwakishaya, 2011).

1.3.5 Plant remedies as an alternative to synthetic anthelmintic

Plants not only grant food and shelter to human beings but have served through centuries as a constant source of medication for the treatment of a variety of diseases. The history of ethno-botanicals is almost as old as human civilization (Sarojini, Kanti, Das, Priyanka, & Kumari, 2012). Search for new and more effective remedies for controlling the diseases of livestock has given rise to the study of plant based remedies (Mathias, 2004). This has also been acknowledged by WHO that estimated that 80% of the people in the developing world or 60% of the human race depend largely on plants for control and treatment of various human and animal diseases (WHO, 2010).

Medicinal plants run the pedestal of health care systems and in many poor rural areas, ethno- botanicals can play an important role in animal production systems and livelihood development and often become the only available means for curing the ailments (Jabbar *et al.,* 2005). All parts of plants may serve as source of medicinally useful components and many plants are known to provide a rich source of botanical anthelmintics, antibacterial and insecticides (Iqbal *et al.,* 2005; John, Githiori, & Thamsborg, 2006).

Medicinal plants nowadays serve as alternative remedies for both livestock and humans. In comparison with synthetic drugs, herbal medicines are economical, easy to consume and are locally available (Raj, Murugamani, & Madhuri, 2011).

1.3.6 Anthelmintic bioactive principles

1.3.6.1 Saponins

The name saponin was derived from soapwort plant, whose root was traditionally used as soap. Saponins are glycosides of triterpenes, streroids (Figure 1.2) and sometimes alkaloids which occur primarily but not exclusively in plants.

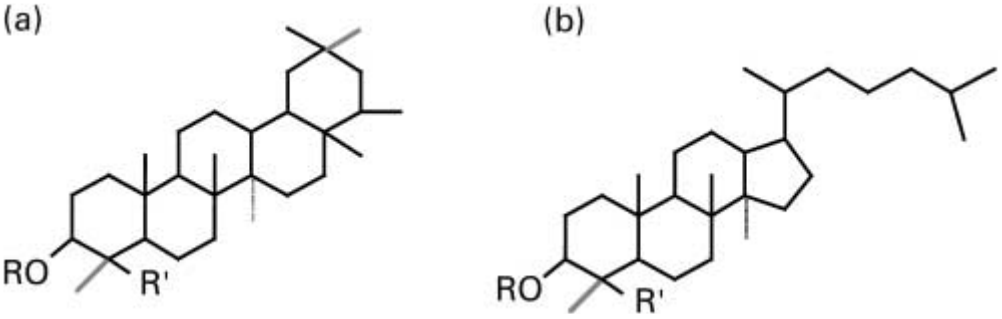

Figure 1.2: Structures of saponins (a) triterpenoid and (b) steroid

A glycone part of saponin is generally oligossccharides and is referred to as a sapogenin. Many of the plants contain little or no saponins while others, the triterpenoid saponins predominate. The oleanane-type of triterpene is the base structure found in the largest variety of medicinal plants (Kareru, 2008).

Saponins have been reported to have anthelmintic properties. Saponins were first reported to kill worms as early as 1962 using an extract from *Albizia anthelmintica* (Kareru, 2008). Other studies have been done to characterize other plants believed to have anthelmintic effects. Work done in Nigeria by Ajayi, Ademola, and Okotie (2008) through larval survival assay revealed that *Aframomum danielli* have anthelminthic activity against gastrointestinal nematodes in sheep. A study done by Deore and Khadabadi (2010) in Maharashta, India investigated the efficacy of *Chlorophytum borivilianum* root tuber against selected worms. From the results, they confirmed the presence of saponin from the TLC analysis of the crude extracts and they concluded that tubers could be used as anthelmintics and this further lead to

confirmation that anthelmintic activity of *C. borivilianum* was due to presence of saponins.

1.3.6.2 Tannins

Tannins are naturally occurring polyphenolic compounds with high molecular weight. They are classified into hydrolysable tannins (HT) and condensed tannins as illustrated in Figure 1.3 (FAO/IAEA, 2000). Condensed tannins are polymeric flavonoids which are most common in forage legumes, trees and shrubs (Min & Hart, 2003). Condensed tannins are relatively stable in the digestive tract of the animal and rarely have toxic effects (Githiori, 2004).

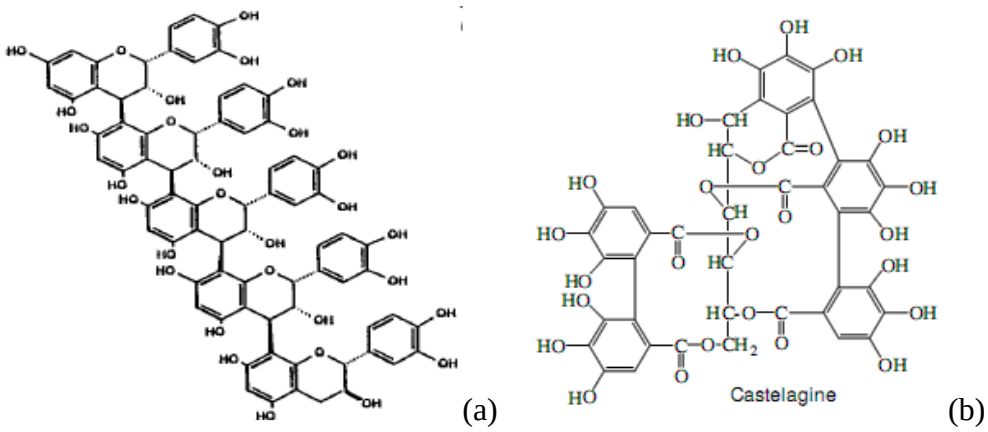

Figure 1.3: Condensed tannins **(a)** and hydrolysable tannins **(b)** (Mueller, 1999)

An *in vitro* anthelmintic activity of *Baliospermum montanum* by Mali and Wadekar (2008) concluded that there was a possibility that tannin was responsible for the anthelmintic activity displayed against *Pheretima posthuma* and *Ascaridia galli*. The anthelmintic activity of condensed tannins against *H. contortus* in sheep has been evidenced by inhibited egg hatching (Zafar *et al.*, 2007). Another study on the effect of condensed tannins in cassava by Netpana *et al.* (2001) concluded that feeding a supplement of cassava hay containing moderate levels of condensed tannins has an effect of reducing nematode egg counts in both cattle and buffaloes.

1.3.7 *Prosopis juliflora*

P. juliflora is a flowering plant in the pea family fabaceae (Quattrocchi, 2000). It is a dry land tree or shrub native to Central America, South America and the Caribbean. It is an evergreen tree with a large crown and an open canopy (Pasiecznik *et al.*, 2001). It is tolerant to arid conditions, fast growing and nitrogen-fixing (Anonymous, 2003; Pasiecznik, Harris, & Smith, 2004). In Kenya it was introduced in the early 1970s to rehabilitate a quarry in Bamburi near Mombasa. It was introduced in Lake Baringo area in the early 1980s through the Fuel wood Afforestation Extension Project (FAEP) (Kariuki, 1993; Lanacuru, 2003). As described by Anonymous, (2003) its height is up to 14m and stem is greenish-brown with strong thorns (Plate 1.1).

Plate 1.1: Pods, leaves and thorns of *Prosopis juliflora*

The leaves of *P. juliflora* plants are compound shape and have high tannin composition (Pasiecznik *et al*., 2001; Mathews & Brand, 2004). It flowers throughout the year with yellow flowers that hang from the branches (Masilamani & Vadivelu, 1997).

1.3.8 Chemical composition

The components of the woody biomass are divided into hemi-cellulose, lignin, cellulose, ash and water. Hemi-cellulose has been estimated as 25%-30%, cellulose 40%-45%, extractives 3%-15% and lignin 11%-28%. Extractive chemicals from woody biomass include sugars, resins, volatile oils, fatty acids, tannins, alcohols and phenols (Goldstein & Villarreal, 1972). Leaves of *P. juliflora* contain alkaloids such as tryptamine, piperidine, phenethylamine and juliprosopine which all of them have antifungal properties (Tapia, Bustos, Astudillo, Theoduloz, & Schmeda-Hirschmann, 2000). Sirmah, Dumarçay, Masson, and Gerardin (2009) identified catechin, gallocatechins, epicatechin, methylgallo-catechins, fatty acids and free sugars from *P. juliflora* bark, whereas the pods contain an important quantity of galactomannans, mannoses, saturated and unsaturated fatty acids and free sugars which are used as a food supplement and medicine for animals and humans (Sirmah *et al.,* 2009).

1.3.9 *P. juliflora* in traditional herbal medicine

Traditional medicine is widely practiced in Kenya, and has been documented by ethnobotanical surveys (Kareru *et al.,* 2007). Many plants of the genus *Prosopis* are known to have medicinal properties and are used in traditional medicine as astringents, in rheumatism and as remedies against scorpion stings and snake bites (Wassel, Rizk, & Abdel-Bary, 1972). Powders from flower extracts mixed with sugars are eaten by pregnant mothers to safeguard them from miscarriage (Garg & Mittal, 2013).

1.3.10 Pharmacological potential of *P. juliflora*

Isolation and structural determination of two new alkaloids, juliprosinene and juliflorinine, from *P. juliflora* showed antibacterial activity against strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Shigella sonnei* (Ahmad, Sultana, & Qazi, 1989). The antimicrobial

activity of benzene insoluble alkaloid extracted from leaves of *P. juliflora* was determined through *in vitro* test and it was found more effective than bacitracin, gentamycin, chloromycetin and trimethoprim against *Staphylococcus aureus, Staphylococcus lactis, Staphylococcus faecalis, Staphylococcus pyogenes* and *Corynebacterium diphtheria* (Sirmah *et al.,* 2009). Almaraz-Abarca *et al.* (2007) showed that pollen of *P. juliflora* provided an important source of flavonoids, which can be considered as natural antioxidants. Mesquite pollen extracts showed antioxidant activity related to the flavonoid concentration in both *in vitro* and *in vivo* systems, with a lower activity in *in vivo* systems. Methanoic extract of *P. juliflora* bark at 100, 200 and 400 mg/kg exhibited significant anti-inflammatory activity in acute and chronic inflammatory models. All the doses of methanolic extract of *P. juliflora* bark showed a dose dependent inhibition against histamine and serotonin induced rat paw edema as compared with control animals. Furthermore, the same dose levels successfully reduced the formation of granulation tissues by cotton pellets in rats (SivaKumar *et al.,* 2009).

1.3.11 Microencapsulation technology

Microencapsulation is a technique by which liquid substances and solid particles of core material are coated with a thin film of protective materials (Hogan, McNamee, Riordan, & Sullivan, 2001). As described by Desai and Park (2005), it is a technique in which bioactive compounds are microencapsulated with a biopolymer. The coating wall protects the core against deterioration, reduces the evaporation of volatile compounds and releases the core material under desired conditions (Kibry, 1991). Several materials have been used as coating wall materials such as ethylcellulose, starches, gum arabic, methylcellulose, gelatin, whey protein maltodextrins, disaccharides, pullunan, and sodium caseinate (Hogan *et al.,* 2001).

1.3.12 Microencapsulation techniques

There are several microencapsulation techniques that are currently available. The current microencapsulation techniques for plant extracts consist of spray drying, emulsion, freeze drying and electrospray.

