

PHYTOCHEMICAL AND *IN VITRO* ANTHELMINTIC STUDIES OF *PROSOPIS JULIFLORA* (SW.) DC (FABACEAE)  
EXTRACTS AGAINST *HAEMONCHUS CONTORTUS*, AN OVINE NEMATODE

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**Abstract**

Gastrointestinal nematode infections in ruminants have direct effects such as mortality, weaknesses, loss of appetite, feeding efficiency and hence decreased productivity. Animal deaths due to nematode infections are common in tropical and subtropical regions where control programs based solely on the use of synthetic anthelmintics are no longer sustainable because of an increased prevalence of gastrointestinal nematode resistance, the slow development of new anthelmintics, high costs to poor farmers and concerns regarding residue in food and the environment. Alternative methods of control are thus required. *Prosopis juliflora* is a fast-growing, drought-resistant tree adapted to poor and saline soils in arid and semi-arid lands of Kenya, inhabited by nomadic pastoralists. Ethanolic extracts of root (REE) and leaf (LEE) of *P. juliflora* were found to exhibit *in vitro* activity against adult *Haemonchus contortus*, with activity comparable to Albendazole, a synthetic anthelmintic drug. The anthelmintic activity of LEE was significantly higher than REE ( $P < 0.05$ ), but lower than ALB. The observed *in vitro* anthelmintic activity was attributed to saponins and condensed tannins and perhaps to alkaloids present in the plant. Phytochemical analysis confirmed presence of tannins, saponins and alkaloids, among other phytoconstituents. These phytochemicals are known to exhibit anthelmintic activity, therefore making the plant a potential candidate for drug development against gastrointestinal nematodes in ruminants.

**Key words:** *Haemonchus contortus*, tannins, saponins, anthelmintic activity, ruminants

## 1.0 Introduction

The economic impact of parasitic gastroenteritis caused by mixed infection with gastrointestinal nematodes (GIN), as a production disease in ruminants lies in direct losses involving mortality due to the clinical form of the disease and also indirect losses due to weaknesses, loss of appetite, decreased feed efficiency, reduced weight gain and decreased productivity. In Kenya, economic loss to the agricultural sector due to *Haemonchus contortus* parasite of small ruminants is estimated at over US\$ 26 million per year (Githiori, 2004). Control programs based on the use of synthetic anthelmintics are no longer sustainable due to high prevalence of gastrointestinal nematode resistance, slow development of new anthelmintics, high costs to poor farmers and concerns regarding residue in food and the environment (Singh *et al.*, 2002). Alternative methods of control such as use of tanniferous plants are thus required for introduction into farm production systems (Niezen *et al.*, 2002). *Prosopis juliflora* (Sw.) DC (Fabaceae) is an evergreen tree native to South America, Central America and the Caribbean. *Prosopis* species are generally fast-growing, drought-resistant, nitrogen-fixing trees or shrubs adapted to poor and saline soils in arid and semi-arid zones. (Pasiecznik *et al.*, 2001).

## 2.0 Materials and Methods

### 2.1 Sample Collection, Preparation and Extraction

Leaves and root bark samples of *P. juliflora*, obtained from Endao, Marigat district, in Baringo county of Kenya were botanically identified and authenticated by a field officer from Kenya Forestry Research Institute, Marigat station and a taxonomist from Botany Department of Jomo Kenyatta University of Agriculture and Technology, where voucher specimens were also deposited. The collected materials were washed thoroughly in water, chopped; air dried for two weeks, pulverized in electric grinder and exhaustively extracted using 80% ethanol. The extracts were concentrated *in vacuo*, dried and stored at 4°C until required for bioassay.

### 2.2 Phytochemical Screening

Phytochemical screening was performed using standard phytochemical procedures (Harborne, 1998) and the extracts were tested for saponins, tannins, flavonoids, alkaloids, triterpenes and sterols.

