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ANTHELMINTIC EFFICACY OF *NAUCLEA LATIFOLIA* EXTRACT AGAINST GASTROINTESTINAL NEMATODES OF SHEEP: *IN VITRO* AND IN *VIVO STUDIES*

I. O Ademola[1]*, B. O Fagbemi[1] and S. O. Idowu[2]

[1]Department of Veterinary Microbiology and Parasitology University of Ibadan, Ibadan. Nigeria.
[2] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria
E-mail: ioademola@yahoo.com, Tel: 234 8032317802

Abstract

Direct effects of *Nauclea latifoli*a extracts on different gastrointestinal nematodes of sheep is described. *In vivo and in* vitro studies were conducted to determine possible anthelmintic effect of leaf extracts of *Nauclea latifolia* toward different ovine gastro intestinal nematodes. A larval development assay was used to investigate *in vitro,* the effect of aqueous and ethanolic extracts of *N. latifolia* towards strongyles larvae. The development and survival of infective larvae (L_3) was assessed and best-fit LC_{50} values were computed by global model of non-linear regression analysis curve-fitting (95% CI). Twenty sheep harbouring naturally acquired gastrointestinal nematodes were treated with oral administration of ethanolic extracts at a dose rate of 125 mg/kg, 250 mg/kg and 500mg/kg to evaluate therapeutic efficacy, *in vivo.*

The presence of the extracts in the cultures decreased the survival of larvae. The LC_{50} of aqueous and ethanolic extract were 0.704 and 0.650 mg/ml respectively and differ significantly ($P<0.05$, paired *t* test). Faecal egg counts (FEC) on day 12 after treatment showed that the extract is effective, relative to control (1-way ANOVA, Dunnett's multiple comparison test), at 500mg/kg against *Haemonchus spp, Trichostrongylus spp (*$p<0.05$), *Strongyloides spp* ($P < 0.01$); at 250mg/kg against *Trichuris spp* ($P < 0.01$) and ineffective against *Oesophagostomum spp* ($p>0.05$). The effect of doses is extremely significant; the day after treatment is sometimes significant while interaction between dose and day after treatment is insignificant (2-way ANOVA).
N. latifolia extract could therefore find application in the control of helminth in livestock, by the ethnoveterinary medicine approach.

Key words: Anthelmintic activities; gastrointestinal nematodes; *Nauclea latifolia*; sheep.

Introduction

The most serious constraints affecting ruminant production in Nigeria is gastrointestinal nematode parasitism, especially *Haemonchus contortus*. This nematode is a bloodsucker and with heavy infection can cause considerable blood loss leading to eventual death if not controlled. Anthelmintic treatment has been the management choice for controlling nematode infection. Chemicals that can be used to remove endoparasites without undesirable side effects on host animals or man are difficult to discover and develop (Prichard, 1990). In recent years, there has been a resurgence of interest in traditional health care practices in the developed developing world. In animal health, this has led to further interest in ethnoveterinary research and development.

Nauclea latifolia is a plant of the family, Rubiaceae native to savannah forest and fringe tropical forests of West Africa (Irvine, 1961). It is called "African quinine in Northern Nigeria, a cold infusion of the bark is taken as a diuretic and anthelmintic. The Fulanis in Nigeria uses the leaf extract to regularly deworm animals. (Adebowale

1993). The ethanolic extract decreased the level of parasitaemia in a dose-dependent manner in mice experimentally infected with a *Trypanosoma brucei brucei.* (Morah, 1998). Different indolo-quinolizidine alkaloids and glycol-alkaloids have been isolated from the root bark. The former has been identified and named anguistine, angustoline. The glyco alkaloids have been identified as cadambine 3-α-dihydro cadambine (Hottellier *et al*, 1975).

The aim of this study was to investigate the possible direct effects of *N. latifoli*a on different gastrointestinal parasites of sheep.

Materials and Methods

Plant collection and extraction

The leaves of *Nauclea latifolia*, (Family: Rubiaceae) were collected around the University of Ibadan, Ibadan, Nigeria. Voucher specimen (22273) was deposited at the herbarium of the Department of Botany and Microbiology, University of Ibadan, Ibadan, Nigeria. Powered whole plant was extracted with 95% ethanol by continuous Soxhlet extraction. The ethanolic extract was concentrated and dried *in vacuo* at 70^0C.

