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Anthelmintic efficacy and safety of selected medicinal plants against mixed gastrointestinal nematodes in artificially infected sheep

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ABSTRACT

There has been widespread resistance to anthelmintics by pathogenic helminths to an extent where there is multi-drug resistance against all major classes of conventional anthelmintics. This world-wide phenomenon calls for urgent search for different approaches to the control of helminthosis including novel anthelmintic products. The purpose of the study was to determine the efficacy and safety of selected plants, which are frequently used in the treatment and control of helminthosis, in artificially infected sheep under controlled laboratory conditions. The selected plant species were, *Albizia anthelmintica* Brongn, *Embelia schimperi* L., *Myrsine africana* L. and *Rapanea melanophloeos* (L.) Mez. Thirty six male Dorper lambs, aged between 6 and 8 months, artificially infected with mixed gastrointestinal nematodes (GIN) under controlled laboratory conditions, were used for the study. Efficacy was determined using percentage fecal egg count reduction test (FECRT %) and percentage total worm count reduction (TWCR %). Safety of the remedies was assessed using health, hematological and biochemical parameters. The FECR % against the mixed gastrointestinal nematodes was -55, 7.6, 34.2, 69.3 and 83.3% for *Albizia anthelmintica*, *Embelia schimperi*, *Rapanea melanophloeos*, albendazole and *Myrsine africana* respectively. TWCR% of 60.7, 44.6, 66, 69.7 and 35.6 percent were recorded for *Albizia anthelmintica*, *Embelia schimperi*, *Myrsine Africana*, *Rapanea melanophloeos*, and albendazole groups respectively. It was concluded that some of the remedies like *M. africana* have good efficacy at safe levels and should further be evaluated to determine the most optimum dosages. The gastrointestinal nematodes used in this study were resistant to albendazole.

Keywords: Medicinal plants, efficacy, gastrointestinal nematodes.

1. INTRODUCTION

The utilization of traditional medicines, mostly derived from plants, in human and veterinary practice is deeply anchored in history. The World Health Organization estimates that approximately 80% of the people in poor countries are reliant on traditional medicine for their basic health care [1]. There has been awakened interest in traditional medicine throughout the world, which mainly includes ethnobotany and the use of remedies of herbal origin. The driving force for this momentum include the perception that "natural is nice", concerns of harmful synthetic drug residues in the ecosystem, and in particular the potential risk of fast emergence of multiple-drug resistant organisms through misuse of conventional drugs.

Due to the awakened interest in traditional remedies, researchers are now more concerned with the discovery of novel compounds of pharmaceutical value in addition to determining the scientific rationale for the usage of the remedies. Instead of relying on trial and error as in random screening procedures, traditional knowledge helps scientists to target plants that may be of medicinal value [2, 3]. There are myriad of plants reported to have anthelmintic properties around the globe [4-8]. Although the majority of the evidence on the anthelmintic activity of these plants is based on anecdotal observations, there is a growing number of controlled studies whose objective is to scientifically validate and quantify the alleged bioactivity. There are two main approaches that have been used in anthelmintic efficacy studies in the past. First is through feeding of plants or their parts to animals with either natural or experimental infections [9]. Second is by testing extracts and concoctions of remedies via *in vivo* and *in vitro* systems [10].

Kenya is rich in medicinal biodiversity which has traditionally been used by practitioners for the management of various diseases and conditions among them helminthosis [8, 11, 12]. However, most of these traditional remedies are yet to be scientifically validated or developed into viable products for the market. The aim of the current study was to determine anthelmintic efficacy in four medicinal plants

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most frequently used to treat and control helminthosis in Loitoktok Sub-county of Kajiado County in Kenya. The four plants studied were *Albizia anthelmintica* Brongn, *Embelia schimperi* L., *Myrsine africana* L. and *Rapanea melanophloeos* (L.) Mez. The first plant belongs to the Fabaceae family while the last three belong to Myrsinaceae. These plants were selected from an earlier ethnopharmacological study conducted in the area involving renowned traditional healers identified through key informants [8].

