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Effects of leaf extracts of *Acanthus montanus* (Acanthaceae) on faecal egg output of *Heligmosomoides bakeri* (Nematoda, Heligmosomatidae) in mice

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Running title: Effects of *Acanthus montanus* on faecal egg output in mice

Abstract

Acanthus montanus Nees T. Anderson (Acanthaceae) is a well-established remedy in folk medicine, effectively treating various ailments, particularly gastrointestinal helminth infections. This study involved seventy (70) worm-free mice intentionally infected with the third larval stage (L_3) of the *Heligmosomoides bakeri* parasite. To assess the effectiveness of treatments, mice were divided into fourteen groups of five, fifteen days post-infection, after the crucial pre-patent period of 13.5 days. Each group received an oral treatment of crude ethanolic extract (CEE), aqueous extract (AQ), or *n*-butanol (BUT) from *A. montanus* leaves. The extracts were given at increasing doses of 1,200 mg/kg, 1,400 mg/kg, 1,700 mg/kg, and 2,000 mg/kg over five days for twelve groups, thereby allowing for a thorough evaluation of their therapeutic potential. Albendazole (ABZ) acted as the positive control, while distilled water (DW) acted as the negative control. Faecal pellets were collected and analysed to evaluate deparasitization rates based on faecal egg output. In all the groups of mice experimentally infected with *H. bakeri* and treated with *A. montanus* extracts, there was a dose-dependent reduction in the daily and mean faecal egg output. The BUT portion caused significant deparasitization at 1,400 mg/kg to 2,000 mg/kg compared to figures from distilled water-treated (DW) control and those from AQ or CEE portions. The deparasitization rates in BUT-treated mice ranged from 69.24±10.35% to 73.63±10.31%, showing no significant difference ($p>0.05$) compared to the 88.91±10.45% rate achieved with albendazole at 10 mg/kg. This study demonstrates that the *n*-butanol extract of *A. montanus* leaves significantly reduces faecal egg output of *H. bakeri* in mice, warranting further investigation into its potential applications for treating infections in livestock and humans.

Key words: *Acanthus montanus* extracts, *Heligmosomoides bakeri,* faecal egg output, deparasitization, anthelmintic, gastrointestinal helminth.

Introduction

Livestock is an essential and frequently underestimated component of livelihood strategies in societies across the globe (Busch, 2023). Approximately 70% of the rural poor rely on livestock to varying degrees. These livestock include cattle, goats, sheep, pigs, poultry, horses, camels, yaks, and llamas. It is estimated that 600 million poor people, including 150 million landless individuals, own livestock (Erdaw, 2023; Collishaw *et al.,* 2023). For many, livestock ownership represents the most critical form of savings available. This is particularly true for pastoralists and impoverished women, who often rely on livestock as their most valuable tangible asset. Livestock are essential for providing a robust safety net during emergencies and significantly reducing vulnerability to financial shocks caused by health crises, crop failures, and other unforeseen risks. (Collishaw *et al.,* 2023). They provide direct benefits such as food, wool, or hides, and increase farm productivity by supplying manure and draught power (Banda and Tanganyika, 2021).

Despite the significant benefits of livestock, the industry still faces numerous challenges that lead to reduced production and economic losses. One major issue is parasitic diseases, particularly helminthiasis, which have been reported to hinder livestock production. This results in a substantial decrease in yields, an increase in cost of production, and ultimately, significant losses of affected animals (Al Amin and Wadhwa, 2023). Managing intestinal nematodes in livestock mainly depends on systemic synthetic anthelmintics. However, the rising reports of resistance to these treatments underscore an urgent need for new solutions (Fissiha and Kinde, 2021; Singh *et al.,* 2023), making helminth control difficult and challenging even with the seemingly potent drugs. Moreover, residues in animal products like meat, milk, and eggs are dangerous to public health (Ghimpețeanu *et al.,* 2022). In many developing countries, smallholder farmers and pastoralists face significant challenges regarding the availability and affordability of systemic medications, which put animal health at risk. This study aimed to assess the anthelmintic potential of *Acanthus montanus* to address helminth-related concerns. This plant may offer a sustainable and environmentally friendly alternative for controlling helminth infections (Woods, 2021).

