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In-Vitro Activity of *Croton macrostachyus* Hochst. Ex Delile against *Haemonchus contortus* (Rudolphi, 1803) Cobb, 1898

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Abstract

This study evaluated the *in vitro* anthelmintic activity of hexane and methanolic extracts of *Croton macrostachyus* against *Haemonchus contortus*. Extracts were tested at concentrations of 25, 50 and 100 mg/ml, with levamisole (10 mg/ml) and DMSO (0.5%) as positive and negative controls, respectively. Both extracts demonstrated dose-dependent efficacy, with the hexane extract exhibiting superior performance. At 100 mg/ml, the hexane extract induced paralysis in 30.00 ± 1.00 minutes and mortality in 103.00 ± 2.65 minutes, compared to 35.00 ± 1.00 and 66.00 ± 1.00 minutes for the methanolic extract. The 25 mg/ml concentration failed to induce mortality with either extract. Although slower than levamisole (which induced paralysis in 8.67 ± 0.58 minutes and mortality in 11.00 ± 1.00 minutes), the hexane extract's rapid paralysis at 100 mg/ml effectively halts parasite blood-feeding. Statistical analysis using the Kruskal-Wallis test confirmed significant differences in both paralysis ($H = 22.60$, $p = 0.002$) and mortality times ($H = 16.35$, $p = 0.006$) across all treatment groups. The low standard deviations across replicates confirm reproducible anthelmintic activity. These findings support the traditional ethnoveterinary use of *C. macrostachyus* in Africa and indicate that the hexane fraction has promising *in vitro* activity against adult *H. contortus*. However, the results should be interpreted as preliminary laboratory evidence rather than confirmation of practical field efficacy. Further studies are required to isolate the active compounds, evaluate safety and dosage, and validate efficacy under *in vivo* and field conditions.

Keywords

Croton macrostachyus, *Haemonchus contortus*, Anthelmintic Activity, Haemonchosis, Traditional Knowledge, Kenya

1. Introduction

Gastrointestinal nematode infections, particularly those caused by *Haemonchus contortus*, remain a primary constraint to small ruminant production in sub-Saharan Africa, causing significant economic losses through anemia, weight loss, reduced productivity and mortality [1]. Conventional control strategies rely heavily on synthetic anthelmintics; however, the widespread emergence of anthelmintic resistance—documented against benzimidazoles, levamisole and macrocyclic lactones in Kenya and across East Africa—has critically undermined their efficacy [2]-[4].

This resistance crisis, coupled with concerns regarding drug residues, high costs and limited accessibility for resource-poor farmers, has intensified the search for sustainable alternative control strategies [5] [6].

Ethnoveterinary medicine represents a promising reservoir of novel anthelmintic compounds. In Kenya and neighboring Ethiopia, *Croton macrostachyus* Hochst. ex Del. (Euphorbiaceae) is extensively documented in ethnobotanical surveys for the treatment of gastrointestinal parasites in livestock [7] [8]. Traditional preparations include aqueous decoctions of leaves, bark or seeds administered orally as vermifuges or purgatives [9]. Preliminary phytochemical investigations indicate that *C. macrostachyus* contains diverse secondary metabolites—including alkaloids, flavonoids, tannins, terpenoids, and saponins—many of which possess documented bioactivity against parasitic organisms [1] [10].

While *C. macrostachyus* has demonstrated *in vitro* anthelmintic activity against *H. contortus* eggs and adult helminths in Ethiopian studies [7], rigorous validation under standardized methodological frameworks remains limited, particularly for Kenyan plant accessions and solvent-specific extracts. Furthermore, comparative efficacy data across extraction polarities (hexane vs. methanol vs. aqueous) are sparse, yet critical for optimizing traditional preparation methods and identifying bioactive fractions.

This study therefore aimed to: 1) evaluate the *in vitro* anthelmintic activity of hexane and methanolic extracts of *C. macrostachyus* leaves and stems against adult *H. contortus* using a motility-based adult helminth mortality assay; 2) compare the dose-response profiles and potency of extracts relative to the synthetic anthelmintic levamisole; and 3) contextualize findings within existing literature on *C. macrostachyus* phytochemistry and anthelmintic mechanisms. We hypothesized that non-polar (hexane) extracts would demonstrate superior anthelmintic activity due to enhanced transcuticular penetration of lipophilic bioactive compounds [7].