2. MATERIALS AND METHODS

2.1 Plant collection and preparation

The plant materials were collected with the help of the traditional healers (THs) from different parts of Loitoktok Sub County and transported to the University of Nairobi. Pieces of the stem bark of *Albizia anthelmintica* were hived off the tree trunks using a matchette in the area between Loitoktok town and Kimana market. These were later chopped into smaller pieces and left to dry under the shade for several days. The seeds of *Embelia schimperi, Myrsine africana and Rapanea melanophloeos* were obtained from Kuku area, foot of Chyulu hills and the foot of Mt. Kilimanjaro respectively. The seeds were also dried under shade in a well aerated enclosure for several days. Representative samples for each plant were collected and placed into a field press for transportation and identification at the University of Nairobi herbarium where voucher specimens were deposited.

The dry plant materials were milled into powder using a laboratory mill (Christy and Norris Ltd, England) and anthelmintic remedies prepared as follows:

1) *Albizia anthelmintica* remedy was prepared by soaking 800 grams of powder in 3.2 litres of water (25% w/v) and shaken vigorously at least 3 times every 24 hours for 72 hours. The mixture was then filtered through a piece of gauze repeatedly until it was clear. The dose to be administered was fixed at 150 ml (equivalent to 3 g of freeze dried extract) from an earlier pre-trial where a dose of 4.4 g of freeze dried powder per lamb caused death from severe respiratory embarrassment but doses below 3 g were tolerated.
2) *Embelia schimperi* remedy was prepared by soaking 600 grams of powder in 2.4 litres of water (25% w/v) and shaken vigorously at least 3 times every 24 hours for 72 hours. The mixture was then filtered through a piece of gauze repeatedly until it was clear. The dose to be given was fixed at 200 ml (equivalent to 8.5 g of freeze dried extract) based on an earlier pre-trial.
3) *Myrsine africana* remedy was prepared by soaking 600 grams of powder in 2.4 litres of water (25%) and shaken vigorously at least 3 times every 24 hours for 72 hours. The mixture was then filtered through a piece of gauze repeatedly until it was clear. The dose to be given was fixed at 200 ml (equivalent to 5.2 g of freeze dried extract) based on an earlier pre-trial.
4) *Rapanea melanophloeos* remedy was prepared by soaking 800 grams of powder in 3.2 litres of water (25% w/v) and shaken vigorously at least 3 times every 24 hours for 72 hours. The mixture was then filtered through a piece of gauze repeatedly until it was clear. The dose to be given was fixed at 200 ml (equivalent to 5 g of freeze dried extract) based on the results of an earlier pre-trial and doses cited by the traditional practitioners.

2.2 Animals used in the study

Thirty (36) male Dorper lambs, aged between 6 and 8 months, were purchased from a ranch in Kiambu County approximately 40 Km North East of the University of Nairobi, Faculty of Veterinary Medicine, where the study was done. The lambs were identified by use of numbered plastic ear tags, weighed and faecal samples collected from the rectum for the screening of endoparasites. About half of them were shedding gastrointestinal (GI) nematode eggs. They were all treated using Closamectin® (combination of closantel and ivermectin by Norbrook, Kenya) at the recommended dosage rate of 200 µg and 5 mg per kilogram body weight for ivermectin and closantel respectively by subcutaneous injection. The lambs were housed in clean dry pens with concrete floors that were regularly cleaned. They were put in groups of four according to their body weights to create harmony and equitable access to feed and water. Unlimited amounts of hay, mineral lick and water were provided. Ewe and lamb mash (Pembe Feeds Ltd) were fed at the rate of 200 g per lamb daily. The health of the lambs was monitored on a daily basis. After two weeks of arrival the lambs were screened again for gastrointestinal nematodes and all were found to be negative. Permission to use animals in the study was granted by the Faculty of Veterinary Medicine Animal Care, Use and Biosafety Committee.

2.3 Helminth cultures, infection and treatment

Mixed Infective larvae (L3) were obtained by culturing faeces from, prescreened naturally parasitized, sheep reared outdoors in an institutional farm in Tigoni, Kiambu County, about 25 Km North West of the experimental site. The faeces were collected directly from the rectum and incubated at about 26 ºC for 10 days. The larvae were harvested using the Baermann technique [13] and stored in bottles containing water. One hundred larvae were identified to determine the proportion of the nematode genera in the culture (Haemonchus: 77%; Trichostrongylus: 20%; Oesophagostomum: 3%). The larvae were used for infection before they were one week of age.

