PHYTOCHEMICAL AND *IN VITRO* ANTIMICROBIAL EVALUATION OF ETHANOLIC EXTRACTS OF *PROSOPIS JULIFLORA (SW.)* DC (FABACEAE)

S. O. Rechab[1], P. G. Kareru[2], H. L. Kutima[3], G. C. Nyagah[4], F. K. Njonge[5], D. W. Gakuya[6] and R. W. Waithaka[7]
[1,2,4,7]Chemistry Department, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
[3]Zoology Department, Jomo Kenyatta University of Agriculture and Technology Nairobi, Kenya
[5]Land Resource and Animal Science Department, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
[6]Department of Clinical Studies, Faculty of Veterinary Medicine, University of Nairobi, Nairobi, Kenya
E-mail: rechabsylvester@gmail.com

Abstract
Prosopis juliflora, a multipurpose dry land tree or shrub introduced to Kenya due to concern about desertification, deforestation and fuelwood shortages, has become invasive, forming dense, impenetrable thickets, associated with unfavorable impacts on human economic activities. It has soothing, astringent, antifungal and antiseptic properties and is commonly used to treat eye conditions, open wounds and dermatological ailments. An assessment of phytochemical composition and antimicrobial activity of ethanolic extract of root(REE) and leaves(LEE) of *P. juliflora* against clinical isolates of *Candida albicans*, *Staphylococcus aureus*(ATCC-25923), *Bacillus subtilis* , *Escherichia coli* (ATCC-25922) and *Pseudomonas aeruginosa* (ATCC-27853) was carried out using paper disc diffusion method. The results of investigation showed that all the extracts had inhibitory effect on the growth of all the isolates. Only chloramphenical, erythromycin and minocycline were effective against all the bacterial strains tested and there was no significant difference ($P > 0.05$) between the activity of REE and LEE at the highest concentration compared to the activity of chloramphenical, erythromycin and minocycline. *S. aureus* showed the lowest susceptibility to most of the conventional drugs while *B. subtilis* was most susceptible to chloramphenical. All the bacterial strains exhibited susceptibility to erythromycin and minocycline while Penicillin, methicillin and ampicillin were the least effective antibiotics. Both LEE and REE exhibited better antifungal activity compared to cotrimoxazole and all the extracts had saponins, tannins and alkaloids; phytochemicals whose antimicrobial properties are well documented. Results from this study strongly validate use of *P. juliflora* in the management of microbial infections.

Key words: Gram negative, gram positive, antimicrobial, phytochemicals

1.0 Introduction

The economic impact of parasitic gastroenteritis caused by mixed infection with several species of stomach and intestinal round worms, 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 because of 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 week, pulverized in electric grinder and exhaustively extracted using 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 procedures (Harborne, 1998) and the extracts were tested for triterpenes, sterols, flavonoids, saponins, tannins and alkaloids.

2.3 Determination of In vitro Anthelmintic Activity

2.3.1 Haemonchus Contortus Egg Recovery

Haemonchus contortus eggs were recovered from faeces according to Hubert and Kerboeuf (1992). Sample of faeces (10–15 g) were collected from sheep experimentally infected with mono-specific larval suspensions of fresh *H. contortus*. 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 µ m 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.

2.3.2 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 50µl was distributed in a 48-well flat-bottomed microtitre plate containing approximately 50 fresh eggs and mixed with the same volume of plant extracts dissolved in PBS having different concentrations (1, 2, 4 and 8 $mgmL^{-1}$).. Albendazole (99.8% pure standard reference) was used as a positive control. The albendazole was dissolved in dimethyl sulfoxide (DMSO) and diluted at concentrations between 2 $mgmL^{-1}$ and 0.03125 $mgmL^{-1}$. The 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. All the eggs and first-stage larvae (L_1) in each plate were counted. 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.

2.3.3 In vitro Adult Mortality Assay (AMA)

Adult *H. contortus* were collected from the abomasum of infected sheep obtained from a local Abattoir. Immediately after slaughter, the abomasum were collected and transported to the laboratory. The parasites were then washed and kept in phosphate buffered saline (PBS, pH: 7.2, 4°C). Ten actively moving worms were placed in Petri dishes containing 10.0, 8.0, 4.0, 2.0, 1.0, 0.5, and 0.25mg/ml 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 0.5, 0.25, 0.125, 0.0625, and 0.03125mg/ml was used as a positive control. 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 5-6 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_{50}) egg hatching 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 Results for Phytochemical Screening

Results for Phytochemical analysis as shown in table 1 revealed that LEE and REE possess alkaloids, tannins, saponins, flavonoids, sterols and triterpenes that could be responsible for its 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). 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 $mgmL^{-1}$ exhibited complete inhibition on egg hatching as shown in figure 1. The ED_{50} values for the extracts and albendazole are as shown in table 2.