In vitro assay

Nematode egg recovery technique

The nematode eggs were isolated as previously described by Hubert and Kerboeuf (1992), using 10 -15 g of faeces collected from a sheep habouring naturally acquired strongyles.

The concentration of eggs was estimated in 50 µl samples and adjusted to 200 - 220 eggs/ml. The egg suspension was diluted with filtrate from the first step of egg extraction that had been centrifuged for five minutes at 100 g to eliminate organic debris to provide bacteria for larval development. To avoid the proliferation of fungi 5µg of amphotericin B was added per ml of suspension.

Plant extracts

Aqueous and ethanolic crude extracts of *N. latifolia* were used. A stock solution of the crude extracts was prepared in turn by dissolving 100 mg of the crude plant extracts in 10ml 20% propylene glycol. Dilution of appropriate aliquots of the stock solutions was made to achieve the concentration in the tubes ranging from 0.25-2.0mg/ml. 20% propylene glycol was added to the control tubes.

Nutritive medium

The nutritive medium was prepared as described by Hubert and Kerboeuf (1992) and composed of Earle's balance salt solution plus yeast extract diluted in saline solution (1 gm of yeast extract/90ml of saline solution) in the proportion 1:9 volume to volume.

Larval development assay

The test was carried out in a 5 ml test tube. 150 µl of nutritive medium was added to 500 µl of egg suspension containing approximately 100 eggs.

The tube was covered and put in an incubator at 27^0C for 48 h (1st stage larvae). 350 µl of the plant extract was added. Three replicates per plant extract concentration or water and propylene glycol (control) were made. All the eggs used for Larval development assay (LDA) were collected from the same sheep and the genus determined at the end of the assay.

The third stage larvae were obtained seven days later. At this time the parasite was counted by separating the larvae into two classes, living third stage larvae (L_3) and dead larvae. The genus of the larvae was determined by larval culture

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Determination of the 50 percent lethal concentration (LC_{50})

In larval development test, the LC_{50} was determined from the concentration-response sigmoidal (variable slope) curve, using the global model of non-linear regression analysis curve-fitting. The relation below gives the larval survival rate parameter:

$$\frac{\text{Number of Living } L_3/\text{ Total number of nematode in wells (plant extract).}}{\text{Number of Living } L_3/\text{ Total number of nematode in control tube (water).}}$$

This was plotted against log concentration. The top, bottom and hillslope of the curves were shared, while best-fit values were computed, with 95% confidence interval, for the unshared parameter (LC_{50}) for each of the extracts (aqueous and ethanolic). In addition, the top was constrained as <1.0, while the bottom was constrained as >0.0 as dictated by the context of the experiment. This curve-fitting analysis was performed by GraphPad Prism for Windows, Version 4.01 (GraphPad Software, 2004).

In vivo test
Animals

Twenty, 4-8 months old weaned West African dwarf lambs (8-13 kg) were bought from local sheep and goat markets.
The sheep had naturally acquired mixed parasitic infection of gastrointestinal nematodes. Infections were confirmed before the beginning of study by collecting faecal samples from the animals, by rectum and the number of nematode egg therein determined by the floatation method; only those animals whose egg counts exceeded 750 eggs per gram of faeces (EPG) were used.
After purchase, the animals were washed with 1% coumaphos, (Asuntol 50, Bayer Germany) solution. The animals were vaccinated against Peste des petits ruminant (PPR) vaccine supplied by the National Veterinary Research institute Vom, Nigeria.

The study lambs were housed for two months before study initiation. After treatment they were penned singly by treatment until the end of the study. No physical contact was possible between sheep from different treatment groups. The sheep were kept on wood shaving and fed with fresh clean (without nematode contamination) grass, wheat bran and water *ad-libitum*. Ethical rules regarding the use of animals were observed. Animals were handled without pain and stress

Treatment and follow-up procedures

Prior to treatment, faecal samples were obtained by rectum from each animal, at least three times at interval of three days. On each occasion the number of eggs in the faeces according to genus was determined by larval culture and identification was by morphological characteristics as described by MAFF, 1986.

On day 0, the sheep were blocked by sex and allocated, within sex to four treatment groups according to complete randomized design taking into consideration their live weight and faecal egg count. The level of excretion as well as genus was determined On day 1 of the experiment group B-D were drenched with a suspension of the ethanolic crude extract at 125, 250 and 500 mg/kg body weight respectively while group A served as undrenched control. Treatment was repeated the second day.