The method by Mugambi and others [14] was used to estimate the number of the larvae but with slight modifications. Briefly, 50 µl of larval suspension was spread in drops onto a glass slide and larvae counted under a microscope. An estimate of the number of larvae in 1 ml of the suspension was arrived at from counts in 10 aliquots. The larval dose per animal was adjusted to 2ml and given orally using a 5 ml syringe. The first infection was at the rate of 3000 L3 per animal 3 weeks after the arrival of the lambs. From the third week post infection the lambs were screened for GI nematode eggs on a weekly basis. Animals found to be negative for GI nematodes 5 weeks post infection or with less than 200 epg were given another dose of 2000 L3 per animal and this was repeated again at the end of the 7th week. All animals were shedding GI nematode eggs by the tenth week since the first infection and hence ready for the treatment.

The lambs were divided into two blocks by FEC from where they were randomly allocated into 6 groups of 6 animals each. Six animals per group is the minimum recommended by the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P) for such efficacy trials [15]. The FEC on the day of treatment ranged from 100 to 37100 epg and the group means from 1417 to 8725 epg (Table 1).

Table 1: Groups of sheep used in the anthelmintic efficacy against artificial infection of mixed gastrointestinal nematodes

Group	Treatment	Parts used (where specified)	Dose/lamb (or as specified)	Mean eggs/gram
A	*Albizia anthelmintica*	Stem bark	150 ml	2067
E	*Embelia schimperi*	Fruits	200 ml	2100
M	*Myrsine Africana*	Fruits	200 ml	2283
R	*Rapanea melanophloeos*	Fruits	200 ml	1417
V	Valbazen® (albendazole)		10 mg/Kg body weight	8725
C	Untreated control		200 ml tap water	2100

2.4 Determination of weights, biochemical and hematological parameters

The lambs were weighed on days 0, 21 and 35; faecal samples were taken directly from the rectum on days 0, 10, 18 and 35 while blood was obtained via jugular venipuncture for hematology and biochemistry on days 0, 8, 21 and 35. Modified McMaster method (Henriksen and Aagaard, 1976) was used to determine the faecal egg counts. Hematological analysis was done using a fully automatic hematology cell-counter (Melet Schloesing Laboratories-BP 508-95528 cergy-Pontoise Cedex – France). The differential cell counts were done manually. Total protein, albumin and aspartate amino transferase (AST) were analyzed from plasma with a spectrophometer (Biomerieux Sa 69280 Marcy iEtoile/France).

2.5 Worm counts and identification

From day 35 post treatment the lambs were humanely slaughtered and the abomasum ligated at both ends and removed. The abomasum was opened along the greater curvature using a blunt tipped pair of scissors and the contents put into a bucket. The mucosa of the abomasum was washed by gently running water into the bucket and the contents adjusted to a volume of 2 litres. A ten percent (10%) aliquot (200 ml) was taken, after thorough mixing, and all worms inside counted under a stereo microscope. They were preserved in 70 % ethanol for identification later using procedure by MAFF [13]. The small and the large intestine were removed separately and each of them opened and mucosa washed into a separate bucket and the above procedure repeated.

2.6 Statistical analysis

The parameters analyzed were Live weight (LWT), faecal egg count (FEC), Total worm count (TWC), packed cell volume (PCV) and other hematological components. Others were total protein (TP), albumin and the enzyme aspartate aminotransferase (AST). The data were subjected to one way analysis of variance using SPSS 17 testing whether there is significance ($P<0.05$) between treatments. Because of a skewed distribution, the analysis of FEC and TWC was on logarithm transformed data [16]. For example, LFEC = $\log_{10}$ (FEC+25); LTWC = $\log_{10}$ (TWC+1).