2. Materials and Methods

2.1. Traditional Knowledge and Plant Material Collection

Thirty-four traditional herbalists from Migori County, Kenya, were interviewed using semi-structured questionnaires to document local plants used for treating human and animal diseases. The ethnobotanical data regarding the use of *C.*

macrostachyus for helminth control were collected from three of these 34 interviewed herbalists, all of whom provided voluntary, informed consent with the assurance of strict data confidentiality. Other cited uses for *C. macrostachyus* were as a hemostatic, anti-thrush, analgesic, laxative and anthelmintic applications. Plant identity was confirmed in the field (Uriri, Migori County) by a taxonomist from the University of Nairobi using the Flora of Kenya [11]. Because the documented traditional ethnoveterinary preparation utilizes aerial plant parts collectively, leaves and stems were harvested, and voucher specimens (BOO 1035 and 1266) were deposited at the University of Nairobi Botany Department Herbarium (NAI). The plant material was shade-dried at ambient temperature (22˚C - 25˚C) for 14 days, milled to a fine powder (≤0.5 mm particle size), and stored in airtight containers at 4˚C until extraction.

2.2. Plant Extraction

The dried plant material was subjected to a sequential extraction protocol to obtain fractions of increasing polarity. Briefly, 100 g of the dried powder was exhaustively extracted in a Soxhlet apparatus using hexane (non-polar) for 8 hours. The remaining defatted plant residue (marc) was subsequently dried and exhaustively extracted with methanol (polar) for an additional 8 hours. This sequential approach ensured that the methanolic fraction represented compounds not solubilized by the initial hexane extraction.

The individual extracts were concentrated under reduced pressure at 40˚C using a rotary evaporator, transferred to pre-weighed vials, and dried to a constant weight under vacuum. The percentage yield for the extraction was calculated as:

Percentage yield = (weight of crude extract obtained/weight of dried plant material used) × 100 (1)

All extracts were stored desiccated at 4˚C. The total combined extraction yield of the sequentially obtained hexane and methanolic fractions was 6.8% (w/w) based on the initial 100 g of dried plant material.

2.3. Collection of *H. contortus*

Mature, intact, actively motile *H. contortus* of comparable size and with no visible damage were collected from naturally infected abomasa of two sheep at a local abattoir to reduce the likelihood that the assay represented a single-host effect. They were identified by a meat inspector and confirmed by a parasitologist. The helminths were washed three times in sterile phosphate-buffered saline (PBS) and maintained there at 37˚C for 2 hours to allow acclimatization and expulsion of gut contents before exposure to test solutions. For each treatment concentration, 10 adult worms were used per Petri dish, and each treatment was replicated three times, giving a total of 30 worms per treatment concentration.

2.4. Preparation of Test Solutions

Crude extracts were dissolved in DMSO and serially diluted with sterile PBS to

final concentrations of 25, 50 and 100 mg/ml. The final DMSO concentration in all assay wells did not exceed 0.5% (v/v). Sterile filter paper discs (6 mm) were impregnated with 50 µL of each test solution.

2.5. In *Vitro* Anthelmintic Assay

Ten adult *H. contortus* helminths were transferred to sterile Petri dishes containing 10 mL of PBS. An impregnated filter disc was added to each dish. The method was selected to allow controlled delivery of a measured extract volume into the assay environment while minimizing direct handling of the worms and ensuring gradual diffusion of the extract into the surrounding PBS. Ombasa *et al.* [12] have used a similar approach in preliminary screening approaches against *H. contortus*. Worms were then incubated at 37˚C for 24 hours. Motility and mortality were recorded using a stereomicroscope. Post-incubation, worms were transferred to fresh PBS for 30 minutes to assess reversibility. Paralysis and death were recorded as separate endpoints. Paralysis was defined as the absence of spontaneous movement and absence of movement after gentle mechanical stimulation for 5 - 6 seconds during the exposure period. To distinguish reversible paralysis from death, worms showing no movement were transferred to fresh PBS and observed for 30 minutes. Death was recorded only when worms failed to recover motility after the 30 min recovery period in fresh PBS and after gentle stimulation. Time to paralysis was therefore recorded as the first time point at which motility ceased during exposure, whereas time to death was recorded after failure to recover movement following transfer to fresh PBS. Each concentration was tested in triplicate. Levamisole hydrochloride (10 mg/mL in PBS) served as the positive control, and 0.5% DMSO in PBS served as the negative control.

2.6. Statistical Analysis

Data were summarized as mean ± standard deviation from three independent replicate Petri dishes per treatment. The experimental unit was the Petri dish containing 10 adult *H. contortus* worms, and the mean time to paralysis and mean time to death were calculated for each replicate before treatment-level means were generated. Treatment effects were analyzed using the Kruskal-Wallis non-parametric test, followed by Dunn's post-hoc multiple comparison test. Statistical significance was accepted at $p < 0.05$, with highly significant differences noted at $p < 0.01$.