The plant *A. montanus* (Nees) T. Anderson, belonging to the Acanthaceae family, is a vigorous perennial shrub characterized by its thinly branched growth habit. It serves as a ground cover and features clusters of oblong to lance-shaped, glossy dark green leaves, which can grow up to 12 inches (30 cm) long (Oshadu, 2014; Oshadu *et al.,* 2022). *A. montanus,* known as "Idumngbe" among the Etulo people of Benue State, is widely found in Nigeria. The traditional use of its leaves by the Etulo natives has demonstrated effective results in the prevention and control of gastrointestinal worms in both children and adults (Oshadu, 2014; Oshadu *et al.,* 2021a, b). While some research has been conducted on the anthelmintic properties of *A. montanus* (Oshadu, 2014; Oshadu *et al.,* 2021a, b), the effects of graded doses of its various fractions (extracts) have not yet been demonstrated *in vivo.* Specifically, there has been no assessment of its impact on faecal egg output in animals and humans. This study decisively investigates the *in vivo* anthelmintic effects of leaf extracts from *A. montanus* on mice infected with *Heligmosomoides bakeri,* a renowned laboratory model for

trichostrongyloid nematodes. Our main objective is to identify the specific fractions that demonstrate significant anthelmintic activity, highlighting the potential of *A. montanus* as an effective agent in the fight against parasitic infections.

Materials and methods

Research design

Fresh leaves of *A. montanus* were collected, air-dried, and subjected to solvent partitioning. Graded doses of the extracts were administered to mice that had been experimentally infected with *H. bakeri* for five consecutive days. Faecal samples from the mice were then analyzed, and the number of eggs per gram of faeces (EPG) was used to determine the rate of deparasitization.

Collection and identification of plant materials

The extensive literature on ethnomedicine and folkloric claims provides compelling evidence for selecting specific plant materials for this study. In March and April, we meticulously collected fresh leaves and stalks of *A. montanus* from a pristine stream in the northern region of Katsina-Ala township, situated within the Katsina-Ala Local Government Area of Benue State, Nigeria, at latitude 7º 10'N and longitude 9º 19'E. This sample was then transported to Zaria, where it underwent rigorous identification and authentication by a skilled plant taxonomist at the Herbarium of Ahmadu Bello University. To further substantiate our findings, a voucher specimen was deposited and assigned the identifier number 7037 (Oshadu, 2014), reinforcing the credibility of our research.

Plant material extraction and preservation

The harvested plant material was air-dried at room temperature for fourteen days until it reached a consistent weight. After drying, it was ground in a wooden mortar to a fine powder and stored in an air-tight nylon bag in a cool, dark place, ready for use (Oshadu, 2014). The method described by Oshadu (2014) and Oshadu *et al.* (2021a, b) was used for extraction and solvent partitioning, yielding Crude Ethanolic Extracts (CEE), *n*-butanol (BUT), and Aqueous (AQ) fractions. In brief, 1 kg (250 g per separating funnel) of the powdered material soaked in 95% ethanol in the ratio 1:6 *w/v* for 72 hours yielded CEE, of which 100 g of the product was suspended in 17.65% methanol and partitioned with an equal volume of chloroform to produce chloroform and aqueous portions. Lastly, the aqueous portion was further partitioned with *n*-butanol in equal volume to obtain the final BUT and AQ fractions. These fractions concentrated to dryness over a water bath were packed in clean, air-tight glass bottles and stored at room temperature in the laboratory hood until used. The chloroform portion was not used in this study. This is because of its extremely low yield.