2.7 Estimation of anthelmintic efficacy

The anthelmintic efficacies were estimated through percentage fecal egg count reduction (FECR %) and post-mortem worm count reduction percentages. The FECR% was calculated as follows: FECR% = $(1-(T_2/T_1 \times C_1/C_2)) \times 100$. Where, *T* and *C* denotes the arithmetic means of the eggs per gram of faeces for the treated and control groups. The subscripts 1 and 2 denotes the counts before and after treatment, respectively [17]. The confidence interval for the albendazole reduction was calculated according to the formula by Coles and others [18] to find out whether there was resistance as follows: 95% CI limits; upper limit = $100[1-\bar{Y}_t/\bar{Y}_c \exp(-2.048\sqrt{Y^2})]$ and lower limit = $100[1-\bar{Y}_t/\bar{Y}_c \exp(+2.048\sqrt{Y^2})]$, Where, $\bar{Y}_t$ and $\bar{Y}_c$ denotes the arithmetic means of the treated and control groups respectively, and Y^2 is the variance of the reduction (log scale).

The percentage total worm count reduction (TWCR%) was calculated using the formula:

$TWCR\% = (1-TWC_t/TWC_c) \times 100$ [15], where the subscripts t and c designate the treatment and the untreated control groups respectively. The same formula was also used for the percentage differential (individual species) worm count reductions.

3. RESULTS AND DISCUSSION

3.1 Effects of anthelmintic treatments on general health of the animals

Some irritation and coughing followed by transient bloating occurred in animals dosed with *A. anthelmintica* preparation but this was over in about 12 hours. However, these animals slightly reduced feed intake during the following week but went back to normal thereafter. A few animals developed bottle jaw in groups A, C and V. One animal in group V died of conditions unrelated to helminthosis (probably of GIT blockage by numerous phytobenzoars that were encountered on post mortem). Otherwise the parameters of general health of all the other animals remained within the normal range.

Transient bloating of sheep treated with aqueous extracts of *A. anthelmintica* in a similar study [10]. In the same study it also caused deaths in mice, infected with *Heligmosomoides polygyrus*, when given at the dose of 33 g/Kg body weight. Moreover, another study reported deaths of all the mice infected with *H. polygyrus* and treated with 5, 10 and 20 g/Kg body weight of methanolic extracts of *A. anthelmintica* [19]. An earlier study related to the current one found *Albizia anthelmintica* to have high amounts of saponins [20]. Saponins (triterpenoids) may have been responsible for the reduction in feed intake, causing nutritional deficiencies, hemolysis and even mortalities in herbivores [21, 22].

3.2 Effects of anthelmintic treatments on fecal egg counts

The fecal egg counts (FEC), before and 10 days post treatment, and FECR% in the artificially infected Dorper lambs are displayed on

Table 2. The FEC varied from 0 to 10,700 in the treated groups while in the untreated control group it varied from 200 to 6,100 on day 10. The FECR% was -55, 7.6, 34.2, 69.3 and 83.3% for *Albizia anthelmintica, Embelia schimperi, Rapanea melanophloeos*, albendazole and *Myrsine africana* respectively.

Table 2: Anthelmintic efficacy against artificial infection of mixed gastrointestinal nematodes in sheep

Group	Arithmetic mean (Range)				
	Pre- treatment (day 0)	**Post- treatment (day 10)**	**FECR %**	**95% CI (Albendazole)**	**Remarks**
A	2067 (100-7400)	2817 (300-10700)	-55		No efficacy
E	2100 (100-7900)	1700 (0-5400)	7.6		Negligible fficacy
M	2283 (100-12500)	333 (200-500)	83.3		Good efficacy
R	1417 (100-3400)	817 (0-1500)	34.2		Some efficacy
V	8725 (200-31700)	2350 (0-8000)	69.3	-560.8 – 75.3	Resistance
C	2100 (100-8900)	1840 (200-6100)	0		Untreated control

Key: A = *Albizia anthelmintica*; E = *Embelia schimperi*; M = *Myrsine africana*; R = *Rapanea melanophloeos*; V = Valbazen (albendazole); C = Untreated control; FECR % = Percentage fecal egg count reduction and CI = Confidence interval for the reduction).