3. Results

Figure 1 summarizes the *in vitro* anthelmintic activity of *C. macrostachyus* hexane and ethanolic extracts against adult *H. contortus*.

The anthelmintic activity of *C. macrostachyus* extracts varied with both extract concentration and solvent fraction. For both hexane and methanolic extracts, increasing the concentration from 25 to 100 mg/ml generally reduced the time required to induce paralysis, indicating a dose-dependent effect. The hexane extract

showed the strongest activity at 100 mg/ml, where paralysis occurred within 30.00 ± 1.00 min and death occurred within 103.00 ± 2.65 min. At lower concentrations, the onset of paralysis was delayed, and complete mortality was not consistently achieved at 25 mg/ml. The methanolic extract also demonstrated anthelmintic activity, although its response pattern differed from that of the hexane fraction. At 100 mg/ml, the methanolic extract induced paralysis within 35.00 ± 1.00 min and mortality within 66.00 ± 1.00 min. At 50 mg/ml and 25 mg/ml, the methanolic extract required longer exposure times to produce paralysis, and the 25 mg/ml concentration did not induce complete mortality during the observation period. The positive control, levamisole, produced the most rapid response, confirming the sensitivity of the adult worm assay. In contrast, the negative control, 0.5% DMSO in PBS, did not induce paralysis or death, confirming that the observed effects were attributable to the plant extracts rather than the solvent vehicle. The uppercase letters shown above the bars in Figure 1 indicate statistical differences among treatments. Treatments sharing the same letter were not significantly different, whereas treatments with different letters differed significantly ($p < 0.05$). The lettering pattern demonstrates that both extract concentration and solvent fraction significantly influenced paralysis and mortality times, supporting a concentration-dependent *in vitro* anthelmintic effect of *C. macrostachyus* extracts.

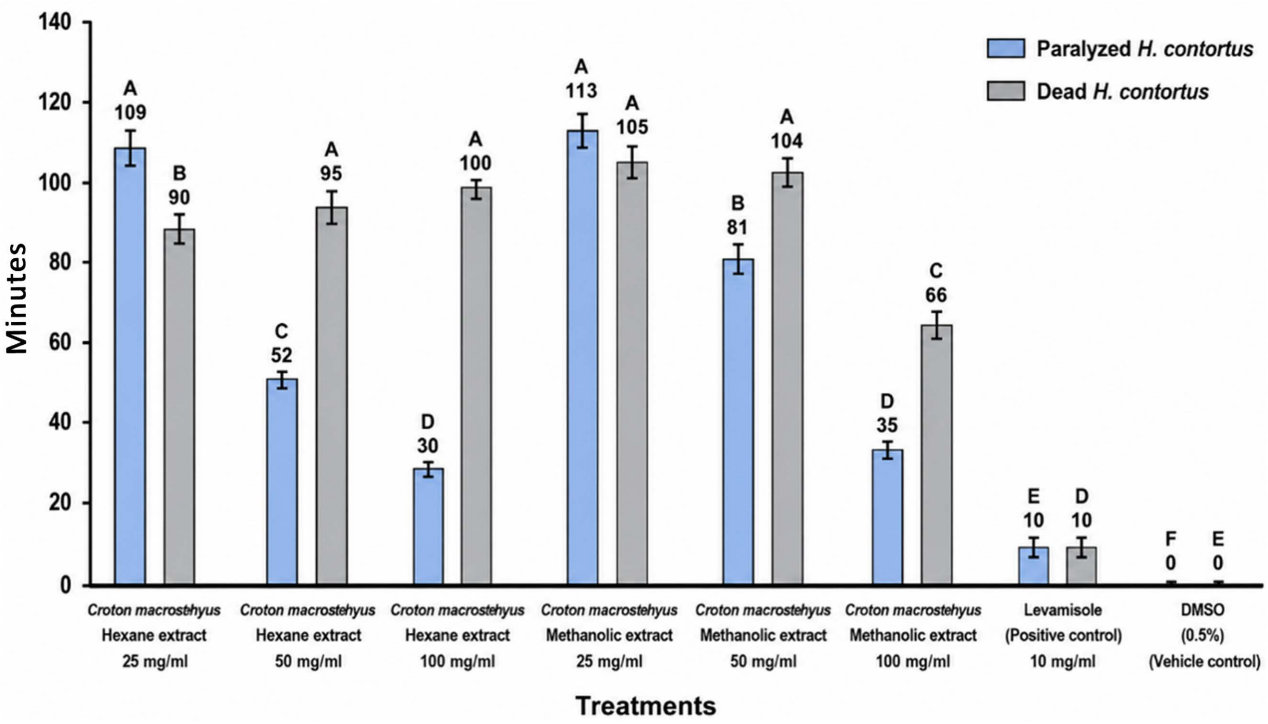

Figure 1. Anthelmintic activity against *Haemonchus contortus*.