Observation of clinical signs and death were undertaken three times daily. The bodyweight of the sheep were recorded weekly. Daily body temperature was recorded from each sheep**.**

Faecal egg counts were performed on each animal on days 0, 3, 6,9,12 after treatment. Individual faecal egg counts were determined according to genus by larval culture to determine the genus of strongyles. Test tube – filter paper technique (Ademola *et al*; 2004) was used for larval culture. Briefly, 400 mg of faeces containing the eggs was weighed, this was smeared evenly and thinly on the upper two third of a prepared rectangular strips of Whatman filter paper with a spatula. The smear strips of filter paper were inserted into a 15ml centrifuge tube containing 2.5 ml distilled water. The unsmeared portion reaches the water at the bottom of the tube. The entire filter paper was thereby constantly moistened through capillarity thus given a humid medium for larval development. The tubes were labelled, covered with aluminum foil (thus reducing evaporation rate), placed in tube rack and incubated at 27^oC for eight days. By this time the parasites have developed to third stage larvae. Two drops of iodine solution

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was then added and mixed .The suspension was then placed in McMaster slide and the larvae identified by the morphological characteristics as described by MAFF, 1986.

Statistical analysis

The LC_{50} values for the aqueous and ethanolic extracts were compared by Students *t* (paired) test. Two-tailed P value less than 0.05 is taken as significant. The mean faecal egg per gram (EPG) on day 12 after treatment was compared between the various doses investigated and for each of the five species of nematodes studied. This was done by 1-way analysis of variance (ANOVA). The relative contribution of dose, day after treatment and any interaction between these two factors, to the overall variation in the data obtained for each species was evaluated by 2-way ANOVA. All the statistical analyses were performed by GraphPad Prism for Windows, Version 4.01 (GraphPad Software, 2004)

Table 1: LC_{50} of *N. latifolia* extracts using global model of non-linear regression curve-fitting.

Extract	**LC_{50}(mg/ml)**		**R^2**
	Best fit value	**95% CI**	
Ethanol	0.650	0.590-0.717	0.9697
Aqueous	0.704	0.634-0.782	0.9442

Constraints:
Top is shared and <1.0, Hillslope is shared, Bottom is shared and >0.0
Best fit value± S.E. (S.E =Standard error):
Bottom = 1.00e-007 ±0.0578, Top = 0.953± 0.058, Hillslope = -2.178 ±0.447
Goodness of fit:
Degrees of freedom = 43, Absolute sum of squares = 0.1359, Sy.x = 0.05622,
R^2 =0.9599

Table 2: Faecal eggs counts per gram (EPG) and percentage reduction in faecal eggs count (*Haemonchus spp*.) for *Nauclea latifolia* treated sheep compared with untreated control

	Group A (=5) control		Group B (n=5) 125 mg/kg		Group C (n=5) 250 mg/kg		Group C (n=5) 500 mg/kg.	
Day	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction
0	3.8± 1.2	-	3.9± 1.0	-	3.8± 0.7	-	3.8± 1.2	-
3	3.9± 0.9	-	3.9± 1.2	-	2.7± 0.9	28.0	1.4± 1.1	60.0
6	3.9± 0.7	-	3.8± 1.8	-	2.7± 1.0	29.5	1.5± 0.5	60.1
9	3.9± 0.5	-	3.9± 0.7	-	2.6± 1.10	30.0	1.5± 0.1	60.0
12	3.9± 0.8	-	3.8± 2.0	-	2.6± 1.10	30.0	1.5± 0.2	60.0

Egg count values are expressed as mean ± standard deviation % reduction are expressed as means.

Table 3: Faecal eggs counts per gram (EPG) and percentage reduction in faecal eggs count (*Trichostrongylus spp)* for *Nauclea latifolia* treated sheep compared with untreated control

	Group A (=5) control		Group B (n=5) 125 mg/kg		Group C (n=5) 250 mg/kg		Group C (n=5) 500 mg/kg.	
Day	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction
0	2.0± 0.6	-	2.0± 0.8	-	2.0± 0.5	-	2.0± 0.9	-
3	2.1± 0.7	-	1.9± 1.2	-	1.1± 0.8	51.5	0.7± 0.5	63.5
6	2.2± 0.8	-	2.0± 0.5	-	1.0± 1.0	54.0	0.7± 0.1	65.0
9	2.1± 1.0	-	2.0± 0.9	-	1.0± 0.5	54.0	0.7± 0.4	65.5
12	2.1± 1.0	-	2.0± 0.7	-	1.0± 0.7	54.0	0.7± 0.3	65.7

Egg count values are expressed as mean ± standard deviation % reduction are expressed as means.