### 2.3 Determination of *In Vitro* Anthelmintic Activity

#### 2.3.1 Egg Hatch Assay (EHA)

##### 2.3.1.1 *Haemonchus Contortus* Egg Recovery

Mixed GIN eggs were recovered from faeces according to Hubert and Kerboeuf (1992). Sample of faeces (10–15 g) were collected from sheep infected with mixed GIN. The faecal samples were suspended in water and cleared of organic debris by filtration through 1 mm and 150 µm sieves. Eggs were collected on a 25 µm sieve and further cleared of organic debris by centrifugation in magnesium sulphate (density 1.10) for five minutes at 1000 × *g*. The supernatant was filtered through 100 µm and 63 µm sieves and the eggs were washed in water and collected on a 25 µm sieve. The concentration of eggs was estimated in 200 µL samples and adjusted to 500 eggs/mL. 5 µg/mL amphotericin B solution (Sigma, Germany) was added to the egg suspension to avoid fungal development. Identification of the mixed GIN using morphological characteristics (Hansen and Perry 1994; Van Wyk *et al.*, 2004) showed that the main species were *Haemonchus contortus*, *Trichostrongylus spp.* and *Oesophagostomum spp.* with an estimated prevalence rate of 60%, 25% and 15% respectively.

##### 2.3.1.2 *In vitro* Ovicidal Activity

The egg hatch assay (EHA) was carried out using the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for determination of anthelmintic resistance (Coles *et al.*, 1992) with modifications that allowed the testing of the natural compounds (Alawa *et al.*, 2003). Egg suspension of 100 µL containing approximately 50 fresh eggs was distributed in a 48-well flat-bottomed microtitre plate and mixed with the same volume of plant extracts dissolved in PBS having different concentrations ranging from 0.03125 to 2 mgmL<sup>-1</sup>. Albendazole dissolved in 0.3% dimethyl sulfoxide (DMSO) and diluted at concentrations between 0.03125 and 2 mgmL<sup>-1</sup> was used as a positive control while negative control plates contained the diluent (PBS or 0.3% DMSO) and the egg solution. The eggs were incubated in this mixture for 48 h at 27°C and 70% relative humidity. After this time a drop of Lugol's iodine solution was added to stop the eggs from hatching. The number of eggs which had not hatched and number of hatched larvae were counted and percentage hatching calculated. There were three replicates for each concentration and control.

### 2.3.2 *In vitro* Adult Mortality Assay (AMA)

Mature *H. contortus* worms were collected from the abomasa of freshly slaughtered sheep at local abattoirs. Immediately after slaughter, the abomasa were slit open, the parasites washed off, kept in phosphate buffered saline (PBS, pH: 7.2, 4°C) and transported to the laboratory. Ten actively moving worms were placed in Petri dishes containing 10.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mgmL<sup>-1</sup> of the root and leaves ethanolic extracts of *P. juliflora* in PBS and PBS alone for the control group in a total volume of 4 ml. Albendazole dissolved in 1% DMSO and diluted in PBS at the concentrations of 10.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mgmL<sup>-1</sup> was used as positive control. The temperature was controlled at 37±1°C) and three replications per each treatment concentration were employed. After 24 hrs, the plant extracts and albendazole was washed away and the parasites suspended in PBS for 30 minutes for possible recovery of the parasite motility. The number of motile (alive) and immotile (dead) worms were counted under dissecting microscope, and recorded for each concentration. Death of worms was ascertained by absence of motility for an observation period of 5-10 seconds.

## 2.4 Data Analysis

Data from EHA and adult mortality assay (AMA) was transformed by probit transformation against the logarithm of extract concentration. The extract concentration required to inhibit 50% (ED<sub>50</sub>) egg hatching or cause 50% mortality was calculated using probit analysis.

## 3.0 Results and Discussion

### 3.1 Extraction Yield

Ethanolic extraction of the roots gave a higher yield of 16.78% as compared to that of the leaves which was 6.94%, an indication that there were more polar compounds in roots as compared to the leaves.

### 3.2 Phytochemical Profile of Ethanolic Extracts

Results for Phytochemical analysis as shown in table 1 revealed that leaf ethanolic extract (LEE) and root ethanolic extract (REE) possess alkaloids, tannins, saponins, flavonoids, sterols and triterpenes. These phytochemical principles could be responsible for its observed pharmacological activity.

Table 1: Phytochemical profile of LEE and REE of *P. juliflora*

Secondary metabolite	LEE	REE
Alkaloids	+	+
Tannins	+	+
Saponins	+	++
Flavonoids	+	+
Sterols/ Triterpenes	+	+

'+' Present, '++' Present in high concentration, **LEE**: Leaf Ethanolic extract; **REE**: Root Ethanolic Extract

### 3.3 Results for *in vitro* Anthelmintic Activity

In the search for natural anthelmintics, *in vitro* tests are used as preliminary studies of plants. In these tests, the plant extracts are directly placed in contact with the eggs, larvae or adult parasites to evaluate the effect on egg hatching, larval development or motility and mortality of adult worms (Hammond *et al.*, 1997).