Experimental animals used in the evaluation of the anthelmintic efficacy of extracts

Healthy albino mice *(Mus musculus)* were used in this study. Ninety, 5-10 weeks of these animals of both sexes, weighing between 15 and 25 g, were acquired and maintained in the Animal House of the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria. They were housed in well-ventilated plastic cages lined with chopped wood shavings under standard conditions for two weeks (Oshadu, 2014; Oshadu *et al.,* 2021b). Within this period, faecal pellets obtained *per rectum* were analysed for helminth eggs and possible protozoan oocysts. The mice were then dewormed with a broad-spectrum anthelmintic (albendazole at 10 mg/kg), preparatory for the experiment.

Preparation of cultures and experimental infection of mice with the infective stage of *H. bakeri*

Faecal samples previously obtained from infected mice with *H. bakeri* were collected and cultured to recover infective larvae (L_3). These were orally inoculated into the experimental animals (Oshadu, 2014; Johnston *et al.,* 2015; Russell *et al.,* 2021). The method described by Oshadu (2014) and Oshadu *et al.* (2021b) was used to count and experimentally infect worm-free mice used in this study with 150 L_3 of *H. bakeri.* Briefly, aliquots of concentrated larval suspension recovered from faecal culture were drawn, and the L_3 repeatedly counted under x4 of the compound microscope and volume adjusted. About 150 L_3 found in 0.1 mL were collected and inoculated directly into the oesophagus of each well-restrained mouse.

Experimental groupings

On the 15th day post-infection (PI), 70 mice positive for eggs of *H. bakeri* were randomly assigned to 14 experimental groups, each containing five mice. Twelve (12) of the groups were treated with increasing doses of CEE, BUT, and AQ fractions of *A. montanus* leaf extracts chosen at a common logarithmic interval of 0.08 (Oshadu, 2014; Oshadu *et al.,* 2021b), administered to mice *per os* for five consecutive days, i.e., on the 18th-22nd day PI (Lai, 2006). Albendazole (ABZ at 10 mg/kg *stat*) served as the positive group while Distilled water (DW at 5 mL/kg *ad lib.*), the negative control group.

Faecal analysis

During the experiment, faecal egg count (FEC) monitoring was observed daily to evaluate the *in vivo* anthelmintic efficacy of the leaf extracts. The modified McMaster technique, as described by Ghafar *et al.* (2021), was used to determine egg per gram of faeces (EPG). In brief, 1 g of mouse faecal pellets was suspended in 14 mL of saturated sodium chloride/ sucrose solution of specific gravity 1.28 and centrifuged at 250 rpm (11 *g*) for 10 minutes. The well-mixed aliquot of the decanted supernatant was charged into both chambers of the McMaster slide, and eggs were counted under a 10X objective. The EPG was therefore calculated by multiplying FEC by 50 (conversion factor).

Evaluation of the anthelmintic efficacy of extracts

At the end of the experiment, the anthelmintic efficacies of the various fractions of the leaf extracts of *A. montanus* were evaluated using the following formula:

$$\%DP = \frac{N-n}{N} \times 100$$ *where:* %DP = percentage deparasitization, N = average EPG in untreated group (DW), and n = average EPG in treated group (extracts).

In this study, a deparasitization rate of ≥70% was considered significant at $p<0.05$ (Oshadu, 2014; Oshadu *et al.,* 2021a, b).

Statistical analysis

The egg per gram of faeces (EPG) obtained in this study was tabulated, and the percentage deparasitization (%DP) calculated. The daily deparasitization graphs were plotted using Microsoft Excel 2013. Data generated were analysed using one-way analysis of variance (ANOVA) and checked with Tukey's Multiple Comparison Post Test (Graphpad Instat®). The results were expressed as Mean ± SEM, and the differences between the mean %DP of treated

and untreated control groups were considered significant at $p<0.05$.