The *Myrsine africana* remedy had the best FECR of 83% even surpassing that of albendazole and the FECR of 59% obtained during the field trial in Loitoktok Sub County [23]. It was also the only group that had significantly (P<0.05) fewer epg than the untreated control group. This FECR is slightly higher but comparable to the FECR of 77% reported in sheep naturally infected with mixed GIN in Samburu County [4]; though, the sheep in the Samburu study had much lower mean pretreatment epg (300) than in the current study (2283). The Samburu study also reported 100% efficacy against Monezia tapeworms [4]. Further, extracts from the fruits of this plant have been found to have good efficacy against the nematodes *Bunostomum trigonocephalum* and *Oesophagostomum columbianum*, and the cestode *Taenia solium* [24]. *Rapanea melanophloeos* and *E. schimperi* (both Myrsinaceae) had insignificant FECR and this result is consistent with another study [5] done in Kenya using Dorper lambs artificially infected with a monoculture of *H. contortus.*

The *A. anthelmintica* group was the only one that had higher mean eggs per gram of feces than its pre-treatment levels and the untreated control. This increase in FEC could possibly have been a result of higher concentration in feces due to the observed transient bloating and reduction in feed intake in the days post treatment of the animals. However, the Samburu study [4] reported high FECR of 89.8% using about 26.5g of the root bark of *A. anthelmintica* per adult sheep naturally infected with mixed GI nematodes while the current study used about 37.5g of stem bark from Loitoktok per animal. The differences in the reported efficacies could be as a result of the variation in the dosages given or the phytochemical composition of the plants obtained from different areas and ecosystems and also the methodologies, and possibly the composition and species of GI nematodes in the study animals [25]. Varying collection and storage conditions of the plant materials have also been found to affect the physical and chemical properties of the plant secondary metabolites (PSM) and probably their bioactivities. Furthermore, seasonal and environmental differences will affect the synthetic pathways of the PSM, which can potentially impact their physical and chemical properties [26].

The FECR for albendazole of 69% with a 95% CI of -560.8 to 75.3 is an indication of resistance by the GIN used to artificially infect the animals in this study. In an earlier field trial, resistance was only suspected because, though the FECR was less than 95%, the upper 95% CI was more than 90% [23]. However, in this case, resistance is confirmed because both conditions, FECR <95% and CI < 90% have been met [18].

3.3 Effect of anthelmintic treatments on live weight, hematological and biochemical parameters

All the groups lost live body weights by a mean of between 0.4 and 2.4 Kg by day 35. However, only the loss by group A (*A. anthelmintica)* was statistically significant (P<0.05) in comparison to the control group. There were no statistically significant changes in all the hematological and biochemical parameters analyzed. However, the PCV levels dropped in all other groups of animals by day 8 except the *M. africana* group that increased marginally by 0.9%. By day 21 of treatment the PCV levels improved marginally except in groups A and R, and again the *M. africana* treatment had the highest improvement of 2.2% of the pretreatment levels.

The improvement of PCV levels in the M. Africana group could mean that apart from reducing GI nematode burdens in the parasitized animals, it could also be having stimulatory effects on the haemopoietic tissue or even other body systems leading to increased resistance and even resilience. Recent evidence suggests that the consumption of medicinal plants or plant extracts could improve the immune response (immunomodulatory effects) of parasitized hosts, by improving the number of specific effector cells [25, 27].

3.4 Effect of anthelmintic treatments on total and differential worm count

The total and differential worm counts and percentage reductions are shown in Table 3. The TWCR% of 60.7, 44.6, 66, 69.7 and 35.6 percent were recorded for groups A, E, M, R and V respectively. All the treatments caused a reduction of TWC but only those of *R. melanophloeos, M. africana* and *A. anthelmintica* were significant (P<0.05). In addition these three treatments had significant activity against *H.contortus* with reductions ranging from 73 to 78%. *A. anthelmintica* (Fabaceae) showed substantial activity against the abomasal *T. axei* but not the intestinal *T. columbriformis* or any other intestinal nematode. Furthermore, *E. schimperi, M. africana and R. melanophloeos* (all Myrsinaceae) had little or no activity at all on the intestinal nematodes.