Table 2: ED_{50} values for EHA

Extract	95% Confidence Limits for Concentration			95% Confidence Limits for log_{10}(concentration)			R^2
	ED_{50} value (mg/ml)	Lower Bound	Upper Bound	ED_{50} value (mg/ml)	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 finding was consistent with other research findings that plant phytochemicals (such as resins, bitter principles, tannins, flavonoids and indolquinolizidine alkaloids) exhibited high anthelmintic activity against *strongyle* nematodes of small ruminant animals by preventing parasite eggs from hatching (Onyeyili *et al*., 2001).

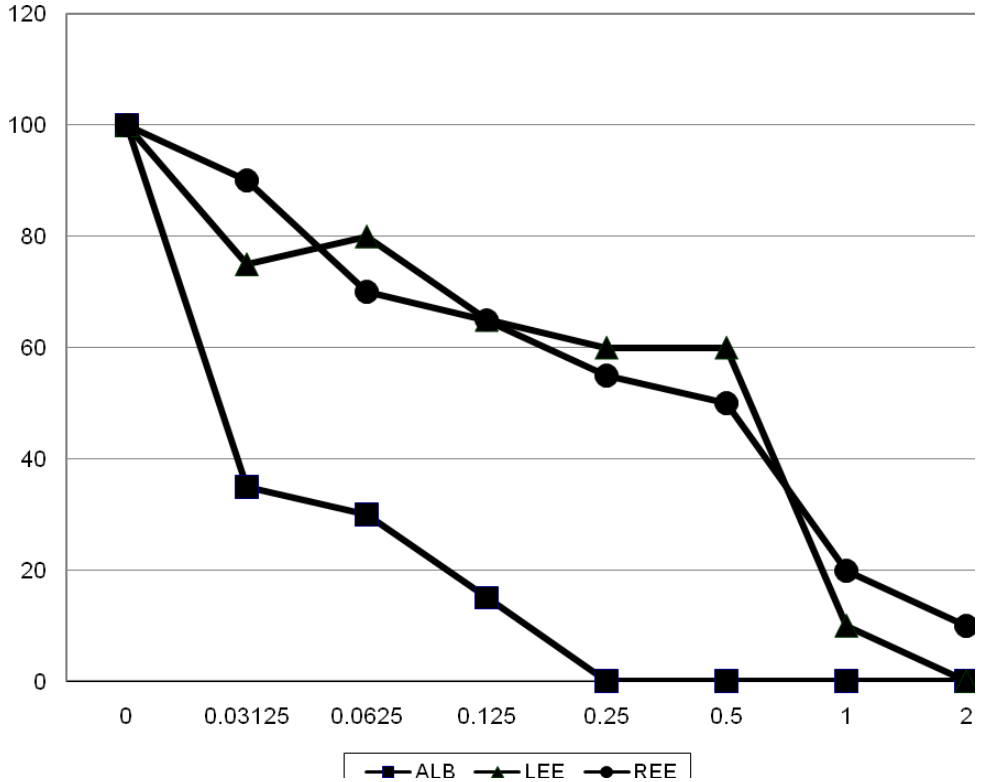

Figure 1: Graph showing mean percentage egg hatching of various concentrations of LEE, REE and ALB after 48 hours

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 higher activity (LD_{50}=0.857 mg/ml) as compared to REE (LD_{50}=1.782 mg/ml). Albendazole had significantly higher activity as compared to the ethanolic extracts. (LD_{50}=0.046mg/ml).