Results

Generally, a decrease in faecal egg output/ egg per gram of faeces (EPG) was seen to be significantly ($p<0.05$) lower in *n*-butanol-treated (BUT) mice compared to other extract-treated groups with resultant increase in percentage deparasitization (Figure I-IV). This is followed by crude ethanolic extract (CEE) and aqueous (AQ) portion. By the end of the experiment, *n*-butanol extract at 2,000 mg/kg produced deparasitization of 94.89% (73.63±10.31) compared to that of albendazole (ABZ), which is 100% (88.91±10.45) at 10 mg/kg. There was, however, no statistically significant difference ($p>0.05$) between extract-treated groups except for *n*-butanol (BUT) at a dose rate between 1,700 and 2,000 mg/kg. However, there was a significant difference ($p<0.01$) between the extract-treated and the DW control groups as well as the extract-treated and ABZ groups ($p<0.05$) (Table I).

Table I: Mean percentage (%) deparasitization in mice infected with 150 L_3 of *H. bakeri* and orally treated for five consecutive days with varying doses of *A. montanus* leaf extracts, distilled water, and albendazole.

	Substance				
Dose (mg/kg)	**CEE**	**BUT**	**AQ**	**DW (5 mL/kg)**	**ABZ (10 mg/kg)**
1,200	42.63±8.75[a]	56.27±8.76[a]	33.64±7.07[a]	0.000±0.00[c]	88.91±10.45[b]
1,400	48.60±8.75[a]	69.24±10.35[b]	38.81±7.42[a]		
1,700	53.56±9.31[a]	71.06±10.41[b]	43.43±7.20[a]		
2,000	59.31±10.89[a]	73.63±10.31[b]	49.95±7.96[a]		

F=5.905 (MStreatment/MSresidual); 5 mL/kg is the minimum convenient volume (MCV) administered to mice; 10 mg/kg is the dose as recommended by the manufacturer; a, b, c differ significantly ($p<0.05$) from one another.

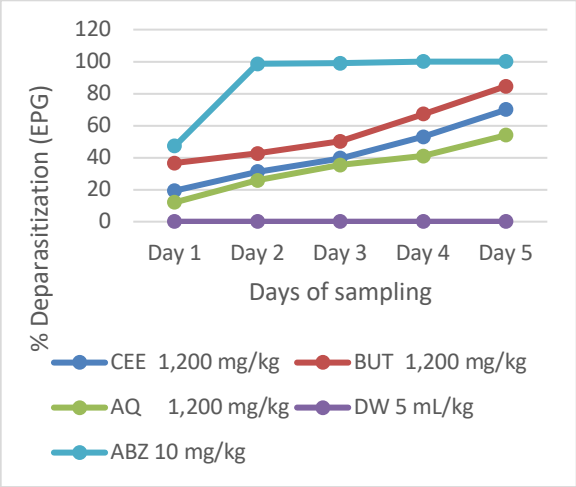

Figure I: Pattern of daily percentage (%) deparasitization in mice infected with 150 L_3 of *H. bakeri* and orally treated for five consecutive days with 1,200 mg/kg of *A. montanus* leaf extracts, distilled water, and albendazole.

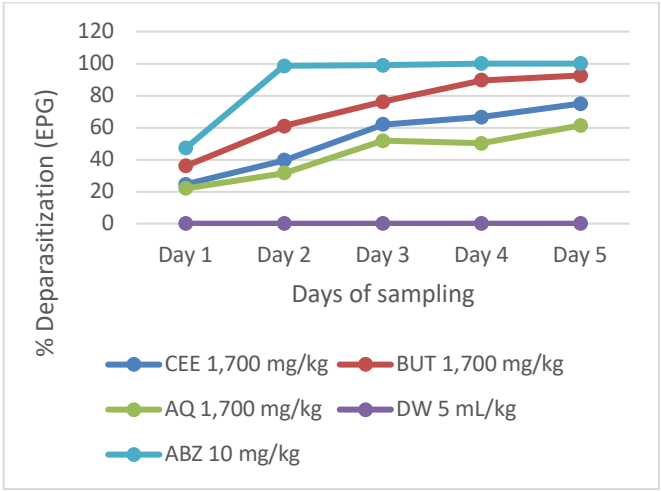

Figure III: Pattern of daily percentage (%) deparasitization in mice infected with 150 L_3 of *H. bakeri* and orally treated for five consecutive days with 1,700 mg/kg of *A. montanus* leaf extracts, distilled water, and albendazole.