1.3.12.1 Spray Drying

Spray drying microencapsulation technique (Figure 1.4) has been in use in chemical and food industry since 1950s (Fang & Bhandari, 2010), this technique is so far one of the oldest and the most widely used technology for microencapsulation. Spray drying technique is normally done by forming particles from dispersion of the active ingredient in the solution that is used as coating agent (Munin & Edwards-Lévy, 2011). The advantages of this kind of technique are low cost, flexible and continuous operation. It is normally preferred in industrial technology. The basic principle of spray drying is based on the homogenization of the core material with the wall material and atomization of this dispersion with a nozzle or spinning wheel in the spray-drying chamber. The atomization promotes fast removal of water. Then, the particles are separated from the drying air and fall into the bottom of the drier (Nedovic, Kalusevica, Manojlovicb, Levica, & Bugarski, 2011).

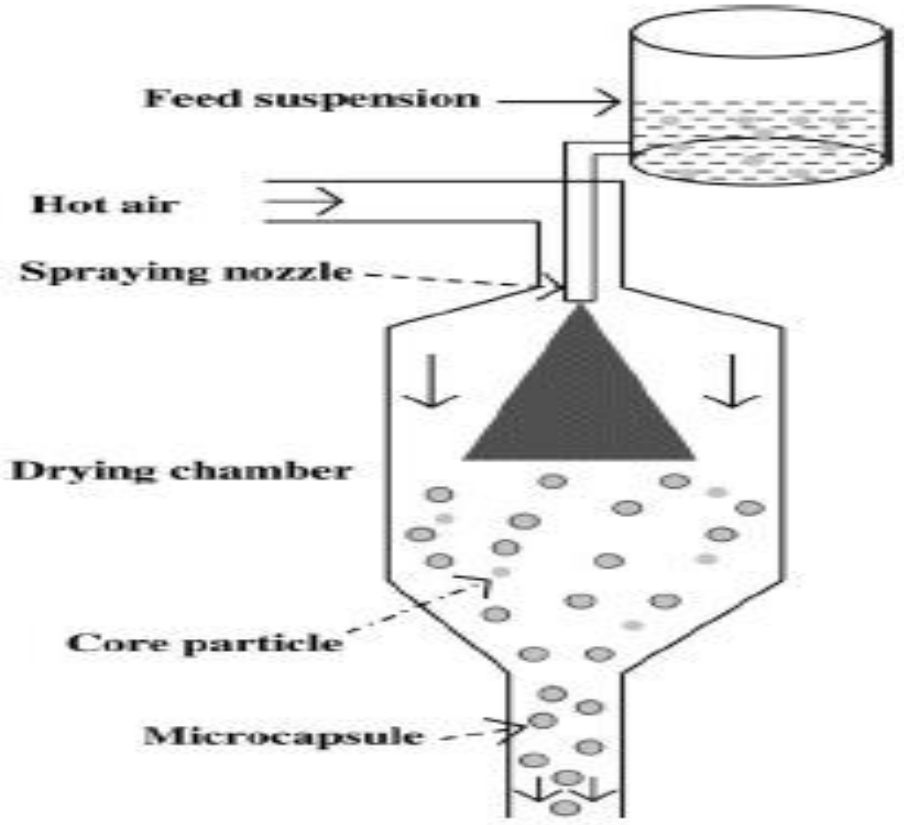

Figure 1.4: Schematic illustration of spray drying technique for microencapsulation (Nedovic *et al.,* 2011)

1.3.12.2 Emulsions

This is a technology which is normally utilized for water soluble active ingredients. Since the emulsification is by dispersing one liquid in a second immiscible liquid as small spherical droplets, an emulsion consists of at least two immiscible liquids which are usually oil and water. If oil droplets are dispersed in an aqueous phase, it is called as oil-in-water emulsion, whereas if water droplets are dispersed in an oil phase it is called water-in-oil emulsion (Fang & Bhandari, 2010).

1.3.12.3 Freeze Drying

Freeze drying is a simple microencapsulation technique for compounds that are soluble in water. It is also referred to as lyophilization. The principle of freeze drying is based on the dehydration of heat-sensitive samples. Freeze-drying performs by freezing the material and declining the surrounding pressure and then adding enough heat (Fang & Bhandari, 2010). The frozen water in the material converts solid phase to gas phase. The resulting microencapsulated compounds are not exposed to high temperature, therefore the freeze-dried products preserve their initial characteristics (Patel, Patel, & Suthar, 2009).

1.3.12.4 Electrospray

Electrospraying is a technique whereby there is atomization of liquid sample. The technique is also known as electrohydrodynamic atomization. The principle of electrospraying is based on the theory of charged droplets; where an electric field applied to a liquid droplet exiting a capillary is able to deform the interface of the droplet (Jaworek, 2008).

1.3.13 Testing for anthelmintic activities of the herbal plant through *in vitro* and *in vivo* studies

In vitro and *in vivo* studies are usually done to assess drug efficacy. *In vivo* test, involves feeding the ruminant animal with herbal extract followed by monitoring helminthes eggs in the animal faeces over time after the drug is administered.

Reduction of faecal egg counts with time is an indication of efficacy (Githiori, 2004; Dawo & Tibbo, 2005; Agaie & Onyeyili, 2007; Krimpen *et al.*, 2008; Burke *et al.*, 2009; Deore & Khadabadi, 2010). *In vitro* studies involve culturing helminth larvae from ruminant faeces either at room temperature or in an incubator. The larvae are then inoculated with herbal extracts and dead larvae are monitored with time (Zafar *et al.*, 2007; Kareru, 2008; Mali & Wadekar, 2008; Sujon *et al.*, 2008; Jesupilla & Palanivelu, 2009; Deore & Khadabadi, 2010).

A study done by Kareru *et al.* (2012) revealed that *E. leptostachya* and *R. rhododendroides* methanol extracts were potent against *H. contortus* adult worms with mortalities of 77% and 54%, respectively. *In vitro* anthelmintic activity of *Terminalia arjuna* bark extracts against *H. contortus* showed a dose dependent anthelmintic activity with an LC_{50} value of 0.646 mg/ml (Zafar *et al.*, 2009). Similar results have also been reported by Ademola and Eloff (2010) on *in vitro* anthelmintic activity of *Combretum molle* acetone extract (LC_{50}=0.866mg/ml) against *H. contortus* eggs. *Aframomum danielli* hexane extract demonstrated lower anthelmintic activity with LC_{50} value of 0.39mg/ml compared to ethanolic extract (LC_{50}=0.033mg/ml) using larval mortality assay (Ademola, Ajayi, & Okotie, 2008).

1.4 Statement of problem

Parasitic conditions caused by occurrence of gastro intestinal nematodes in ruminants are one of the greatest threats to livestock production worldwide. Gastrointestinal nematodes especially *H. contortus* have shown resistance towards all classes of synthetic drugs globally (Artho, Schnyder, Kohler, Torgerson, & Hertzberg, 2007). Mba, Ogunrinade, and Dina (1992) reported resistance of thiabendazole in strongyles of sheep in Ibadan, Nigeria. Similarly, anthelmintic resistance of cattle strongyles to albendazole, levamisole and morantel was reported by Fashanu and Fagbemi (2003) in Southwestern Nigeria. These drugs have long been considered for controlling this problem but high prices, scarcity in remote areas, side effects, chemical residues in products (Shagal, Kubmarawa, & Barminas, 2013) and degradation of active compounds before reaching circulation system has hindered their effectiveness. Therefore, application of microencapsulation technology on *P. juliflora* extracts is

considered as a promising alternative for the control of these nematodes in small ruminants.

1.5 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. Parasitic conditions caused by helminthes are one of the main problem affecting small ruminants in Kenya. The economic loss due to parasitic diseases in goats and sheep was estimated at Ksh 2.6 billion annually (Kareru, 2008). Synthetic anthelmintics have long been considered the only effective way of controlling this problem but high prices, scarcity in remote areas, side effects, chemical residues in products, development of resistance of the targeted parasites (Artho *et al.,* 2007) and degradation of active compounds in the gastrointestinal tract before reaching circulation system has been a major challenge to their effectiveness. This has awakened the interest of microencapsulated herbal medicinal extracts as an alternative source for control of these parasitic helminthes. Therefore, in respect to the significance of use of traditional herbal medicine in primary health care, Kenya government in 1979-1983 developed a strategic plan. This plan was developed to evaluate the functions and extent of usefulness of traditional medicine (NCAPD, 2005). A key goal of Kenyans sustainable development goals (SDG) and vision 2030 is to supply adequate livestock health care for all.

The present study was conducted to evaluate the anthelmintic activity of microencapsulated *P. juliflora* extracts against *H. contortus* by *in vitro* tests. Microencapsulation technique was used to entrap the bioactive compounds from the herbal drug.

1.6 Hypothesis

1.6.1 Null hypothesis

Microencapsulated *Prosopis juliflora* roots and leaves ethanolic extracts does not have anthelminthic activity against *Haemonchus contortus* of sheep.

1.7 Objectives of the study

1.7.1 General objective

The general objective of this study was to evaluate the anthelmintic activity of microencapsulated *P. juliflora* extracts against *H. contortus* of sheep.

1.7.2 Specific objectives

1. To determine the phytochemical substances and quantify saponins, tannins and alkaloids in roots and leaves of *P. juliflora* plant.
2. To determine *in vitro* ovicidal activity of microencapsulated *P. juliflora* extracts against *H. contortus* eggs.
3. To determine *in vitro* larvicidal activity of microencapsulated *P. juliflora* extracts.
4. To determine the *in vitro* anthelmintic activity of microencapsulated *P. juliflora* extract against adult *H. contortus* parasite.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study site

Plant samples were collected from Marigat area of Baringo County (Figure 2.1) and research conducted at JKUAT Zoology, Chemistry and Botany laboratories.