#### 3.3.1 Egg Hatch Assay (EHA)

The EHA revealed that there was no statistically significant difference in the activities of both LEE and REE (p>0.05). However, in comparison to ALB, the difference in activity was significantly different (p<0.05). ALB concentrations of 0.25 to 2 mgmL<sup>-1</sup> exhibited complete inhibition on egg hatching as shown in figure 1. Complete inhibition on egg

hatching was also exhibited by LEE at the highest concentration of 2 mgmL<sup>-1</sup>. The ED<sub>50</sub> values for the extracts and albendazole are as shown in table 2.

Table 2: ED<sub>50</sub> Values for EHA

Extract	95% Confidence Limits for Concentration			95% Confidence Limits for log <sub>10</sub> (concentration)			R <sup>2</sup>
	ED <sub>50</sub> value (mgmL <sup>-1</sup> )	Lower Bound	Upper Bound	ED <sub>50</sub> value (mgmL <sup>-1</sup> )	Lower Bound	Upper Bound	
ALB	0.023	0.013	0.038	-1.631	-1.88	-1.423	0.71
LEE	0.307	0.216	0.443	-0.513	-0.666	-0.353	0.641
REE	0.364	0.256	0.525	-0.439	-0.592	-0.28	0.906

The observed inhibitory effect on helminthes egg hatching was due to various principles present in *P. juliflora* extracts and this observation is consistent with other research findings that plant phytochemicals such as resins, bitter principles, tannins, flavonoids and indolquinolizidine alkaloids exhibit high anthelmintic activity against *strongyle* nematodes of small ruminant animals by preventing parasite eggs from hatching (Onyeyili *et al.*, 2001).

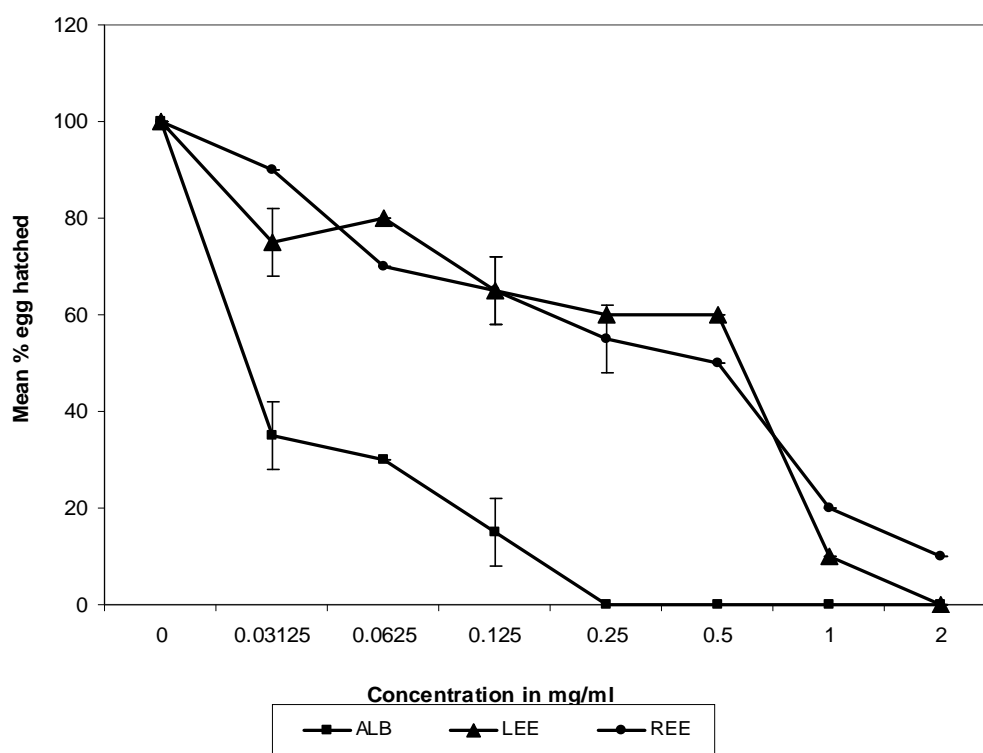


Figure 1: Graph showing mean percentage egg hatching of various concentrations of LEE, REE and ALB after 48 hours