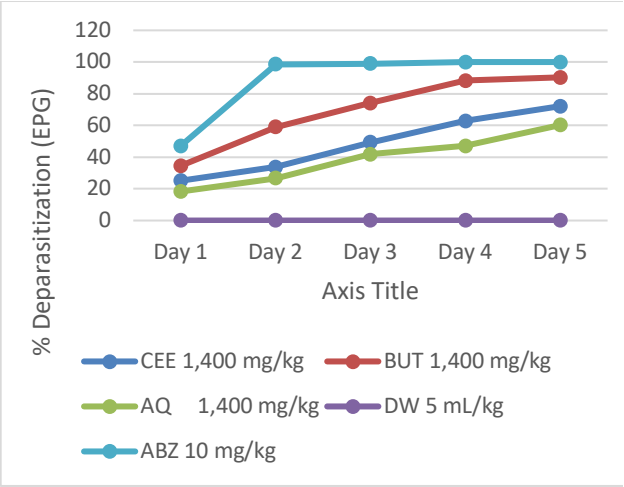

Figure II: Pattern of daily percentage (%) deparasitization in mice infected with 150 L_3 of *H. bakeri* and orally treated for five consecutive days with 1,400 mg/kg of *A. montanus* leaf extracts, distilled water, and albendazole.

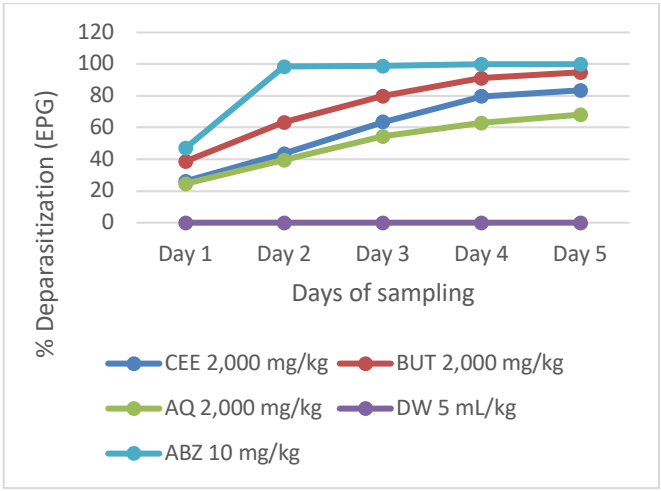

Figure IV: Pattern of daily percentage (%) deparasitization in mice infected with 150 L_3 of *H. bakeri* and orally treated for five consecutive days with 2,000 mg/kg of *A. montanus* leaf extracts, distilled water, and albendazole.