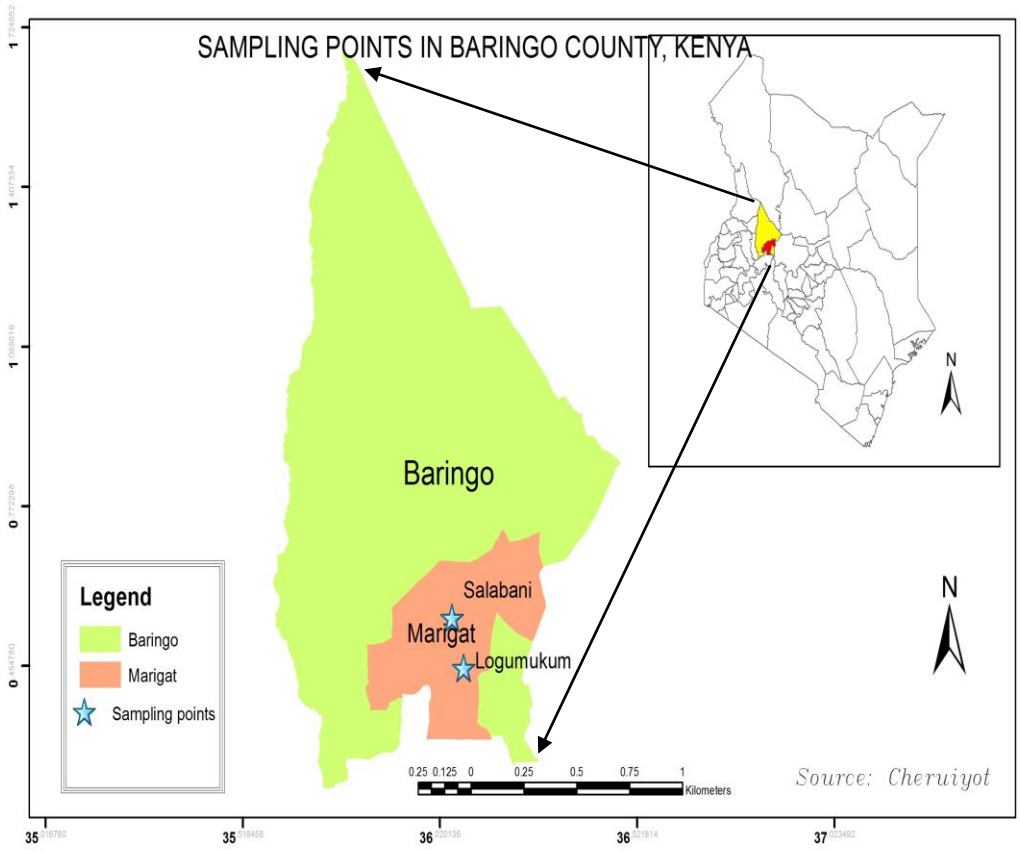

Figure 2.1: Map of Kenya showing sampling points in Marigat, Baringo County

2.2 Experimental design and procedure

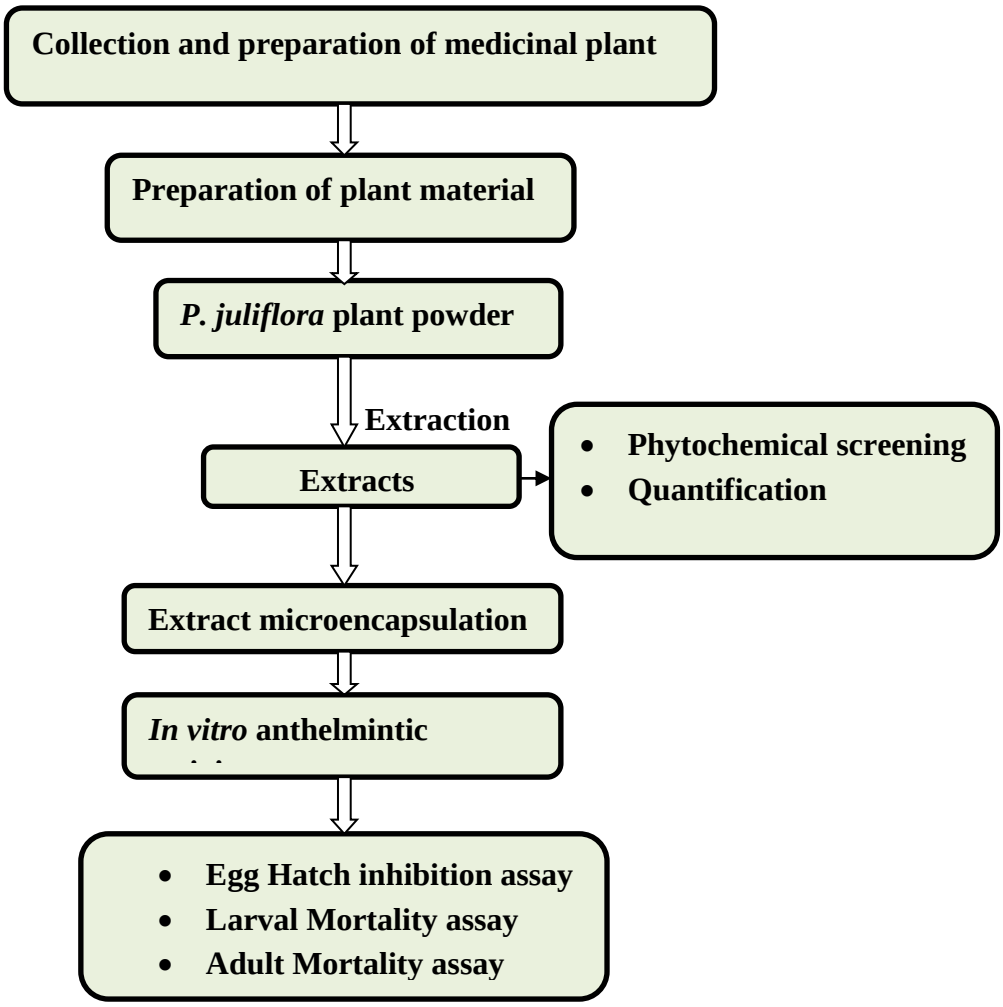

Figure 2.2: Schematic experimental design

Schematic experimental design for the study is presented in Figure 2.2. The design played a significant role in the study since it was used to plan and conduct experiments. It outlined on collection of medicinal plant, plant preparation, extraction, phytochemical screening, quantification, microencapsulation and *in vitro* anthelmintic activity.

2.3 Sample collection and preparation

One kilogram each of *P. juliflora* leaves and roots (Plate 2.1) were collected from Marigat in Baringo County, Kenya. The samples were packed in pre-sterilized polythene bags and transported by vehicle to JKUAT where it was authenticated by botanists. The plant samples were washed in water, chopped and shade dried as illustrated in Plate 2.1 for three weeks. Once completely dried, grinding into fine powder was done by a mechanical grinder manufactured by Jomo Kenyatta University of Agriculture and Technology, Mechanical Engineering Department.

Plate 2.1: Shade drying of (a) roots and (b) leaves of *P. juliflora*

2.4 Ethanolic extraction

Fine ground powder of *P. juliflora* leaves and roots samples were weighed, extracted with ethanol and residues filtered using Whatman filter paper No.1. The extracts were concentrated using a rotary evaporator (BUCHI R-200, Labortechnik Switzerland) at 45ºc and stored at 4°C until required for phytochemical screening, microencapsulation and bioassay.

2.5 Determination of phytochemical and quantification of saponins, tannins and alkaloids in roots and leaves

2.5.1 Phytochemical screening

Extracts were subjected to phytochemical screening as described by method of Harborne (1998).

2.5.1.1 Test for Saponins

Half a gram of plant extract was added into 5 ml of distilled water and shaken vigorously. Formation of a stable emulsion on addition of three drops of olive oil indicated the presence of saponins (Trease & Evans, 1996)

2.5.1.2 Test for tannins

In this test, 0.5ml of 5% ferric chloride solution was added to 0.5ml of the sample solution. A dark-green colour indicated the presence of tannins (Kokate, 1996).

2.5.1.3 Test for alkaloids

Mayer's test: In this test, 1ml of Mayer's reagent (potassium mercuric iodine) was added to 1ml of the test solution and observed for a white precipitate, which is a positive indicator for the presence of alkaloids (Sadasivam, 1996).

2.5.1.4 Test for flavonoids

To 1 ml of Crude extract, 3 drops of ammonia solution was added followed by 0.5ml of concentrated hydrochloric acid. The formation of pale brown colouration indicated the presence of flavonoids (Tiwari, 2011).

2.5.1.5 Test for Sterol

To test the presence of sterols, 2 ml of acetic anhydride was added to 2 ml of the sample solution and kept in ice. This was followed by careful drop-wise addition of 1 ml concentrated sulphuric acid by the sides of the test tube while still kept in ice. A brown ring at the interface was taken as an indicator for the presence of phytosterols. In Salkowski's test, 1 ml of chloroform was added to 1 ml of sample solution followed by careful drop-wise addition of concentrated sulphuric acid by the sides of

the test tube to form a lower layer. A red-brown colour at the interface was taken as the indicator for the presence of a steroidal ring (Siddiqui & Ali, 1997).

2.5.2 Quantification of saponins, tannins and alkaloids in the herbal drug

Saponins, tannins and alkaloids from *P. juliflora* were quantified using established standard procedures as described in section (2.5.2.1, 2.5.2.2 and 2.5.2.3). They were quantified since they are the main phytochemicals responsible for anthelmintic effect as reported by Danquah, Koffuor, Annan, and Ketor (2012). Plant sterols are responsible for blocking the absorption of dietary and endogenously derived cholesterol from the gut (Lagarda *et al.,* 2006). As described by Karpe (2014) flavonoids are the main compounds that act as primary free anti-oxidant scavenging radicals. Therefore, it was of less importance to quantify the two phytochemicals.

2.5.2.1 Quantification of saponins

The procedure of Obadoni and Ochuko (2001) was used to determine the amount of saponins in the herbal drug. Ten grams of sample was taken in 250 ml of 20% aqueous ethanol and heated over water bath for 4 hrs with continuous stirring at about 55°C. The mixture was then filtered and the residue re-extracted with another 200 ml of 20% aqueous ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was then transferred into a 250 ml separation funnel and 20 ml of diethyl ether added and shaken vigorously. The aqueous layer was recovered while the diethyl ether layer discarded. The purification process was repeated. Sixty ml of n-butanol was then added. Combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight and saponin content was then calculated as percentage using the following formula:

$$\% \text{ Yield } = \frac{\text{Weight of saponins extracts}}{\text{Weight of plant sample}} \times 100\%$$

2.5.2.2 Quantification of tannins

Dried (finely ground) plant material (200 mg) was weighed and transferred into a 50ml glass beaker. Ten ml of aqueous acetone (70%) was then added and extracted for 20 minutes at room temperature. The contents of the beaker were transferred to centrifuge tubes and subjected to centrifugation at 3000 rpm for 10 minutes. The supernatant was collected, divided into two equal portions and kept on ice and then analyzed for total phenols and non-tannin phenols (McDonald, Prenzler, Autolovich, & Robards, 2001).

Folin-Ciocalteu method described by Otang *et al*., (2012) was used for the determination of total phenols in the supernatant. 0.3 ml of the first portion of the supernatant was taken in a test tube and 2.2 ml of distilled water added followed by 1.25 ml of Folin-Ciocalteu reagent and then 6.25 ml of sodium carbonate solution. The tube was vortexed and absorbance recorded at 725 nm after 40 minutes in a UV spectrophotometer (UV-1800 Shimadzu). The amount of total phenols was calculated as tannic acid equivalent from the calibration curve and recorded as x, expressed on a dry matter basis.

Non-tannin phenols were analysed by first removal of tannin from the tannin-containing extracts using polyvinyl polypyrrolidone (PVPP). Four ml of PVPP was added to 3.0 ml of the second tannin-containing portion of the extract in tubes and then vortex and kept on ice. The contents in the tube were then centrifuged at 3000 rpm for 10 min and the supernatant collected and absorbance recorded at 725 nm. This was recorded as y, expressed on a dry matter basis. The difference between total phenols and non-tannin phenols (x-y) was taken as the amount of tannins equivalent.

