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Ovicidal and Larvicidal Effects of Crude Ethanolic Extracts of *Acanthus Montanus* (Acanthaceae) on *Heligmosomoides Bakeri* (Nematoda, Heligmosomatidae)

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ABSTRACT

Objective: An in vitro study was carried out to determine the ovicidal and larvicidal activity of crude ethanolic extracts of Acanthus montanus (Acanthaceae) leaves on the eggs and the third larval (L3) stage of Heligmosomoides bakeri.

Methods: Four different concentrations of the extracts (by serial dilution from 1,000 mg/mL to 125 mg/mL) were compared with albendazole as the positive control (serially diluted from the recommended dose of 15 mg/mL to 1.875mg/mL) and distilled water serving as the negative control respectively, in the bioassay. The extract activity was dose-dependent.

Results: At 250 mg/mL, there was 100% inhibition of egg hatch comparable to albendazole at 7.5 mg/mL. There was zero percent inhibition of eggs in the distilled water control culture. The activity of the extracts on the L3 larvae shows that at the concentration of 125 mg/mL, the larvicidal effect was 100%, one-hour post treatment compared to albendazole at 15 mg/mL (97.51%). However, the negative control showed an increase in Larvicidal activity from 22.5% to 40% in one hour and fifteen hours respectively.

Conclusion: It was therefore, concluded that crude ethanolic extracts of Acanthus montanus leaf has significant ovicidal and larvicidal properties against Heligmosomoides bakeri egg and third larval stage.

KEYWORDS: *Acanthus montanus; extract; Heligmosomoides bakeri; larvicidal, ovicidal*

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Introduction

Parasitic diseases have been a significant constraint to livestock productivity across all agro-ecological zones and production systems in Africa, and gastrointestinal (GI) nematodes remain a major challenge of economic importance in domesticated livestock throughout the world (Prichard, 1994). Helminthosis, especially parasitic gastroenteritis (PGE) constitutes a serious health problem to the productivity of small ruminants in Nigeria. Parasitic gastroenteritis of small ruminants is a complex of diseases attributed to many nematode parasites especially species of the genera *Haemonchus, Trichostrongylus, Oesophagostomum* and *Strongyloides* (Soulsby, 1982; Chiejina, 1986; Nwosu *et al.,* 1996). The disease is associated with enormous economic losses due to the associated morbidity, mortality and cost of treatment, and other control measures (Schillhorn van Veen, 1973; Akerejola *et al.,* 1979; Chiejina, 1987).

Even though modern synthetic medicines are effective in reducing disease burdens, they are also associated with a number of side effects. Crude drugs though less effective, are relatively free from side effects (Mali and Mehta, 2008). A large number of medicinal plants are claimed to possess anthelmintic properties in traditional systems of medicine and are also utilized by ethnic groups worldwide. Following the folk claims, several medicinal plants have been scrutinized for this activity using various *in vitro* and *in vivo* methods (Mali and Mehta, 2007 a,b). *Acanthus montanus* commonly called *"mountain thistle"* has been employed in folk medicine by the Igede people of Benue State, Nigeria for treatment of different kinds of ailments (Igoli *et al.,* 2005), and positive results seem to be associated with the folkloric use of the leaves of this plant by the Etulo natives in Benue State, Nigeria, in the treatment and control of gastrointestinal worms in children and adults (Agishi, 2004). Adamu *et al.* (2010) reported that *A. montanus* might have anthelmintic property. Although several studies (Agishi, 2004; Adamu *et al.,* 2010)have been conducted on the

anthelmintic properties of *A. montanus* leaf extract, no study has established the ovicidal and larvicidal properties of ethanolic extract of this plant on the eggs and the third larval stage of *H. bakeri.* In the present study, *in vitro* assay was performed to evaluate the ovicidal and larvicidal properties of crude ethanolic extracts of the leaves of *A. montanus* respectively on the eggs and larvae of *Heligmosomoides bakeri*, the trichostrongyloid model of the laboratory mouse, with the view to determining the minimum concentration that has significant anthelmintic activity.