Discussion

The extensive literature on ethnomedicine and its folkloric claims, as well as earlier reports of anthelmintic efficacy (Oshadu, 2014; Oshadu *et al.,* 2021a, b), warranted the selection and use of *Acanthus montanus* in this study. The median lethal dose (LD_{50}) of the extract was previously determined to be >10,000 mg/kg (Oshadu, 2014; Oshadu *et al.,* 2022). It was observed in this study that all the extracts produced anthelmintic effects that culminated in the reduction of faecal egg output. The *n*-butanol extract produced significant faecal egg count reduction (FECR) from the third day of faecal analysis, reaching 94.89% by the end of the experiment for 2.0 mg/kg, with the mean percentage of 73.63±10.31. According to the World Association for the Advancement of Veterinary Parasitology (WAAVP), the primary parameter for drug efficacy is the reduction in either parasite excretion or parasite counts, and a minimum efficacy of 90% is required against non-coccidial gastrointestinal protozoa (Geurden *et al.,* 2014; Kaplan, 2020) and over 90% in gastrointestinal nematodes when the FECR test is conducted with the view of determining anthelmintic resistance in nematodes of veterinary importance (Beugnet *et al.,* 2022; Cole *et al.,* 1992). Ahmed *et al.* (2023) reported several plants with anthelmintic activities across the globe, some of which have neurogenous-cholinergic effects (Ekawardhani *et al.,* 2021; Tchetan *et al.,* 2022). This is true for most systemic synthetic anthelmintics (Oshadu, 2014). The benzimidazole/ probenzimidazole anthelmintics, which include drugs such as albendazole, mebendazole, thiabendazole, fenbedazole, and flubendazole, for instance, act by interfering with the polymerization of microtubules of the parasite (Oshadu, 2014; Chai *et al.,* 2021; Jacob *et al.,* 2022). These drugs bind to the parasite's protein tubulin, causing it to die by starvation (Oshadu, 2014; Laxmikeshav *et al.,* 2022). The second group of anthelmintics referred to as the tetrahydropyrimidines/ imidazothiazoles group affect acetylcholine neuro-transmission by interfering with nicotinic acetylcholine receptors of nematodes muscle cells and produce contraction and spastic paralysis (Oshadu, 2014; Laxmikeshav *et al.,* 2022). Examples of these drugs include levamisole, pyrantel, morantel, and oxantel. The macrocyclic lactones or avermectins/ milbemycins, the third group of anthelmintics, interact with chloride channels on helminths' gamma-aminobutyric acid (GABA) receptor complexes. They also inhibit pharyngeal pumping; thereby interfering with the parasites' feeding, movement, and reproductive ability in susceptible nematodes. This results in their paralysis and eventual elimination from the host (Chai *et al.,* 2021; Jacob *et al.,* 2022). Drugs in this category include ivermectin, abamectin, doramectin, eprinomectin, selamectin, milbemycin oxime, and moxidectin (Oshadu, 2014).

Anthelmintics act in diverse ways depending on the parasite and its potential host's physiology. Most of these drugs act on the worm's tegument or cuticle (Oshadu, 2014; Roy *et al.,* 2021), the primary parasite-host interface. The tegument and/or cuticular structures provide surface area for absorption of the host's nutrients. This structure also enables the parasite to perceive its microenvironment in the host's body (Gaillard *et al.,* 2021; Chai *et al.,* 2021). The dose-dependent reduction of faecal egg count/ egg per gram of faeces as seen in this study is probably because the adult *H. bakeri* parasite has been affected by the extracts. The secondary metabolites reported in earlier studies (Oshadu, 2014; Oshadu *et al.,* 2021b) may be responsible for the profound anthelmintic effects on the parasite, singly or

in synergy. Moreover, tannins, especially condensed tannins, abundant in the *A. montanus* plant, could be responsible for these activities (Oshadu, 2014; Oshadu *et al.,* 2021 b). Tannins are polyphenolic chemical compounds known for disrupting oxidative phosphorylation in helminth parasites, thereby inhibiting the utilization of energy (Koopmann *et al.,* 2020; Mal and Pal, 2021). In a previous study (Oshadu *et al.,* 2021b), a large amount of tannins was detected in the *n*-butanol leaf extracts of *A. montanus.* It is, therefore, logical to conclude that these polyphenolic chemical compounds were responsible for the low faecal egg output of the *H. bakeri* parasite in this study. Tannins can also bind to free proteins in the guts of the host animals (Oshadu, 2014; Sillanpää *et al.,* 2023), thereby exerting their anthelmintic effect. This phenomenon can also occur on the parasites' glycoprotein on their cuticle (Greiffer *et al.,* 2022). These mechanisms of action may thus cause death, hence the reduction in faecal egg output of the parasite, due ultimately to the digestion of the parasite and its proteinaceous ova.

In conclusion, the mechanism of action responsible for this profound anthelmintic activity on the adult parasite, with the resultant decrease in faecal output observed in this study, should therefore be investigated in natural infection in livestock and probably in humans.

Conflict of Interest

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

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