2.5.2.3 Quantification of alkaloids

Total alkaloids content was determined by the alkaline precipitation gravimetric method described by Harborne, (1998) and Otang *et al.,* (2012). Five grams of the sample was weighed into a 250 ml beaker and 200 ml of 20% acetic acid in ethanol added, covered and allowed to stand for 4 hours. This was then filtered and the volume reduced to one quarter of the original volume using water bath. Concentrated ammonium hydroxide was added drop-wise to the extract until the precipitation was

complete. The whole solution was allowed to settle and the precipitate collected and washed with dilute ammonium hydroxide and then filtered. The residue collected was alkaloids which was dried, weighed and calculated using the following formula.

$$\% \text{ alkaloid content} = \frac{\text{weight of alkaloid precipitated}}{\text{weight of sample extracted}} \times 100\%$$

2.5.3 Fourier Transform Infrared Spectrophotometer (FTIR)

This analysis was done to confirm alkaloids and saponins isolated from the plant through quantification process. Plant powder, crude saponins and alkaloids extracts (1mg) were mixed with 25mg of dry spectroscopic grade potassium bromide (KBr) and mixed by grinding using mortar and pestle and compressed into a thin translucent pellet. The powdered sample of each plant specimen was loaded in FTIR spectroscope (Shimadzu 8400). The FTIR spectra absorbance values were recorded in the range of 4000-500 cm^{-1} to determine the functional groups. Presence of OH, C-H, C=C, C=O and N-H functional groups confirms the presence of alkaloids and saponins in the samples (Kareru, 2008; Sasidharan, Torey, Yeng, & Latha, 2010).

2.6 Preparation of *P. juliflora* extracts microspheres

Microspheres of *P. juliflora* extract were prepared by emulsion solvent evaporation method (Chowdary, 2011). Ten grams of ethyl cellulose polymer was weighed and dissolved in 100 ml of ethanol to form a homogenous solution. Calculated quantity of *P. juliflora* extract was then added to the homogenous polymer solution and mixed thoroughly using a magnetic stirrer. The resulting mixture was added to 200 ml of aqueous mucilage of sodium carboxymethly cellulose (CMC) to emulsify the added dispersion as fine droplets. The ethanol solvent was then removed by evaporation during the continuous stirring at room temperature for three hours to produce spherical microspheres. The microencapsulated microspheres from leaves and roots were prepared in drugs to polymer ratios of 1:2, 1:1 and 2:1. Microspheres were subjected to drying and their weights determined. Percentage yield of the prepared microspheres was then calculated using the following formula:

Percentage yield = $\frac{\text{Weight of microsphere x 100}}{\text{Weight of the polymer + Drug}}$

2.7 *In vitro* drug released from *P. juliflora* microsphere

Drug released from *P. juliflora* microsphere was done according to the procedure described by Chowdary (2011). Microencapsulated *P. juliflora* extracts on concentrations ranging from 0.0625 mg/ml to a maximum of 2 mg/ml and in drug to polymer ratios of 1:2, 1:1 and 2:1 were redispersed on 2.0 ml of Phosphate Buffered Saline (PBS, pH 7.4) and kept in an incubator at 37 ^{0}C for 48 hours without agitation. The supernatant was collected and filtered after centrifugation at 35,000 rpm for 30 min. The released drug was kept at 4°C until required for egg hatch assay and larval mortality assay.

2.8 Determination of *in vitro* ovicidal activity of encapsulated *P. juliflora* extracts against *H. contortus* eggs

2.8.1 Egg hatch assay

Egg hatch assay was conducted according to World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles, Geerts, Klei, Tailor, & Waller, 1992). Mature adult parasites of *H. contortus* were collected directly from abomasum of naturally infected sheep slaughtered from Ruiru slaughter house in Kiambu County, Kenya. The worms were picked using forceps from the abomasum and transferred into universal bottles containing were in PBS (pH 7.4). The samples were transported to JKUAT zoology laboratory. Identification and separation of adult mature female were done according to the method by Hansen and Perry (1994) using a microscope. The females were washed with distilled water severally and gently crushed using pestle and mortar to liberate eggs as described by Simon, Jegede, & Nafarnda (2012). The concentration of eggs in 200 µl solution was quantified using a McMaster slide. The microencapsulated and non-microencapsulated *P. juliflora* extracts of leaves and roots were used as active treatment. The non-microencapsulated extracts were dissolved in dimethylsulfoxide (DMSO) to improve its solubility in PBS. Solution of (2 mg/ml) was taken for preparation of final concentrations ranging from 0.0625 to 2.0 mg/ml (Rechab *et al.,* 2014). Albendazole (Sigma Aldrich, USA) at concentration of 0.0625 to 2.0 mg/ml

was utilized as positive control and was dissolved in DMSO, while untreated eggs in PBS were used as negative control. The test was conducted in 96-well flat-bottomed microtitre plate (Iwaki, Asahi Glass Company, Japan). Egg suspension of (200 µl: 100 eggs) was pipette into each well, and eggs were incubated for 48 hours at 27ºC and 70 % relative humidity. Each concentration was done in triplicate. After 48 hours of incubation, a drop of lugol's iodine was added to stop further hatching. Hatched larvae (live or dead) and unhatched eggs were counted using a compound microscope at X40 magnification with the help of a counter (Rechab *et al.*, 2014). The egg-hatch inhibition rates assessed were calculated by the following formula.

$$\% \text{ egg hatch inhibition} = \frac{\text{Total number of eggs - Number of hatched larvae}}{\text{Total number of eggs}} \times 100$$

2.9 Determination of *in vitro* larvicidal activity of microencapsulated *P. juliflora* extracts

2.9.1 Larval mortality assay

Larval mortality assay was conducted according to World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles *et al.*, 1992). Collection of adult parasite, egg recovery, concentration of eggs and hatching was done just as indicated in section 2.8.1. Amphotericin B (5 µg/ml) from Sigma, Germany was added to the egg suspension to control growth of bacteria and fungi. After 48 hours of incubation, 20 µl of nutritive media (comprising of 1g yeast in 90 ml of normal saline and10 ml earle's balanced salt) was added into each well. Two hundred microliters from microencapsulated and non-microencapsulated *P. juliflora* extracts of concentrations ranging from 0.0625 to 2.0 mg/ml were added to respective plates. Albendazole (Sigma Aldrich, USA) at concentration of 0.0625 to 2.0 mg/ml was utilized as positive control and dissolved just as indicated in section 2.8.1, while untreated larvae in PBS were used as negative control. There were three replicates for each extract concentration and control. The plates were further incubated for 24 hours (total of 3 days). Counting of all larvae in each well was done

under an inverted microscope. The percentage of mortality of the larvae was determined using the following formula:

$$\text{Mortality \%} = \frac{\text{Number of dead larvae}}{\text{Number of larvae in culture}} \times 100$$

2.10 Determination of *in vitro* anthelmintic activity of encapsulated *P. juliflora* extract against adult *H. contortus* parasite

2.10.1 Adult mortality assay

Adult mortality assay was conducted according to the procedure by Eguale, Tilahun, Debella, Feleke, and Makonnen, (2007). Adult *H. contortus* were collected and prepared as described in section 2.8.1**.** Ten actively moving worms were placed in petri dishes with microencapsulated and non-microencapsulated *P. juliflora* extracts and those with PBS alone (to serve as a negative control group). Albendazole (Sigma Aldrich, USA) was dissolved in DMSO in a concentration ranging from 0.0625 to 10.0 mg/ml and used as a positive control. Each concentrations and in different ratios were tested in triplicate. The motility of each worm was observed after 24 hours and the treated worms were kept for 30 minutes in the lukewarm fresh PBS to observe the recovery of motility. Finally, the number of motile (alive) and immotile (dead) worms were counted under dissecting microscope and recorded. Lack of motility was an indicator of parasite mortality. Percentage mortality was calculated as the total number of dead worms divided by the total number of worms per petri dish and multiplied by a hundred.

2.11 Data analysis

Results obtained from the study were analyzed using Microsoft office excel and statistical packages for social science (SPSS) software version 21. Mean percentage egg hatch inhibition rates, larval and adult mortality from encapsulated (leaves and roots) extracts, albendazole (ALB) and ethanolic extracts of leaves (LEE) and roots (REE) at different concentrations and ratios were compared using one-way ANOVA test at $p<0.05$ significant levels. The concentration required to inhibit 50% (IC_{50}) for

ovicidal and (EC_{50}) values for both (larvicidal and adult mortality) were determined using the regression line of probit according to the $\log_{10}$ of the extract concentration.

CHAPTER THREE

3.0 RESULTS AND DISCUSSION

3.1 Ethanol extraction yields

The mean percentage extraction yields of crude ethanolic extracts of *P. juliflora* are recorded in table 3.1.

Table 3.1: Mean percentage (w/w) yields (n=3) of ethanolic extracts of *P. juliflora*

Plant part	Extraction yield in %
Leaves	5.6±0.47
Roots	11.2±0.16

Each of the yields were derived from a mass of 100g of crude plant powder and a sample size, n=3 for each sample. Roots of *P. juliflora* gave the highest crude extract yield (11.2%) while leaves gave the lowest yield (5.24%). The difference in extraction yields could possibly be as a result of roots having more phytochemical principles than leaves. A study done Wamburu *et al.,* (2013) on leaves of *P. juliflora* reported an ethanolic extract yield of 6.94%.

3.2 Phytochemical screening and quantification of saponins, tannins and alkaloids in roots and leaves of *P. juliflora* plant

3.2.1 Phytochemical screening

Results obtained from phytochemicals screening of leaves and roots ethanolic extracts of *P. juliflora* are presented in Table 3.2.

Table 3.2: Phytochemical screening of leaves and roots ethanolic extracts of *P. juliflora*

Secondary metabolite screened		Saponins	Tannins	Flavonoids	Alkaloids	Sterols
Plant part	Leaves	+	+	+	+	+
	Roots	+	+	+	+	+

'+' Indicates the presence of phytochemicals

Phytochemical screening of *P. juliflora* leaf and root ethanolic extract tested positive for saponins, tannins, flavonoids, alkaloids and sterols. Among these compounds alkaloids, flavonoids, saponins and tannins are important secondary metabolites which could be responsible for medicinal value of plants. Plants have an almost limitless ability to synthesize substances most of which are secondary metabolites of which at least 12,000 compounds have been isolated (Ghoshal, Prasad, & Lakshm, 2006). Phytochemical substances play an important role in plant defense against microorganism, stress as well as interspecies protections. These plant components have been used as drugs for many years. Thus, phytochemical screening serves as the initial step in predicting the types of potential active compounds from plants (Chew *et al*., 2011). Saponins and tannins are the phytochemicals responsible for anthelmintic effect as reported by Danquah *et al.* (2012). They uncouple oxidative phosphorylation of the parasite thus hindering energy productions which cause parasite death. Saponins were first reported to kill worms as early as 1962 using an extract from *Albizia anthelmintica.* An *in vitro* anthelmintic activity of *Baliospermum montanum* by Mali and Wadekar (2008) concluded that there was a possibility that tannin was responsible for the anthelmintic activity displayed against *Pheretima posthuma* and *Ascaridia galli.* Tannins also have been reported to have anthelmintic activity against *Ascaris suum* (Brunet, 2008). This is because tannins have the capacity to bind to proteins and inactivate many mechanisms including the

nutrient availability of the larvae (Ghoshal *et al.*, 2006). This may impair vital process like feeding, reproduction of the parasite and disrupt the integrity of the cuticle (Bachaya *et al.*, 2009). Alkaloids on the other hand are a diverse low-molecular weight nitrogen containing compounds and are mostly derived from amino acids and found in about 20% of plant species. As secondary metabolites, alkaloids are thought to play a significant role in protecting the plant against herbivores and pathogens. Owing to their potent biological activity approximately 12000 known alkaloids have been exploited as pharmaceutical stimulants and narcotics (Van Beek, Verpoorte, Svendsen, Leeuwenberg, & Bisset, 1984). *In vivo* anthelmintic activity of *Coriandrum sativum* is considered to be as a result of alkaloids as reported by Eguale *et al.* (2007). Plant sterols which chemically resemble cholesterol have been shown to block the absorption of dietary and endogenously derived cholesterol from the gut (Lagarda *et al.*, 2006). The main function of sterols as reported by Lagarda *et al.*, (2006) is to inhibit the uptake of dietary and endogenously produced cholesterol from the gut.