Table 4: Faecal eggs counts per gram (EPG) and percentage reduction in faecal eggs count (*Oesophagostomum spp*.) for *Nauclea latifolia* treated sheep compared with untreated control.

	Group A (=5) control		Group B (n=5) 125mg/kg		Group C (n=5) 250mg/kg		Group C (n=5) 500mg/kg.	
Day	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction
0	1.2± 0.5	-	1.2± 0.9	-	1.2± 0.7	-	1.2± 0.6	-
3	1.3± 0.1	-	1.1± 0.5	5.5	0.8± 0.5	35.0	0.4± 0.2	64.0
6	1.2± 0.7	-	1.1± 0.7	6.3	0.7± 0.6	36.0	0.4± 0.1	64.5
9	1.2± 1.0	-	1.0± 0.9	6.5	0.8± 0.1	36.4	0.4± 0.1	64.5
12	1.2± 1.9	-	1.1± 0.6	6.3	0.8± 0.2	36.3	0.4± 0.1	64.6

Egg count values are expressed as mean ± standard deviation. % reduction are expressed as means.

Table 5: Faecal eggs counts per gram (EPG) and percentage reduction in faecal eggs count (*Strongyloides spp.)* for *Nauclea latifolia* treated sheep compared with untreated control.

	Group A(=5) control		Group B (n=5) 125mg/kg		Group C (n=5) 250mg/kg		Group C (n=5) 500mg/kg.	
Day	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction
0	1.0± 0.3	-	1.0± 0.6	-	1.1± 0.5	-	1.0± 0.3	-
3	1.0±	-	1.0± 0.2	-	0.6± 0.3	62.5	-	100.0
6	1.0± 0.5	-	0.8± 0.5	21.4	0.6± 0.2	64.5	-	100.0
9	1.0± 0.2	-	0.8± 0.5	23.1	0.6± 0.1	64.5	-	100.0
12	1.0± 0.5	-	0.8± 0.4	23.5	0.6± 0.4	64.4	-	100.0

Egg count values are expressed as mean ± standard deviation. % reduction are expressed as means.

Table 6: **Faecal eggs counts per gram (EPG) and percentage reduction in faecal eggs count (*Trichuris spp.)* for *Nauclea latifolia* treated sheep compared with untreated control.**

	Group A (n=5) Control		Group B (n=5) 125mg/kg		Group C (n=5) 250mg/kg		Group C (n=5) 500mg/kg.	
Day	Mean count x 10^3	% Reduction	Meancount x 10^3	% Reduction	Mean count x 10^3	% Reduction	Mean count x 10^3	% Reduction
0	0.6± 0.5	-	0.6± 0.3	-	0.6± 0.5	-	0.7± 0.3	-
3	0.6± 0.2	-	0.4± 0.3	14.5	0.2± 0.2	55.7	-	100.0
6	0.6± 0.1	-	0.4± 0.1	16.1	0.2± 0.1	66.0	-	100.0
9	0.6± 0.1	-	0.4± 0.1	16.0	0.2± 0.2	60.1	-	100.0
12	0.6± 0.1	-	0.4± 0.2	16.0	0.2± 0.1	60.0	-	100.0

Egg count values are expressed as mean ± standard deviation % Reduction are expressed as means.

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Table 7: Dunnett's multiple comparison test comparing the effect of various doses of *N. latifolia* extract with the control, showing the relative sensitivity of different species of gastrointestinal sheep nematodes**.**

Comparisons	P value
Haemonchus contortus	
Control vs 125mg/kg	P>0.05
Control vs 250mg/kg	P>0.05
Control vs 500mg/kg	P<0.05
Trichostrongylus spp	
Control vs 125mg/kg	P>0.05
Control vs 250mg/kg	P>0.05
Control vs 500mg/kg	P<0.05
Oesophagostomum	
Control vs 125mg/kg	P>0.05
Control vs 250mg/kg	P>0.05
Control vs 500mg/kg	P>0.05
Strongyloides spp	
Control vs 125mg/kg	P>0.05
Control vs 250mg/kg	P>0.05
Control vs 500mg/kg	P<0.01
Trichuris spp	
Control vs 125mg/kg	P>0.05
Control vs 250mg/kg	P<0.01
Control vs 500mg/kg	P<0.01

Table 8: Two-way ANOVA showing the relative contribution to variation in faecal egg count (FEC) by treatment (doses), day (after treatment) and interaction between the two factors.