### 3.3.2 Adult Mortality Assay (AMA)

Adult mortality assay revealed that both LEE and REE exhibited anthelmintic activity in a concentration dependent manner as shown in figure 2. However, LEE had a significantly higher activity as compared to REE (Table 3). Albendazole (ALB) had significantly higher activity as compared to the ethanolic extracts (LD<sub>50</sub>=0.046 mgmL<sup>-1</sup>). Leaf ethanolic extract exhibited complete mortality of *H. contortus* at concentrations of 8 and 10 mgmL<sup>-1</sup> while ALB showed total mortality even at a lower concentration of 0.25 mgmL<sup>-1</sup>. Both LEE and REE did not exhibit mortality at concentrations between 0.03125 and 0.125 mgmL<sup>-1</sup>.

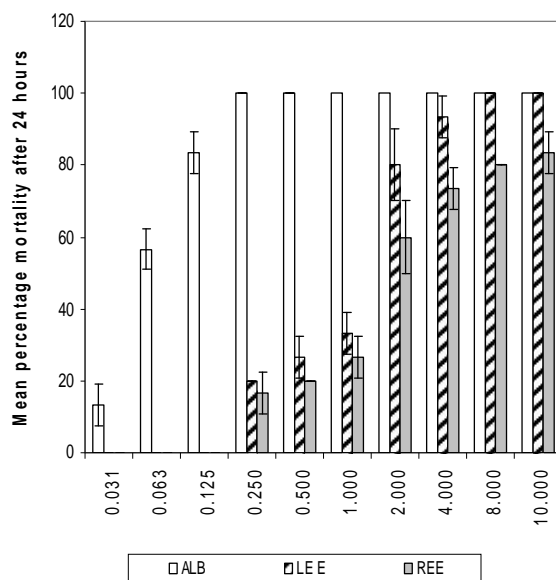


Figure 2: Graph showing mean percentage mortalities of *H. contortus* subjected to various concentrations of Albendazole (ALB) and ethanolic extracts of leaves (LEE) and roots (REE)

Table 3: ED<sub>50</sub> values for AMA

Extract	95% Confidence Limits for Concentration			95% Confidence Limits for log <sub>10</sub> (concentration)			R <sup>2</sup>
	ED <sub>50</sub> value (mg/ml)	Lower Bound	Upper Bound	ED <sub>50</sub> value (mg/ml)	Lower Bound	Upper Bound	
ALB	0.046	0.033	0.065	-1.333	-1.487	-1.186	0.942
LEE	0.857	0.635	1.144	-0.067	-0.197	0.058	0.843
REE	1.782	1.357	2.336	0.251	0.133	0.368	0.918

The anthelmintic activity of LEE and REE may be attributed to presence of phytochemicals such as saponins, tannins and alkaloids. Min *et al.* (2003) reported that Condensed tannins might diffuse through the external surfaces such as eggshells and bind to egg proteins thus inhibiting egg hatching and larval development. Saponins destabilize membranes and increase cell permeability by combining with membrane-associated sterols (Gee and Johnson, 1988) while alkaloids may improve tonicity of the gastrointestinal tract and thus expel the worms or may have a direct effect on the nervous system of nematodes. Other phytochemicals like flavonoids and oleanane type triterpenes may also have their independent or synergistic effects (Brantner *et al.*, 1996). The use of botanical anthelmintics has been proposed as an alternative strategy for the control of gastrointestinal nematode infections in order to reduce the dependence on chemical anthelmintic treatments and to delay the selection and the transmission of anthelmintic resistances in worm populations (Hoste *et al.*, 2006).

#### 4.0 Conclusion

In the present study, we found that most of the biologically active phytochemicals were present in the ethanolic extracts of *Prosopis juliflora* and its medicinal properties could be due to the presence of these phytochemicals acting either independently or synergistically. This study, therefore, has provided biochemical basis for the use of extracts from *P. juliflora* in the treatment and prevention of helminth infections. However, further studies are recommended to isolate and elucidate the active components responsible for the observed activity.

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