3.2.2 Quantitative phytochemical analysis of alkaloids, saponins and tannins in roots and leaves of *Prosopis juliflora* plant

The amounts of alkaloids, saponins and tannins isolated from plant were presented in Table 3.3. The results obtained from this study clearly revealed that leaves and roots of *P. juliflora* contained some proportions of alkaloids, saponins and tannins. The percentage mean value of saponins present in roots had the highest value of 5.9% and lowest in leaves with 2.4%. Saponins contents on roots and leaves varied significantly ($t= 9.984$, $P = 0.010$). On the other hand alkaloid contents in roots and leaves varied significantly ($t = 13.116$, $P = 0.006$) with the highest occurrence in leaves (3.7%) as compared with roots (1.7%).

Table 3.3: Percentage composition of phytochemicals in roots and leaves of *P. juliflora* plant

Phytochemicals	Concentration in %		
	Roots	leaves	p –value
Alkaloids	1.7 ± 0.05	3.7 ± 0.31	.006**
Saponins	5.9 ± 0.20	2.4 ± 0.40	.010**
Tannins	1.8 ± 0.25	0.17±0.05	.010**

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

Even though phytochemical screening results (Table 3.2) showed the presence of tannins in both roots and leaves of *P. juliflora* plant their amounts were too little with roots and leaves having 1.8% and 0.17% respectively (Table 3.3). The nature of tannin in form of dried ground powder makes them difficult to extract due to increased fixing of tannin compounds in the vacuoles or surface wax of the plant. On the other hand, extraction using freshly collected plant materials, they are irreversibly bound to other polymers within the cell (Harborne, 1998). The tannin content in roots and leaves also varied significantly (t = 9.714, P = 0.010). These results are in agreement with the study done by Rechab *et al.* (2014) on *P. juliflora* (roots and leaves) ethanolic extracts which showed significant difference (P< 0.05) on tannin content.

3.2.3 FT-IR results for crude extracts, crude saponins and alkaloids of *P. juliflora* roots and leaves

The presence of saponins and tannins from *P. juliflora* (leaves and roots) has been confirmed through phytochemical screening and Fourier Transform Infrared Spectrophotometer (FTIR) analysis as described by Wamburu *et al.* (2013). The results for crude extracts, crude saponins and alkaloids of *P. juliflora* (roots and leaves) were illustrated (Appendix 1) and spectra values summarized in Table 3.4.

Table 3.4: Infrared spectra (cm^{-1}) values from crude extracts, crude saponins and alkaloids of *P. juliflora* (roots and leaves)

Sample type	Peak values		Functional group
	Leaves	Roots	
Crude saponins	3330.8	3423.4	O-H Group
	3382.9		
	2964.0	2931.6	C-H Group stretching
		2860.2	
	1635.5	1649.0	C=C (Triterpenoids saponins)
		1703.0	C=O Group
Crude alkaloids	3413.8		O-H Group
	2923.9	2922.0	C-H Group stretching
	2854.5	2852.5	
	1651.0	1649.0	C=C Group
	1733.9	1733.9	C=O Group
	3413.8	3440.8	N-H (Alkaloid)
Plant ethanolic extract	3334.7		O-H Group
	2962.5		C-H Group stretching
	2922.0		
	1643.2	1629.7	C=C (Triterpenoid saponins)
		1535.2	N-H (Alkaloid)
		3413.8	

The FT-IR gives broad peaks at 3330.8, 3334.7, 3413.8 and 3423.4 cm (Table 3.4) which indicated the presence of OH stretching (Muruganantham, 2009). The C-H stretching was observed at 2964.0 to 2852.5 cm, which showed the presence of saponins. The peaks obtained at 1733.9 to 1703.0 cm indicated the presence of C=O stretching. On the other hand the peaks obtained at 1651.0 to 1629.7 cm indicated the presence of C=C stretching in aromatic system found in the extracts. The OH, C-H,

C=C and C=O infrared absorptions observed in ethanolic extracts and crude saponins are suggestive of oleanane triterpenoid saponins (Kareru, 2008) and are characterized by the C=O infrared absorbance due to oleanolic acid/ester. The presence of OH and N-H functional groups also indicated the presence of alkaloid. The FT-IR analysis results of plant crude extracts, crude saponins and alkaloids of *P. juliflora* roots and leaves validated the presence of saponins and alkaloids from the plant.

3.3 Yield of microencapsulated *P. juliflora* extract

The percentage yields of the six preparations ranged from 79.1% to 90.7%, as shown in Table 3.5. These higher percentage yields show that this method is appropriate for adoption in the formulation of microencapsulated *P. juliflora* extracts.

Table 3.5: Percentage microencapsulation yield (n=3) of *Prosopis juliflora* extracts

Plant part	Ratio (Drug : Polymer)	microencapsulation yield in %
Roots	2:1	90.7 ± 1.7
	1:1	84.2 ± 5.2
	1:2	79.4 ± 1.7
Leaves	2:1	87.9 ± 1.5
	1:1	81.0 ± 3.6
	1:2	79.1 ± 3.2

Higher microencapsulation yields were obtained when the ratio of drug: polymer was 2:1 while the yields were low for drug: polymer ration of 1:2. The microencapsulation technology applied in the current study is a well-known process used to preserve active agents (Nedovic *et al.,* 2011). In addition, the usage of microencapsulation technology on natural compounds has gained great interest in order to maximize its potential therapeutic benefits (Munin & Edwards-Lévy, 2011).

3.4 Determination of *in vitro* ovicidal effects of microencapsulated *P. juliflora* extracts against *H. contortus* eggs

3.4.1 Egg Hatch Inhibition (EHI) Assay

Mean inhibition percentage of egg hatching on microencapsulated extracts was presented in figure 3.1.and Appendix III (Table 1). Microencapsulated leaves and roots ethanolic extracts in different concentrations and ratios were evaluated for their anthelmintic activity *in vitro*. In egg hatch inhibition assay, a concentration dependent response with all the candidate anthelmintics was observed (Figure 3.1). The inhibition concentration (IC_{50}) estimates of different microencapsulated (roots and leaves), ethanolic (root and leaf) extracts and albendazole have been presented in Table 3.6.

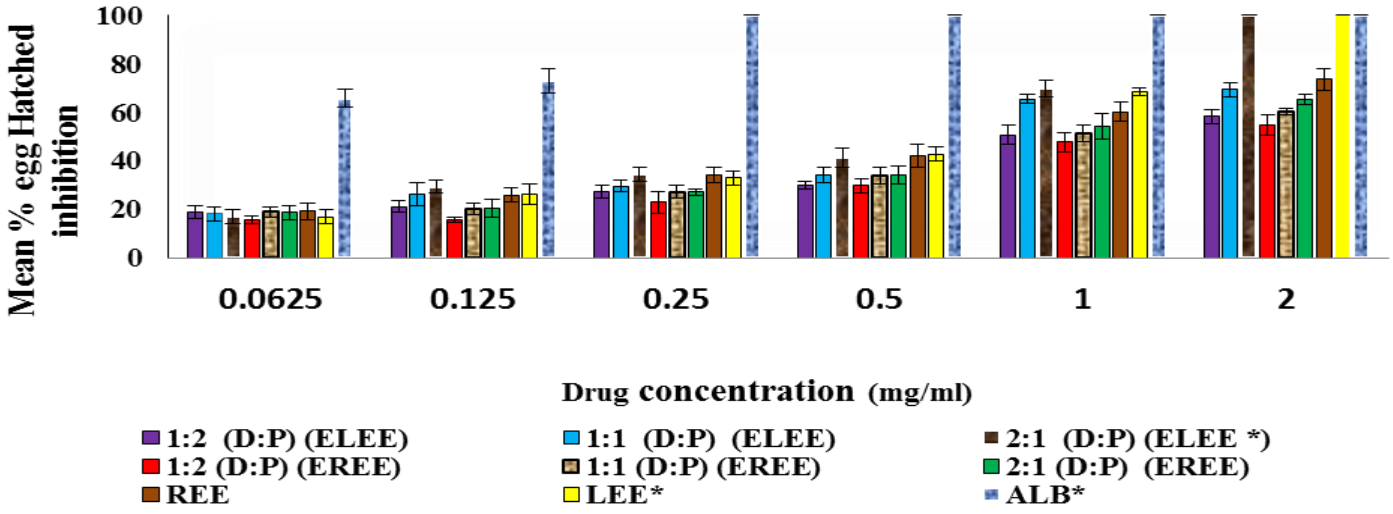

Figure 3.1: Mean % inhibition of egg hatching of microencapsulated *P. juliflora* extracts at different (concentrations and ratios) against *H. contortus* eggs

D: P**: Drug: Polymer,** ELEE**: Encapsulated Leave Ethanolic Extract,** EREE**: Encapsulated Root Ethanolic Extract,** ALB**: Albendazole,** LEE**: Leave Ethanolic Extract;** REE**: Root Ethanolic Extract**

The results obtained from the study indicated that there was a statistically significant difference in egg hatch inhibition at different concentrations (ANOVA, F= 244.938, P= 0.00) and ratios (ANOVA, F= 335.427, P= 0.00) of microencapsulated roots and leaves ethanolic extract of *P. juliflora*. This difference is due to variation in proportion of polymer with respect to amount of drug. Microencapsulated leaf ethanolic extract at (2:1) drug: polymer ratio, leaf ethanolic extract and albendazole exhibited 100% inhibition of egg hatchability at 2.0 mg/ml. The higher drug concentration resulted in higher egg hatch inhibition compared with lower concentration suggesting a concentration dependent response. However, the results showed no statistically significant difference (t= 1.235, P= 0.272) in egg hatch inhibition on both leaf and root ethanolic extracts in comparison with albendazole which showed a significant difference (t=8.359, P= 0.000) in activity. These results are in agreement with previous studies on *in vitro* ovicidal activity of (roots and leaves) ethanolic extract of *P. juliflora* (Rechab *et al.,* 2014) which showed no significance difference (P> 0.05). In this study, egg hatch assay was used to scientifically validate the anthelmintic potential of microencapsulated *P. juliflora* extracts. The egg hatch assay was developed for the diagnosis of benzimidazole resistant helminths. This test has, however, been used for screening of plants compounds for their anthelmintic activity (Min, & Hart, 2003; Iqbal *et al.,* 2005; Hamad, Iqbal, & Muhammad, 2013).