Materials and Methods

Plant material collection and identification

Fresh leaves of *A. montanus* with stalks were collected/ harvested in the months of March and April along a stream in northern part of Katsina-Ala township of Katsina-Ala Local Government Area of Benue State. Katsina-Ala town is located on latitude 7º 10' N and longitude 9º 19' E in the middle belt (Guinea Savannah) of Nigeria. A sample of the plant was brought to Zaria and was identified/ authenticated by a plant taxonomist at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, where a voucher specimen was deposited and assigned a voucher number 7037.

Preparation and preservation of extracts

The harvested plant materials were air-dried at room temperature until a constant weight was obtained, then pounded in a wooden mortar with pestle. The pulverised product weighing about 6 kg was stored in an air-tight nylon bag under cool, dark conditions at room temperature until use.

During extraction, 1 kg (250 g per separating funnel) of the pulverized product was soaked in 95% ethanol (EtOH) in the ratio 1:6 *w/v* in four separating funnels (pre-plugged with cotton wool) for 72 hours. The extracted solution collected in excess of the solvent was transferred in evaporating dishes and concentrated to dryness over water baths at 60ºC. The dried extract obtained after concentration was then weighed to determine the percentage yield and stored at 4ºC until used.

Preparation of stock solutions and serial dilutions of test substances

Crude ethanolic extracts (CEE) of leaves of *A. montanus* weighing 10 g was dissolved 10 mL of distilled water to obtain a 1,000 mg/mL stock solution. Serial dilutions of this stock was done to obtain 10 mL each of 500 mg/mL, 250 mg/mL, 125 mg/mL, 62.5 mg/mL, 31.25 mg/mL, 15.625 mg/mL and 7.8125 mg/mL concentrations of CEE. Similarly, one bolus of Albendazole (Albidol®), 150 mg was crushed with mortar and pestle and dissolved in 10 mL of distilled water (DW) to obtain 15 mg/mL concentration stock solution. This stock was equally serially diluted to obtain 10 mL each of 7.5 mg/mL, 3.75 mg/mL and 1.875 mg/mL concentrations of Albendazole (ABZ).

Faecal sample collection, coproculture and recovery of infective L_3 of *Heligmosomoides bakeri*

Faecal pellets were collected from infected laboratory mice, *Mus musculus* a trichostrongyloid nematode, *Heligmosomoides bakeri* whose life cycle is 14 days. The modified method of Burren (1980) was used for coproculture to obtain clean infective larvae used for the *in vitro* anthelmintic trials.

Egg hatch inhibition assay

Infected mice *(Mus musculus)* were placed in clean well fenestrated plastic cages for 12 hours to shed faecal pellets. The mice were provided drinking water *ad libitum* but no feed was prvided in the cages. Faecal pellets were scrapped using a small spatula into 50 mL centrifuge tubes and mashed into smooth paste with applicator stick. The tubes were then filled with distilled water and spun at 250 rpm (11 g) for two minutes. After decanting the supernatant, the re-suspended slurry was filtered into other 50 mL tubes through a double layer of gauze and centrifuged as described previously. The sediments were plated in Petri dishes (90 x 15 mm) that were lined with moistened Whatman 40 filter paper. The cultures were moistened with distilled water and the various concentrations of Albendazole (Albidol®) and CEE whenever the cultures were getting dried during the seven-day incubation period. After 7 days, the culture was flooded with distilled water and the Whatman filter paper was gently lifted to recover the infective (L_3) larvae. This was placed into a clean 30 mL tube and allowed to stand for 30 minutes to concentrate the larvae to the bottom. The supernatant was carefully pipetted out. Larval count was done at x10 objective of light microscope by pipetting several aliquots spread on clean microscope slides until the tubes were empty. Where necessary, drops of distilled water was added to reduce opacity of the slides. Ovicidal effect was measured in terms of percentage Egg Hatch Inhibition (%EHI) and was calculated using the formula:

$$\%EHI = \frac{N-n}{N} \times 100$$

Where: N = number larvae in Distilled water culture

n = number larvae in Test substance (Jegede *et al.,* 2009).

Percentage Egg hatch inhibition of 70 and above was considered significant ($p<0.05$) in this bioassay (Enejoh *et al.,* 2014).