Source of variation	% of Total variation	P value	P value summary*
Haemonchus spp			
Interaction	8.66	0.3030	NS
Dose	36.28	P<0.0001	ES
Day	6.62	0.0345	S
Trichostrongylus spp			
Interaction	7.63	0.5474	NS
Dose	30.01	P<0.0001	ES
Day	6.03	0.0833	NS
Oesophagostomum spp			
Interaction	4.48	0.9574	NS
Dose	17.09	0.0008	ES
Day	4.80	0.2754	NS
Strongyloides spp			
Interaction	10.54	0.0558	NS
Dose	39.42	P<0.0001	ES
Day	11.81	0.0002	ES
Trichuris spp			
Interaction	11.00	0.0650	NS
Dose	30.51	P<0.0001	ES
Day	17.37	P<0.0001	ES

- NS =Not significant, S= Significant, ES= Extremely significant

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Results

Larval development assays.

There was no evidence that *N. latifolia* extract affects larval development following the incubation period, however, the extract killed the infective stage larvae (L_3) in a concentration-dependent manner. The calculated LC_{50} of aqueous extract of *N. latifolia* was 0.704mg/ml while the ethanolic extract was 0.650mg/ml with a statistically significant difference ($P<0.05$).

In vivo study

There was no visible clinical sign in all the animals treated with the ethanolic extract of the plant. The nematode eggs recovered during the study were identified as*, Haemonchus sp., Trichostrongylus sp., Oesophagostomum sp., Trichuris sp and Strongyloides sp. N. latifolia* extract administered orally reduced the faecal egg counts (FEC) (Table 2-6). Dunnet's multiple comparison post-test reveals the relative sensitivity of different species and level of significance of the effect of different treatments (doses) relative to the control (Table 7). Efficacy is at 500mg/kg against *Haemonchus spp, Trichostrongylus spp (*$P < 0.05$), *Strongyloides spp* ($P < 0.01$); at 250mg/kg against *Trichuris spp* ($P < 0.01$) and ineffective against *Oesophagostomum spp* ($P > 0.05$). The relative contribution of doses, day after treatment and any interaction between the two factors to the overall variation in the data was evaluated by 2-way ANOVA (Table 8). The effect of doses is extremely significant; the day after treatment is sometimes significant while interaction between dose and day after treatment is insignificant.

Discussion

N. latifolia extracts were shown by larval development assay to exhibit anthelmintic activity. Although the *in-vitro* assay provided no evidence that *N. latifolia* extract affected larval development, it killed the infective larvae in a concentration-dependent manner. Aqueous extract demonstrated a lower activity (0.704 mg/ml) compared with the ethanolic extract (0.650mg/ml). The ethanolic extract was more active than the aqueous extract ($P < 0.05$, Table 1). The activity of the aqueous extract is comparable with the activity shown by aqueous extract of *Khaya senegalensis* against infective larvae of sheep nematodes, with LC_{50} of 0.69 mg/mL as recently reported (Ademola et al*;* 2004).

The *in-vivo* data shows the relative sensitivities of the different species of sheep nematodes to the extract at the different doses investigated. The effect of the doses relative to the control was evaluated on day 12 after treatment, at the 5% probability level o f significance (Tables 2-7). Data analysis by 2-way ANOVA reveals the overall pattern of the results, when interpreted with respect to dose, day after treatment and possible interaction (Table 8). Interaction between the factors is insignificant for all the nematode species. The dose is a highly significant factor for the efficacy of the extract against all the nematodes, while day after treatment is also highly significant for *Strongyloides spp. and Trichuris spp.* The day after treatment is also a significant factor for *Haemonchus spp* but an insignificant factor for *Trichostrongylys spp* and *Oesophagostomum spp.*

The extract of *N. latifolia* has shown a broad spectrum of action against sheep nematodes that could become significant in anthelmintic therapy in livestock. This assertion is corrroborated by the fact that the problem of anthelmintic resistance by nematodes and increasing concern over the presence of drug residues in animal products, when pure compounds are administered, has led to a resurgence of interest in the use of phytomedicines, in form of extracts containing mixture of compounds (Athanasiadou, 2001).

Further researches are ongoing in our laboratories on the rational use and possible application of the extract in anthelmintic therapy in livestock, by the ethnoveterinary medicine approach.

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