Table 3.6: IC_{50} values for *in vitro* Egg Hatch Inhibition Assay (EHI)

Sample type	95% confidence limits for concentration (mg/ml)		
	IC_{50} values (mg/ml)	Lower Boundary	Upper Boundary
EREE(1:2)D:P	1.206	0.910	1.792
EREE(1:1)D:P	0.877	0.683	1.205
EREE(2:1)D:P	0.741	0.599	0.950
ELEE(1:2)D:P	0.949	0.725	1.358
ELEE(1:1)D:P	0.616	0.302	1.667
ELEE(2:1)D:P	0.355	0.024	1.378
REE	0.646	0.463	1.074
LEE	0.493	0.296	1.310
ALB	0.053	0.000	0.092

KEY:

D: P: **Drug: Polymer,** ELEE**: Encapsulated Leave Ethanolic Extract,** EREE**: Encapsulated Root Ethanolic Extract,** ALB**: Albendazole,** LEE**: Leave Ethanolic Extract;** REE**: Root Ethanolic Extract**

Microencapsulated leave extracts in different ratios showed a higher activity with IC_{50} values ranging from 0.355 to 0.949 mg/ml as compared with microencapsulated root extract with IC_{50} values from 0.741 to 1.206 mg/ml. This is attributed to the difference in proportions of the active components that were responsible for antihelmintic activity. A study done by Eguale *et al.* (2007) on water extracts of *Hedera helix* showed IC_{50} value of 0.12 mg/ml respectively when tested against *H. contortus* eggs. Methanolic extract of *Ziziphus mucronata bark* showed an activity with IC_{50} value of 3.9 mg/ml while aqueous extract had an IC_{50} value of 14.7 mg/ml on egg hatch inhibition test (Olivier, John, Simon, Kipyegon, & Naomi, 2017).Thus, extracts from these plant had a relatively lower activity compared with *P. juliflora ethanolic* extracts in the current study. Albendazole showed comparable results with

that of *in vitro* anthelmintic activities of four Ethiopian medicinal plants against *H. contortus* which had IC_{50} value of 0.04 mg/ml (Eguale *et al.*, 2006). A previous study by Olivier *et al.*, (2017) on aqueous and methanolic extracts of *Z. mucronata* Barks against *H. Contortus* showed IC_{50} value of 0.045 mg/ml. This value is close to that reported in the current study which showed IC_{50} value of 0.053 (Table 3.6).

3.5 Determination of *in vitro* larvicidal activity of microencapsulated *P. juliflora* extracts

3.5.1 Larval Mortality Assay (LMA)

The mean percentage larval mortality of *H. contortus* at different concentrations and ratios of plant extracts (mg/ml) and albendazole (mg/ml) are presented in Figure 3.2 and Appendix III (Table 2).

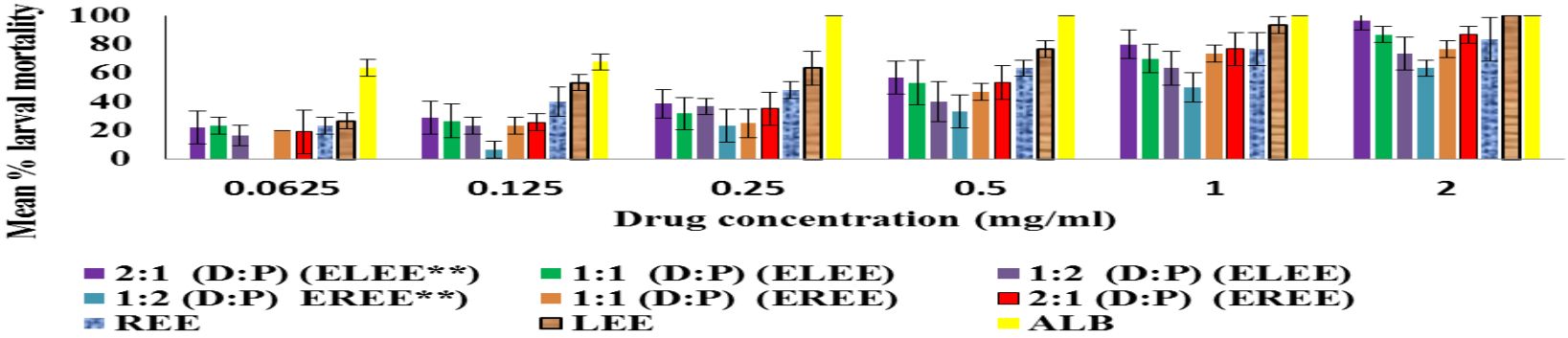

Figure 3.2: *In vitro H. contortus* larvicidal activity in various ratios of encapsulated (leaves and root) extracts, Albendazole (ALB) and ethanolic extracts of leaves (LEE) and roots (REE)

D: P: **Drug: Polymer,** ELEE**: Encapsulated Leave Ethanolic Extract,** EREE**: Encapsulated Root Ethanolic Extract,** ALB**: Albendazole,** LEE**: Leave Ethanolic Extract;** REE**: Root Ethanolic Extract**

In the present study, microencapsulated and non-microencapsulated extracts of *P. juliflora* (roots and leaves) in different concentration and ratios showed variable effect on mortality of larvae of *H. contortus*. The highest larval mortality observed on microencapsulated extracts was from microencapsulated leaves ethanolic extracts in drug to polymer ratio of 2:1 (96.7%) (Figure 3.2), while the least effective encapsulated extract was from encapsulated root ethanolic extracts in drug to polymer ratio of 1:2 (63.3%) at the maximum tested concentration of 2mg/ml. In general, all the microencapsulated and ethanolic extracts of *P. juliflora* (leaves and roots) used in the current study induced over 50% mortality of larval at the highest concentration used (2mg/ml). Albendazole required a maximum concentration of 0.25 mg/ml to induce 100% larval mortality (Figure 3.2). There was a significant difference ($p< 0.05$) in activity among different drug concentration. This is indicated by the fact that increasing the concentration of the plant extracts increases the proportion of the chemical ingredient with anthelmintic potential in the plant extract thus activity increased (Debella, 2002). Similar observation have also been made by Lullman, Morh, & Bieger (1993) who demonstrated that the receptors get saturated with increasing concentration of active ingredient. It is likely that at higher concentration all binding receptors on the larvae were occupied thus leading to hyper-polarisation of membranes limiting excitation and impulse transmission causing flaccid paralysis of larvae muscles (Wasswa & Olila, 2006).

The main advantages of using *in vitro* assays to screen the anti-parasitic properties of plant extracts include low costs and rapid turnover that allows the screening of plants at large scale. Also, these tests measure the effect of anthelmintic activity directly on the processes of egg hatching and larval development without interfering with the internal physiological functions of the host (Githiori *et al*., 2004). Several studies have been carried out in different parts of the world to evaluate the anthelmintic activity of medicinal plants against different nematode species of farm animal *in vitro* (Bizimenyera, Githiori, Eloff, & Swan, 2006). The effective concentration required to induce 50% (EC) larval mortality as calculated by probit analysis and presented in Table 3.7.

Table 3.7: EC_{50} values for *in vitro* larvicidal activity of microencapsulated *P. juliflora* extracts

Sample type	95% confidence limits for concentration (mg/ml)		
	EC_{50} values (mg/ml)	Lower Boundary	Upper Boundary
EREE(1:2)D:P	1.023	0.727	1.664
EREE(1:1)D:P	0.490	0.290	0.948
EREE(2:1)D:P	0.361	0.300	0.434
ELEE(1:2)D:P	0.573	0.455	0.746
ELEE(1:1)D:P	0.378	0.241	0.607
ELEE(2:1)D:P	0.287	0.177	0.450
REE	0.250	0.196	0.311
LEE	0.140	0.092	0.192
ALB	0.056	0.001	0.094

KEY:

D: P: **Drug: Polymer,** ELEE**: Encapsulated Leave Ethanolic Extract,** EREE**: Encapsulated Root Ethanolic Extract,** ALB**: Albendazole,** LEE**: Leave Ethanolic Extract;** REE**: Root Ethanolic Extract**

The highest EC_{50} values (0.287 mg/ml) for the microencapsulated *P. juliflora* (leaves and roots) extracts against larval development was revealed by microencapsulated leaves ethanolic extract in drug to polymer ratio of 2:1. The lowest EC_{50} values (1.023 mg/ml) were recorded on microencapsulated root ethanolic extract in drug to polymer ratio of 1:2. There was a significant difference ($t = 4.991$, $P = 0.000$) between egg hatch inhibition and larval development inhibition. Larval development inhibition of microencapsulated (roots and leaves) extracts showed a better activity with EC_{50} values (Table 3.7) of 1.023, 0.49, 0.361 and 0.573, 0.378, 0.287 mg/ml respectively. The possible explanation of higher activity on eggs against larva could be due to the difference in structure of the egg shell and cuticle of larvae of *H. contortus* through which absorption of chemicals take place (Eguale *et al.,* 2006)

3.6 Determination of *in vitro* anthelmintic activity of microencapsulated *P. juliflora* extract against adult *H. contortus* parasite

3.6.1 Adult Mortality Assay (AMA)

The *in vitro* adult mortality assay tests results for microencapsulated *P. juliflora* in different concentration and ratios are presented in Figure 3.3 and Appendix III (Table 3).

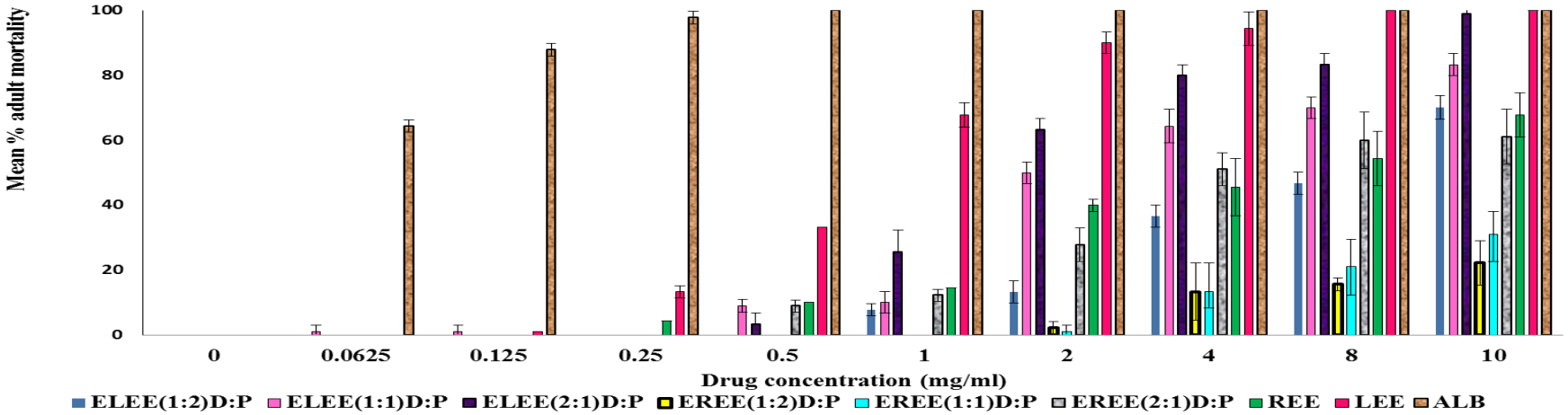

Figure 3.3: Adult mortality assay (AMA) in various ratios and concentrations of microencapsulated (leaves and root) extracts, Albendazole (ALB) and ethanolic extracts of leaves (LEE) and roots (REE)

D: P: **Drug: Polymer,** ELEE**: Encapsulated Leave Ethanolic Extract,** EREE**: Encapsulated Root Ethanolic Extract,** ALB**: Albendazole,** LEE**: Leave Ethanolic Extract;** REE**: Root Ethanolic Extract**

Microencapsulated and non-microencapsulated extracts of *P. juliflora* in different concentration and ratios showed variable effect on mortality of *H. contortus* adults. At the maximum tested concentration of 10 mg/ml (Figure 3.3), the highest (99.0 %) adult mortality was from microencapsulated leave ethanolic extracts in drug to polymer ratio of (2:1) while the least (22.2 %) was from microencapsulated root ethanolic extracts in drug to polymer ratio of (1:2). Albendazole required a maximum concentration of 0.5 mg/ml (Figure 3.3) to induce 100% adult mortality.