Larval inhibition test

Faecal pellets were cultured as described previously in distilled water and harvested on the 7th day into 30 mL test tube and allowed to sediment under standard laboratory conditions. Aliquots (0.5 mL) of the sediment was placed on microscope slides and were repeatedly counted and the mean count ascertained. For this test, 0.1 mL of fluid containing 40 L_3 *H. bakeri* were pipetted into 13 clean micro-titre wells. To each corresponding well, 0.1 mL of distilled water and the test substances previously prepared were placed and monitored after one hour and then 15 hours and observations recorded. A larva was considered dead if there was no movement within three seconds at x10 objective of the light microscope. Percentage larvicidal effect (%LE) of test substances was calculated using the formula:

$$LE\ (\%) = \frac{Number\ of\ larvae\ dead}{Total\ number\ counted} \times 100$$

Larvicidal effect of 70% and above was considered significant at $p<0.05$ (Enejoh *et al.,* 2014).

Data analysis

The egg hatch and L_3 larval counts data generated were summarized to Tables and expressed in percentages

(percentage Egg Hatch Inhibition and Larvicidal Effect). One-way analysis of variance (ANOVA), Tukey's Multiple Comparison Post Test and Chi-Square (Graphpad Prism 6) were used for data analysis. The difference between extract-treated and the control cultures was considered significant at $p<0.05$.

Results

Egg hatch inhibition

The percentage egg hatch inhibition according to the various concentrations of CEE of *A. montanus,* ABZ and DW is as shown in Table 1. Concentrations of 500 and 1000 mg/mL of CEE was observed to induce 100% egg hatch inhibition. Similar rates of egg hatch inhibition were induced by 7.5 and 15 mg/mL ABZ. There was zero percent inhibition of eggs in the DW control culture.

Table 1: Percentage of *Heligmosomoides bakeri* egg hatch in various concentrations of Crude Ethanolic Leaf Extracts of *Acanthus montanus*, Albendazole and Distilled water.

Substance	No. larvae hatched	% Egg hatch inhibition
DW (control)	143	0
ABZ (Albidol®)		
15 mg/Ml	0	100.0
7.5 mg/Ml	0	100.0
3.75 mg/Ml	2	98.60
1.875 mg/Ml	4	77.20
CEE		
1000 mg/Ml	0	100.0
500 mg/Ml	0	100.0
250 mg/Ml	8	94.41
125 mg/Ml	16	88.81

Statistically, there was no significant difference ($p>0.05$) between extract-treated groups, but a significant difference ($p<0.05$) in egg hatch inhibition between extract treated groups and the Distilled water culture. But using Kappa statistic, this research was able to report that there was a fair agreement in the percentage of egg hatch inhibition by the various concentrations of Crude Ethanolic Leaf Extracts of *Acanthus montanus* and Albendazole, which is statistically significant ($p<0.05$), as shown in Table 2.

Table 2: Measure of Agreement in the Egg Hatch Inhibition of *Heligmosomoides bakeri* eggs by various concentrations of Crude Ethanolic Leaf Extracts of *Acanthus montanus* and Albendazole

Symmetric Measures

	Value	Asymp. Std. Error[a]	Appox. T[b]	Appox. Sig.
Measure of Agreement Kappa N of Valid Cases	.3334	.111	2.000	.046

a. Not assuming the null hypothesis.
b. Using the asymptotic standard error assuming the null hypothesis.

Larval inhibition

Following one hour of exposing the culture to the test substances, ABZ at 15 mg/mL was observed to induce 87.5% LE and a concentration of 1.875 mg/mL observed to induce 72.5% LE, while extract concentrations within the range of 125 to 1000 mg/mL were discovered to induce 100% LE in comparison to 22.5% LE obtained in DW control wells. However, at 15-hour post exposure of the culture to the test substances, the extract at all concentrations were found to induce 100% LE while ABZ produced 92.5% LE at a concentration of 15 mg/mL and 77.5% at a concentration of 1.875% respectively. All concentrations of extracts induced 100% mortality of larvae (Table 2).

