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

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

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

I. Introduction

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

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

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

II. Materials And Methods

2.1 Collection and preparation of medicinal plant material

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

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

2.2 Sourcing, housing and grouping of animals

Sheep (male and female stock of 6-12 months) weighing between 13-26.5 kg were bought from Kariobangi livestock market in Nairobi, Kenya. They were transported to J.K.U.A.T. and left to graze and acclimatize for a month. The animals were housed in pre-designed animal shed in the university's animal farm consisting of five cubicles and a feed storage cubicle with each cubicle having a meshed window for ventilation and raised wooden floor, a wooden feeding trough and watering bucket. The animals were then grouped according to the various test concentrations of 500mg/kg, 1,500mg/kg and 4,500mg/kg for the extract treatment groups, 10mg/kg for the treated control group (Albendazole) and untreated control group. Each group consisted of two males and two females and the females were confirmed to be non-pregnant. The animals were randomly selected and assigned to the various groups so that the individual weights in each group were as close as possible. There was no physical contact between animals in different cubicles.

2.3 Pre-treatment procedure

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

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

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

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

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

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

III. Results And Discussion

3.1 Feacal egg count results

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

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

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

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

There were no signs of toxicity such as salivation, diarrhea and skin reaction in all the groups throughout the study period apart from reduced feed intake in the initiation stage of the experiment. The herbal extract mixture showed a time-dependent but not dose-dependent *in vivo* anthelmintic activity. Albendazole and the herbal extract mixture showed a general positive *in vivo* anthelmintic activity compared to untreated control. The high faecal egg counts could be attributed to the high presence of adult parasites in reproductive stages in the host (Worku *et al.*, 2009). There was a general increase in FEC in all the animal groups on day 3, with a general drop from day 7 except for treatment group at 1500mg/kg b.w. This increase in FEC could have been due to reduced faecal output hence, a virtually higher faecal nematode egg concentration (Githiori, 2008). This was caused by general reduction in the animals' feed intake by day 0 post treatment. This may be attributed to a shorter period of acclimatization to the commercial feed leading to poor palatability despite supplementing the commercial feed with grass. However, the feed intake had improved by day 5 post-treatment after mineral supplementation was introduced on day 4 post-treatment.

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

other reason for this observation could have been due to early saturation of the aqueous solutions of the individual plants during preparation of the extracts. With a peak faecal egg reduction of 84%, the 500mg/kg b.w. dose passed the threshold FEC reduction.

Wood *et al.* (1995) reports that any anthelmintic product that reduces FEC by less than 80% during FECR test trial should be considered insufficiently active as a curative agent. Githiori (2008) considered FEC and total worm count (TWC) reductions greater than or equal to 70% biologically significant based on the same guideline. The 500mg/kg b.w. dose may not have been affected by slow drug metabolism and this could provide direction on the maximum dose necessary to produce better *in vivo* anthelmintic effects.

The herbal extract mixture formula exhibited *in vivo* anthelmintic activity against mixed gastrointestinal nematodes. This could be attributed to the mixture of polar phytochemicals present in *E. leptostachya* and *P. juliflora* which are soluble in aqueous medium. Phytochemical studies done on the two plants confirm the presence of alkaloids, flavonoids, saponins, tannins and sterols/triterpenes from the leaf aqueous extracts of P. juliflora (Wamburu *et al.*, 2013) while root aqueous extracts of *E. leptostachya* have tested positive for sterols/triterpenes, glycosides, saponins and tannins (Kareru, 2008; Kareru *et al.*, 2012). Condensed tannins can impair vital processes such as feeding and reproduction of the parasite or may bind and disrupt the integrity of the parasite's cuticle (Dave *et al.*, 2009; Zafar *et al.*, 2009). It is reported that monodesmoside saponins have shown to destabilize membranes and increase cell permeability by combining with membrane-associated sterols (Geidam *et al.*, 2007; Ademola *et al.*, 2008; Ademola and Eloff, 2010) and producing changes in cell morphology leading to cytolysis (Geidam *et al.*, 2007). Alkaloids may improve tonicity of the gastrointestinal tract and thus expel the worms or may have a direct effect on the nervous system of the nematodes (Ademola *et al.*, 2008; Lateef *et al.*, 2003). Albendazole works by interference with polymerization of microtubule, where the drug binds to the protein tubulin of the parasite leading to death by starvation (Kareru *et al*., 2012; Lalchhandama, 2009). The other phytochemicals in the two plants like flavonoids and oleanane type triterpenes may have had their independent or synergistic anthelmintic effects (Zafar *et al.*, 2009).

3.2 Results of live body weights of the experimental sheep

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

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

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

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

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

Presence of tannins in the leaf and root aqueous extracts of *P. juliflora* and *E. leptostachya* respectively is reported (Kareru *et al.*, 2012; Wamburu *et al.*, 2013). Because of their reactivity with plant proteins as they are being chewed by ruminant animals, condensed tannins partially protect animals against rumen degradation of proteins, and so increase the flow of amino acids to the small intestines hence, increasing their absorption (FAO, 2011; Min and Hart, 2003; Zafar *et al.*, 2002). This increase may help to counteract the losses of protein attributed to gastrointestinal nematode infection and for immune response to nematode parasites (Barry *et al.*, 2001; Niezen *et al.*, 2002; Zafar *et al.*, 2002; Min and Hart, 2003).

The herbal extract mixture treated groups could have benefitted from better utilization of proteins in the presence of tannins or the weight gains could have been as a result of improved feeding or both or due to better nutrient utilization due to lower worm load. Weight gains in the Albendazole-treated group could have been due to maximization of nutrient utilization as a result of lower worm load. However, determination of total worm count will be essential in future work with this extract mixture to validate weight gains as a result of lower worm loads as EPG counts cannot reliably be used to make conclusions on worm loads.

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

From **Table 3**, there were no significant changes (P>0.05) in the packed cell volumes (PCVs) by day 19 PT compared to day 0 PT except for the extract mixture dose of 1500mg/kg b.w. whose PCV significantly dropped (P<0.05). All the animals recorded pre- and post-treatment PCV values that were within the permissible range of between 24-45% (Research Animal Resources, University of Minnesota, 2013; Worku *et al.*, 2009) for experimental sheep.

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

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

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

All the groups recorded improved PCV values (thus, reduced degree of anaemia) except the doses at 1500mg/kg b.w. and 4500mg/kg b.w. Anaemia, following administration of an agent, could be as a result of lysis of blood cells and/or inhibition of blood cell synthesis by the active constituents of the extract (Orisakwe *et al.*, 2003). The higher doses of the extract mixture (1500 and 4500mg/kg b.w.) could have had this effect on the experimental animals thus, recording decreased PCV values. Besides the negative effects worms have on increasing anaemia, the lower percentage increase on mean PCV of the treated animals at 500mg/kg b.w. compared to the controls and percentage PCV drops at 1500mg/kg b.w. and 4500mg/kg b.w. groups may be indicators of the drug effect on the PCV values of sheep. This may also suggest that the two doses whose PCVs dropped may have had a negative stimulatory effect on the hemopoietic system or the animals may have increased water intake (Githiori, 2008). As no abnormal adverse effects were observed during this study after administration of the herbal drug, water intake may not have contributed to this effect as

no abnormal water intake was observed in all the groups under the same experimental conditions. Each worm is responsible for a daily loss of blood of about 0.05ml through ingestion and seepage from lesions (Eguale *et al.*, 2007). PCV values are directly related to anaemia, correlated with high FEC and parasite burdens (Dawo and Tibbo, 2005; Worku *et al.*, 2009).

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

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

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