Table 3: Effect of anthelmintic treatment on the total and differential worm counts in sheep artificially infected with mixed gastrointestinal nematodes

Nematode species	C	A	E	M	R	V
Haemonchus contortus	809	222 (73)	422 (48)	198 (76)	176 (78)	630 (22)
Trichostrongylus axei	8	3 (63)	0 (100)	10 (-25)	5 (38)	1 (84)
Trichostrongylus columbriformis	21	49 (-127)	31 (-45)	42 (-95)	15 (32)	0 (100)
Oesophagostomum columbianum	147	112 (24)	93 (57)	85 (43)	103 (30)	3 (98)
Mean nematode worm count	986	389 (61)	546 (45)	336 (66)	299 (70)	634 (36)

Key: A = *Albizia anthelmintica*; E = *Embelia schimperi*; M = *Myrsine africana*; R = *Rapanea melanophloeos*; V = Valbazen (albendazole); C = Untreated control
Numbers in brackets represent the percentage total worm count reduction (TWCR%)

The high TWCR (70%) for *R. melanophloeos* compared to its low faecal egg count reduction of 34% on day 10 can only be speculated. One possible reason would be that the active phytochemicals in this plant only exert a quick effect on the worms like paralyzing them but no long-term effect on the surviving ones which then continue laying eggs normally or probably have a selective effect on the male worms. The former explanation is further supported by the fact that FEC for this treatment group increased faster than all the others and by day 35 it had a mean FEC of 4100 against the runners up (group A) with 2450 epg.

Embelia schimperi (Myrsinaceae) also showed some activity on *H. contortus, T. axei and O. columbianum.* It is only *R. melanophloeos* that had activity across all the nematode species. The significant TWCR by *A. anthelmintica* further supports the earlier observation that the negative FECR value could have been a result of concentration in faeces due to the reduced feed intake other than increased fecundity by the GI nematodes. The differences in activity of various plant remedies on different nematode species can be explained by the possible variations in the active phytochemicals in different plants species [20].

Furthermore, other issues like bioavailability of the active compounds at different parts of the gastrointestinal tract (GIT), the parasite specificity and the host-plant interactions could explain why some plants are more active against specific parasite species and not others [25]. This might be related to the parasite predilection site or the bioavailability of the compound in the different parts of the GIT of the parasitized host [6]. Condensed tannins, for example, have been found to form complexes with macromolecules, such as proteins [26]. Due to physiological conditions in the abomasum, tannins are expected to be in complexes and hence unavailable to exert their anthelmintic activity there but may do so in the intestine, though poorly, when animals are on high protein diets [28].

The TWCR value of only 36% for albendazole further confirms its resistance by the GI nematodes used in this study and especially by *H. contortus* whose reduction by albendazole was only 22%. According to the criterion set by the WAAVP, resistance is declared when TWCR is less than 90% [15]. There has been widespread resistance to anthelmintics by both human and animal pathogenic helminths to an extent where there is multi-drug resistance against all major classes of conventional anthelmintics. In fact, it is now considered a global phenomenon in GI nematodes of farm animals [29, 30]. In Kenya, resistance has mainly been reported in institutional farms which also happen to be the source of breeding stock for other smaller farms with potential danger of spreading this problem [31]. The source of the GI nematode parasites used to infect sheep in this study is an institutional farm just like the ones mentioned by the previous authors.

4. CONCLUSION

The results of this study have clearly shown that some of the plant anthelmintic remedies used in Loitoktok sub County of Kajiado County like *M. africana* have good efficacy at safe levels and could continue to be used with satisfactory outcomes. However, some of them like *A. anthelmintica* may be toxic at the same levels that they could be effective against GI nematodes. The GI nematodes used in this study were resistant to albendazole and that the farm of origin should be advised accordingly. Further studies are necessary to properly evaluate any possible adverse effects and the most optimum dosages, especially against such resistant strains of GI nematodes, for the very efficacious *M. africana*. The actual phytochemicals responsible for this anthelmintic activity and their possible modes of action also need to be further investigated with a view of formulating a novel anthelmintic product.

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