The present study on adult mortality assay indicate that there was a significant difference in mean percentage adult mortality of *H. contortus* at different concentrations ($F=60.185$, $P< 0.05$). These results are in agreement with a study by Suteky *et al.*, (2011) on *in vitro* anthelmintic activity of *Melastoma malabatricum* extract on *H. contortus in vitro* which showed a significant difference ($P< 0.05$). The results also indicate that there were significant differences ($F=225.781$, $P< 0.05$) in mean percentage adult mortality at different ratios of microencapsulated (roots and leaves) of *P. juliflora* extracts. The EC_{50} values for adult mortality assay are shown in Table 3.8.

Table 3.8: EC_{50} values for *H. contortus* Adult Mortality

Sample type	95% confidence limits for concentration (mg/ml)		
	EC_{50} values (mg/ml)	Lower Boundary	Upper Boundary
EREE(1:2)D:P	26.874	17.811	56.785
EREE(1:1)D:P	16.758	12.944	25.732
EREE(2:1)D:P	5.179	4.361	6.295
ELEE(1:2)D:P	6.625	5.717	7.858
ELEE(1:1)D:P	3.007	2.147	4.360
ELEE(2:1)D:P	1.950	1.434	2.614
REE	4.847	4.017	6.005.
LEE	0.713	0.636	0.799
SALB	0.047	0.034	0.058

KEY:

D: P: **Drug: Polymer,** ELEE**: Encapsulated Leave Ethanolic Extract,** EREE**: Encapsulated Root Ethanolic Extract,** ALB**: Albendazole,** LEE**: Leave Ethanolic Extract;** REE**: Root Ethanolic Extract**

The EC_{50} values from microencapsulated leaf and root extract ranged from 1.950 to a maximum of 26.874 mg/ml. These values are relatively high as compared with albendazole and leaf ethanolic extracts (0.047 and 0.713 mg/ml) respectively. This implies that the microencapsulated drug has an extended release time of over 24 hours thus lowering the activity on adults. The adult mortality observed from the present study can be attributed to combination of active compounds resulting in a

synergistic effect on the parasite (Rates, 2001). As described by Brunet, Fourquaux, & Hoste (2011), tannins have the affinity to bind to proteins in the parasite causing changes in its cuticle architecture as well as degeneration of the musculature and intestinal cells. Adult mortality assay is the most convenient test developed to detect anthelmintic efficacy in nematodes (Craven, Bjorn, Barnes, Henriksen, & Nansen, 1999). In this assay, worms are exposed to varying concentrations of drugs and observed for their inhibited mortality at different concentration. *Haemonchus contortus, Ostertagia circumcincta* and *Trichostrongylus colubriformis* have been used for the *in vitro* anthelmintic activity of plant extracts (Akhtar *et al.,* 2000). Although the adult nematodes are the major target for chemotherapy, the main challenge with dealing with gastrointestinal nematode adults is raising them through continuous culture to adult stage (Geary, Sangster, & Thompson, 1999).

The present study showed that *P. juliflora* extracts were more effective against eggs and larvae as compared to adults of *H. contortus*. The possible explanation could be due to the difference in structure of the egg shell and cuticle of larvae of *H. contortus* through which absorption of chemicals take place (Eguale *et al.,* 2006). From these findings, it is evident that, activity of the plant extract against one developmental stage of the parasite may not be effective against other developmental stage.

CHAPTER FOUR

4.0 CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

All the study objectives were met and from the results it can be concluded that:

1. Phytochemical screening of *P. juliflora* (roots and leaves) extracts revealed the presence of alkaloids, saponins and tannins and these could be responsible for the observed anthelmintic activity.
2. The present study showed a significant activity in larval development inhibition in comparison with egg hatch inhibition. This is attributed to the difference in structure of the egg shell and cuticle of larvae of *H. contortus* through which absorption of chemicals take place.
3. The EC_{50} values for microencapsulated leaf and root extracts were relatively high as compared with albendazole and leaf ethanolic extracts. The results suggest that, the active ingredients responsible for adult mortality were slowly released from microcapsules.
4. The results of this study suggested that microencapsulated *P. juliflora* extracts had a significant activity on eggs, larvae and adults of *H. contortus* parasite, with highest activity being on eggs and larvae. Therefore microencapsulated *P. juliflora* roots and leaves ethanolic extracts have anthelminthic activity against *H. contortus* of sheep.

4.2 Recommendations

From the results and conclusions of the current study the following recommendations can be made:

1. Microencapsulation technology is a potential novel approach for enhancing extract stability and can therefore be applied in development of microencapsulated herbal extract for use in *in vivo* studies for control of *H. contortus* in sheep.

2. Studies should be carried out in order to isolate, identify, characterize, purify and microencapsulate the bioactive compounds from *P. juliflora* with an intention of increasing the anthelmintic activity.

3. Further characterization of the microencapsulated extract should also be done to ascertain its long term stability and kinetics of release in ruminant gastrointestinal tract.

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APPENDICES

Appendix I: FT-IR results of plant crude extracts, crude saponins and alkaloids of *P. juliflora* roots and leaves