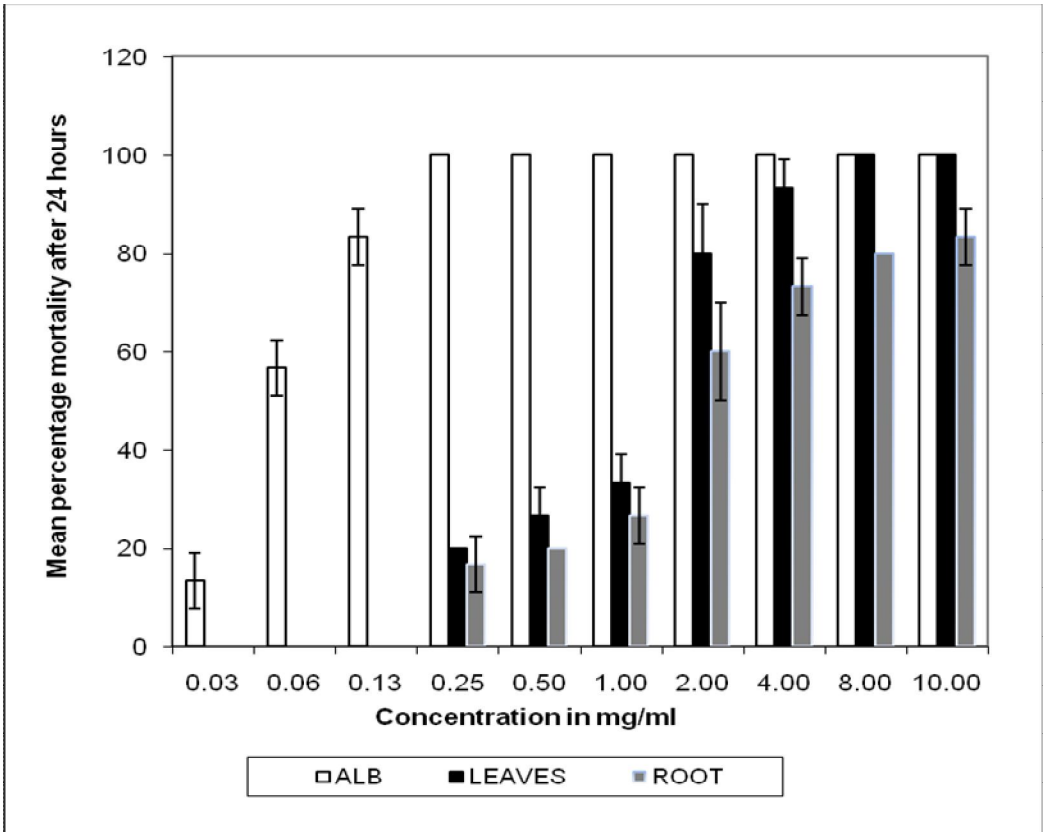

Figure 2: Graph showing mean percentage mortalities of H. contortus subjected to various concentrations of Albendazole and ethanolic extracts of leaves and roots

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 faecal 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 oleane 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).

Acknowledgement
The authors acknowledge Jomo Kenyatta University of Agriculture and Technology for funding the project.

References

Alawa, C. B. F., Adamu, A. M., Gefu, J. O., Ajanusi, O. J., Abdou, P. A., Chiezey, N. P., Alawa, J. N. and Bowman, D. D. (2003). In vitro screening of two medicinal plants (*Vernonia amygdalina* and *Annona senegalensis*) for anthelmintic activity. *Veterinary Parasitology,* **1** (13): pp 73–81.

Brantner, A., Males, Z., Pepeljak S. and Antolic A. (1996). Antibacterial activity of *Paliurus spina-christi* Mill (Christ's thorn). *Journal of Ethnopharmacology*, **52**: pp 119–122.

Coles, G. C., Bauer, C., Borgsteede, F. H., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P. J., (1992). World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology,* **44**: pp 35–44.

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

Githiori, J. B. (2004). Evaluation of anthelmintic properties of ethnoveterinary plants preparations used as livestock dewormers by pastoralist and small holder farmer in Kenya. Doctoral dissertation, Department of Biomedical Sciences and Veterinary Public Health, SLU. Acta Universitatis Agricultural sulciae, p. 76.

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

Harborne, J.B (1998). Phytochemical Methods: A guide to Modern Techniques of Plant Analysis. 3rd edition. Chapman and Hall Ltd, London pp 279.

Hoste, H., Jackson, F., Athanasiadou, S., Thamsborg, S. M. and Hoskin, S. O. (2006). The effects of tannin-rich plants on parasitic nematodes in ruminants. *Trends Parasitology,* **22:** pp 253–261.

Min, B. R., Barry, T. N., Attwood, G. T. and McNabb, W. C**.** (2003). The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. *Animal Feed Science and Technology*, **106:** pp 3-19.

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

Onyeyili, P. A., Nwosu, C. O., Amin, J. D. and Jibike, G. I. (2001). Anthelmintic activity of crude aqueous extract of *Nauclea latifolia* stem bark against ovine nematodes. *Fitoterapia*, **72:** pp 12-21.

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

Singh, D., Swarnkar, C. P. and Khan, F. A. (2002). Anthelmintic resistance in Gastrointestinal nematodes in livestock in India. *Journal of Veterinary Parasitology,* **16**: pp 115-130.

Hubert, J. and Kerbouef, D. (1992). A microlarval development assay for the detection of anthelmintic resistance in sheep nematodes. *Veterinary Record,* **130**: pp 442–446