Table 3. Percentage inhibition of *Heligmosomoides bakeri* larvae in various concentrations of Crude Ethanolic Leaf Extracts of *Acanthus montanus*, Albendazole and Distilled water after one hour and 15 hours treatment.

Substance	After 1 hour			After 15 hours		
	Motile	Dead	LE (%)	Motile	Dead	LE (%)
DW (control)	31	9	22.5	24	16	40
ABZ (Albidol®)						
15 mg/mL	5	35	87.5	3	37	92.5
7.5 mg/mL	7	33	82.5	5	35	87.5
3.75 mg/mL	10	30	75	7	33	82.5
1.875 mg/mL	11	29	72.5	9	31	77.5
CEE						
1000 mg/mL	0	40	100	0	40	100
500 mg/mL	0	40	100	0	40	100
250 mg/mL	0	40	100	0	40	100
125 mg/mL	0	40	100	0	40	100

Though there is a significant difference ($p<0.05$) in egg larvicidal activity between extract treated groups, anthelminth treated group to the Distilled water culture, there is no agreement between the percentage of larvicidal effect by the various concentrations of Crude Ethanolic Leaf Extracts of *Acanthus montanus* and Albendazole ($p>0.05$) as shown in table 4.

Table 4: Measure of Agreement in the Larvicidal activity on *Heligmosomoides bakeri* larvae by various concentrations of Crude Ethanolic Leaf Extracts of *Acanthus montanus* and Albendazole.

Symmetric Measures

		Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Interval by Interval	Pearson's R	.984	.011	7.937	.016[c]
Ordinal by Ordinal	Spearman Correlation	1.000	.000[c]		
Measure of Agreement	Kappa	-.143	.115	-.894	.371
N of Valid Cases		4			

a. Not assuming the null hypothesis.
b. Using the asymptotic standard error assuming the null hypothesis.
c. Based on normal approximation.

Discussion

Generally, the extracts of *A. montanus* was observed to have significant effect onembryonic development and hatching rate of *H. bakeri.* Similar findings were demonstrated by Pone *et al.* (2010) with the extracts of *Canthium mannii.* The ovicidal activities of the extracts of *A. montanus* observed in this study could be due to the fact that the active compounds penetrated the egg shell and stopped the segmentation of blastomeres or paralysed the larvae inside embryonated egg as opined in an earlier study (Pone *et al.,* 2011). Adamu *et al.*

(2010) demonstrated same with aqueous extract of *A. montanus.* Phytochemicals and other secondary metabolites such as glycosides, steroides, sterols, alkaloides, flavonoids, triterpenes and polyphenols (Githiori *et al.,* 2006) may be responsible for these actions. Extracts of *A. montanus* likewise showed larvicidal activity. This profound larvicidal activity of the crude ethanolic leaf extract could be attributed to the aforementioned secondary metabolites which may create unfavorable conditions to the survival of the larvae. Polyphenols, for instance are uncouplers of oxidative phosphorylation in helminth parasites (Bate-Smith, 1962), thereby interfering with their energy generation. Another possible anthelmintic effect of these compound is that they can bind to lycoprotein on the cuticle of the parasite (Thompson and Geary, 1995), and may thus cause death. This may explain why the plant's (*A. montanus*) aerial parts are used as vermifuge by the Etulo natives of Benue State, Nigeria.

Conclusion

From the above results, it was concluded that crude ethanolic extracts of *A. montanus* have potent anthelmintic activity against eggs and third larval stage of *H. bakeri*. Further experiments incorporating *in vivo* model studies are required to establish the effectiveness and pharmacological rationale for the use of *A. montanus* extracts as anthelmintic potential. Further studies to isolate other active constituent(s) from extracts to establish mechanism of action are also required.

Conflict of interest

The authors declare that they do not have conflict of interest.

Author's Contributions

D.O. developed the theoretical concept, G.A.I and J.O. developed and structured study design and result interpretation while P.N. and M.S. developed the methodological framework. A.W. and M. worked on the data and statistical analysis and D.O. and G.A.I. authors contributed to the final version of the manuscript. J.O. supervised the project.

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