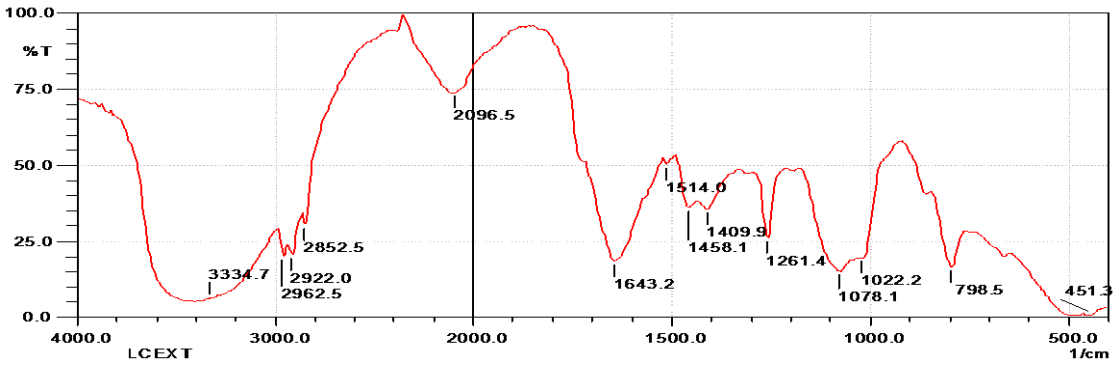

Figure 1: FT-IR Spectra for leaves ethanolic extract

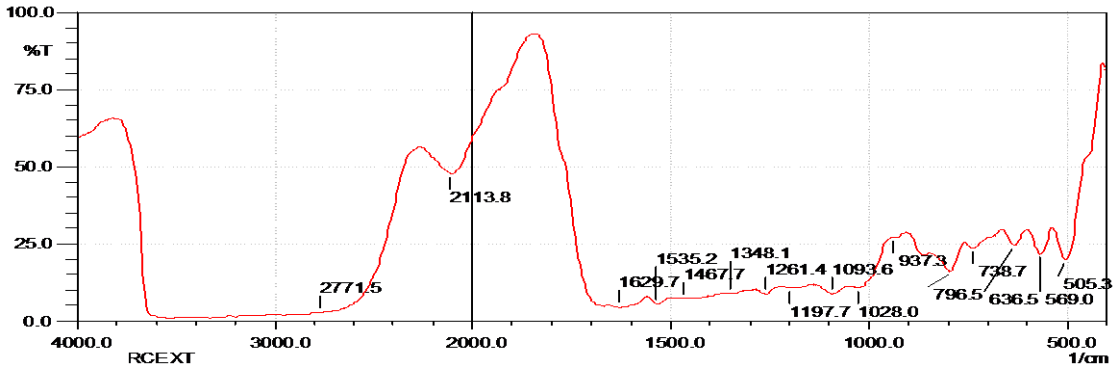

Figure 2: FT-IR Spectra for root ethanolic extract

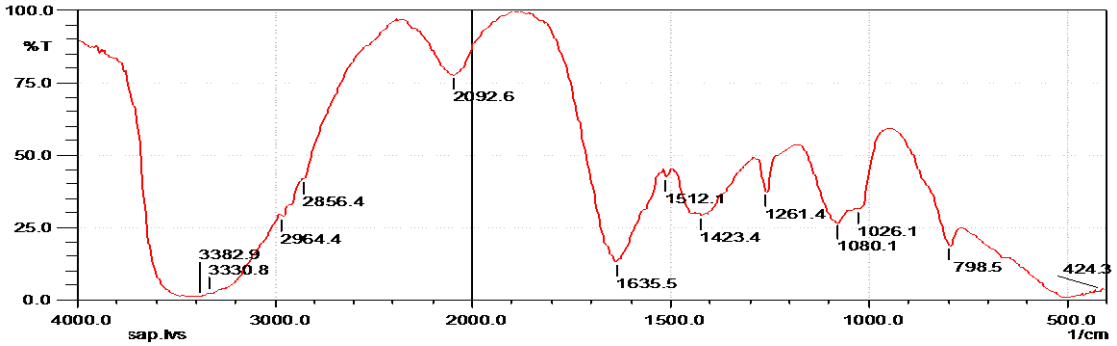

Figure 3: FT-IR Spectra for crude saponins leaves extract

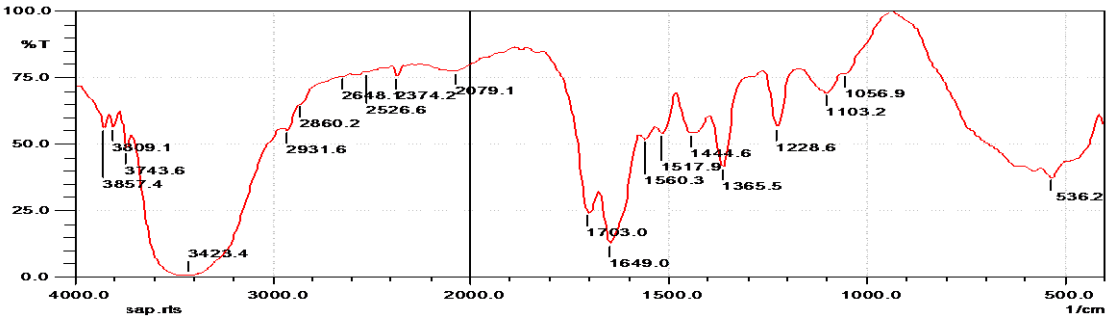

Figure 4: FT-IR Spectra for crude saponins roots extract

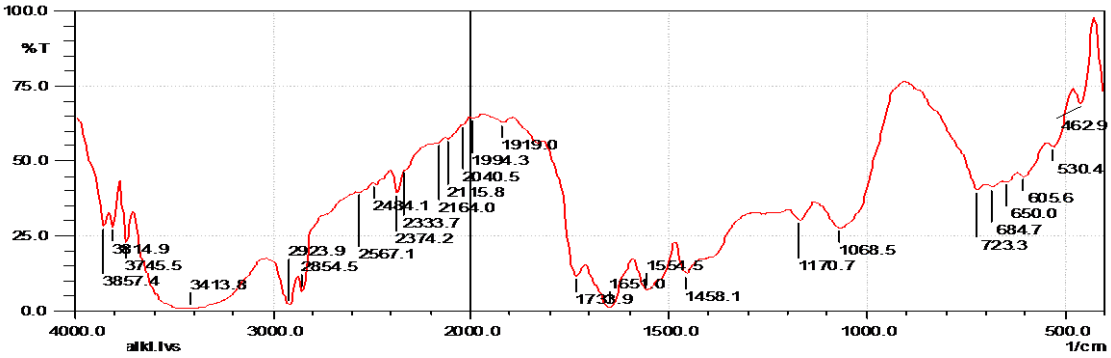

Figure 5: FT-IR Spectra for crude alkaloid leaves extract

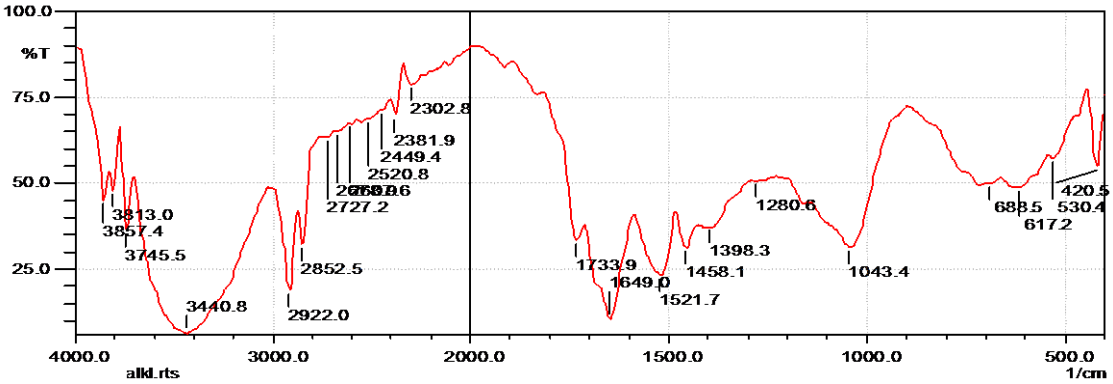

Figure 6: FT-IR Spectra for crude alkaloid roots extract

Appendix II: Standard tannic acid curve for the determination of total tannins

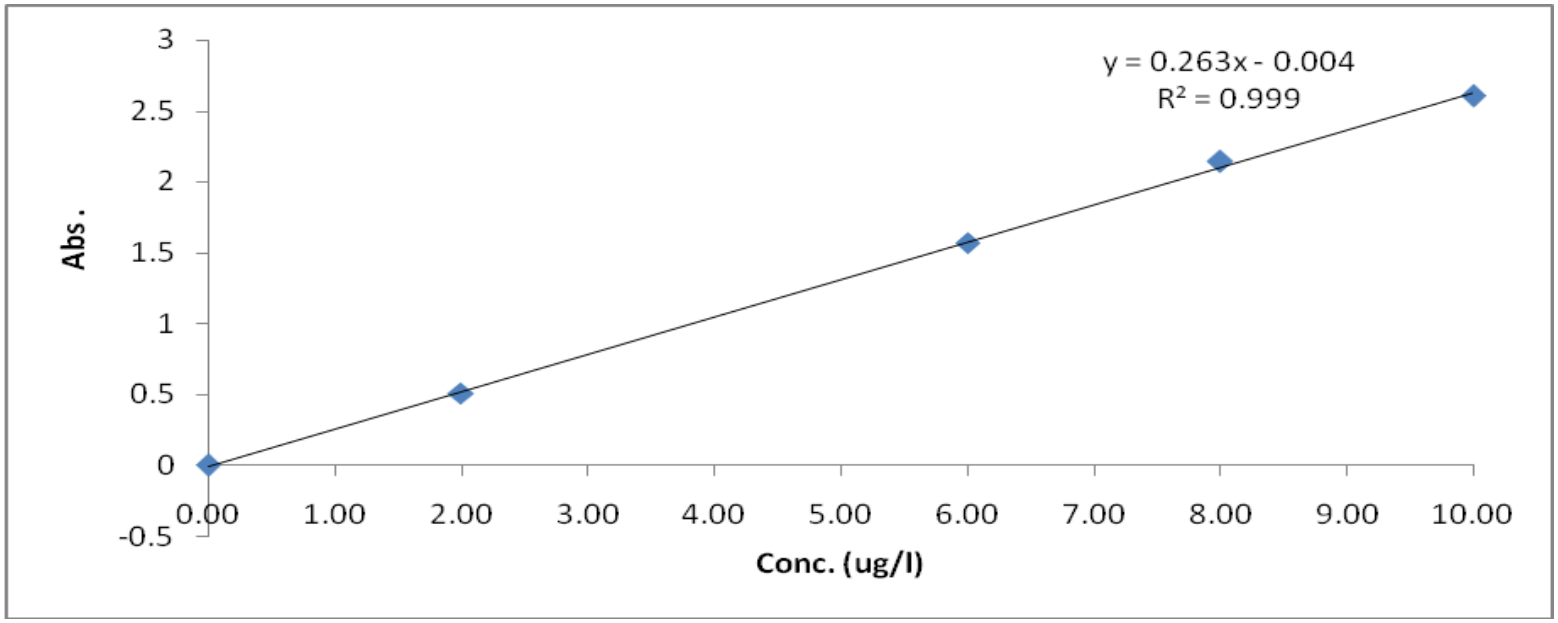

Figure 7: Standard curve

Appendix III: Anthelmintic activity results in (% mean ±S.D.)

Table 1: *In vitro* egg-hatch inhibition assay (EHA) in various ratios of microencapsulated (leaves and root) extracts, Albendazole (ALB) and ethanolic extracts of leaves (LEE) and roots (REE)

	SAMPLE TYPE								
Drug concentration in (mg/ml)	**1:2 (D:P) (ELEE)**	**1:1 (D:P) (ELEE)**	**2:1 (D:P) (ELEE)**	**1:2 (D:P) (EREE)**	**1:1 (D:P) (EREE)**	**2:1 (D:P) (EREE)**	**REE**	**LEE**	**ALB**
0.0625	18.8±2.8	18.12±2.9	17.06±2.8	15.62±1.5	19±2.1	18.72±3.0	19.28±3.5	16.94±3.0	65.7±3.7
0.125	21.18±2.5	26.4±4.8	29.26±2.7	15.8±1.1	20.38±2.4	20.38±3.9	25.96±3.0	26.1±4.2	73.12±5
0.25	27.3±2.4	29.5±2.4	34.48±2.8	22.84±4.5	27.12±2.6	27.12±1.4	34.14±3.4	32.88±2.8	100.0±0.0
0.5	30.06±1.5	34.36±3.1	41.42±3.9	29.7±2.7	34.02±3.3	34.02±3.8	42.28±4.7	42.68±2.9	100.0±0.0
1	50.7±4.1	65.6±2	69.7±3.5	47.84±3.9	51.32±3.2	54.22±5.5	60.24±4.1	68.3±1.6	100.0±0.0
2	58.34±3.0	69.4±2.8	100±0.0	54.6±4.2	60.3±1.1	65.3±2.0	73.68±4.6	100.0±0.0	100.0±0.0

Table 2: *In vitro* larvicidal activity in various ratios of microencapsulated (leaves and root) extracts, Albendazole (ALB) and ethanolic extracts of leaves (LEE) and roots (REE)

	SAMPLE TYPE								
Drug concentration in(mg/ml)	**2:1 (D:P) (ELEE)**	**1:1 (D:P) (ELEE)**	**1:2 (D:P) (ELEE)**	**1:2 (D:P) EREE)**	**1:1 (D:P) (EREE)**	**2:1 (D:P) (EREE)**	**REE**	**LEE**	**ALB**
0.0625	22.0±11.6	23.3±5.8	16.7±7.1	0.0±0.0	20.0±0.0	19.0±15.3	23.3±5.7	26.7±5.7	63.7±5.7
0.125	29.0±11.5	26.7±11.5	23.3±5.8	6.7±5.7	23.3±5.8	25.7±5.8	40.0±10	53.3±5.8	67.7±5.8
0.25	38.7±10.0	31.7±11.3	36.7±5.8	23.3±11.5	25.0±10.0	35.3±11.4	48.0±5.7	63.3±11.5	100.0±0.0
0.5	56.7±11.5	53.3±15.3	40.0±14.1	33.3±11.5	46.7±5.8	53.3±11.8	63.3±5.7	76.7±5.7	100.0±0.0
1.0	80.0±10.0	70.0±10.0	63.3±11.5	50.0±10.0	73.3±5.8	76.7±11.5	76.7±11.5	93.3±5.7	100.0±0.0
2.0	96.7±5.8	86.7±5.7	73.3±11.5	63.3±5.8	76.7±5.7	86.7±5.8	83.3±15.3	100.0±0.0	100.0±0.0

Table 3: *In vitro* Adult Mortality Assay (AMA)

Drug concentration In (mg/ml)	1:2 (D:P) (ELEE)	1:1 (D:P) (ELEE)	2:1 (D:P) (ELEE)	1:2 (D:P) (EREE)	1:1 (D:P) (EREE)	2:1 (D:P) (EREE)	REE	LEE	ALB
0.0625	0±0.0	1.1±1.9	0±0.0	0±0.0	0±0.0	0±0.0	0± 0.0	0±0.0	64.4±1.9
0.125	0±0.0	1.1±1.9	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	1.1±1.9	87.8±2.0
0.25	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	4.4±1.9	13.3±3.3	97.8±1.9
0.5	0±0.0	8.9±2	3.3±3.5	0±0.0	0±0.0	8.9±1.9	10±0.0	33.3±6.6	100±0.0
1.0	7.8±1.9	10±3.3	25.5±6.9	0±0.0	0±0.0	12.2±1.9	14.4±3.8	67.8±5.1	100±0.0
2.0	13.3±3.4	50±3.3	63.3±3.4	2.2±1.9	1.1±1.9	27.8±5.1	40±3.3	90±6.7	100±0.0
4.0	36.7±3.4	64.4±5.1	80±3.3	13.3±8.8	13.3±8.8	51.1±5.1	45.6±5.1	94.4±5.1	100±0.0
8.0	46.7±3.4	70±3.3	83.3±3.4	15.6±2.0	21.1±8.4	60±8.8	54.4±5.1	100±0.0	100±0.0
10.0	70.2±3.6	83.3±3.4	99±1.7	22.2±6.9	31.1±6.9	61.1±8.4	67.8±5.0	100±0.0	100±0.0