4. Discussion

This study evaluated the anthelmintic activity of *C. macrostachyus* extracts against *H. contortus*, and the findings demonstrate dose-dependent efficacy that varies

according to solvent polarity. The hexane extract exhibited superior anthelmintic activity compared to the methanolic extract, with paralysis times decreasing from 110 minutes at 25 mg/ml to 30 minutes at 100 mg/ml, while mortality was achieved within 100 - 105 minutes at the highest concentration. In contrast, the methanolic extract required 110 - 116 minutes for paralysis at 25 mg/ml and 35 minutes at 100 mg/ml, with mortality occurring between 66 - 106 minutes depending on concentration. These results indicate that non-polar compounds extracted by hexane possess greater anthelmintic potency than polar compounds extracted by methanol.

The superior efficacy of the hexane extract aligns with findings from Eguale *et al.* [6], who demonstrated that hydro-alcoholic extracts of *C. macrostachyus* seeds exhibited significant dose-dependent mortality of adult *H. contortus*, achieving 90% mortality at 8 mg/ml concentration after 24 hours of exposure. The researchers attributed this enhanced activity to the presence of lipophilic compounds that facilitate transcuticular absorption into the parasite body, as anthelmintic drugs primarily penetrate nematodes through diffusion across the cuticle rather than oral ingestion [13].

Phytochemical analyses confirm that *C. macrostachyus* contains alkaloids, flavonoids, polyphenols, tannins, terpenoids, and saponins [14]. Among these, Eguale *et al.* [6] identified alkaloids and flavonoids as predominant constituents, suggesting their potential role in anthelmintic activity. The presence of these bioactive compounds supports the traditional use of *C. macrostachyus* in Ethiopian ethnoveterinary medicine for treating gastrointestinal parasites in livestock [6]. The plant has been extensively documented in ethnoveterinary surveys across Ethiopia, where various parts including seeds, bark and leaves are employed as vermifuges and purgatives [3].

Dose-response relationship observed in this study corroborates previous research demonstrating concentration-dependent anthelmintic effects of *C. macrostachyus*. Eguale *et al.* [6] reported that both aqueous and hydro-alcoholic extracts inhibited egg hatching at concentrations as low as 0.5 mg/ml, with ED50 values of 0.1 mg/ml and 0.32 mg/ml respectively. The current findings extend this knowledge by demonstrating that adult helminth paralysis and mortality also follow concentration-dependent patterns, with higher concentrations producing faster effects. This consistency across different developmental stages strengthens the evidence for *C. macrostachyus* as a broad-spectrum anthelmintic agent.

However, when compared to the synthetic anthelmintic levamisole, which induced paralysis within ~9 minutes (mean 8.67 min) and mortality within 10 - 12 minutes at 10 mg/ml. This temporal disparity highlights a critical limitation of botanical anthelmintics in clinical settings where rapid parasite elimination is necessary to prevent ongoing blood-feeding and host damage. The positive control's superior efficacy underscores the challenge facing phytotherapeutic approaches: while plant extracts offer potential alternatives to synthetic drugs, particularly in resource-limited settings where anthelmintic resistance is emerging [5], their slower

onset of action may limit their utility in acute infections requiring immediate intervention.

The negative control (DMSO 0.5%) showed no anthelmintic activity, confirming that the observed effects were attributable to the plant extracts rather than the solvent. This validates the experimental design and supports the conclusion that bioactive compounds within *C. macrostachyus* are responsible for the anthelmintic activity. The absence of effect in the negative control group also indicates that the concentrations of solvent used were not toxic to the parasites, eliminating confounding variables in the interpretation of results.

The variation in efficacy between hexane and methanolic extracts has important implications for traditional preparation methods. In Ethiopian traditional medicine, *C. macrostachyus* is typically administered as aqueous decoctions or crude preparations [3]. The current findings suggest that such preparations may not extract the full spectrum of anthelmintic compounds, particularly the lipophilic constituents that demonstrated superior activity. This observation aligns with Eguale *et al.* [6], who found that hydro-alcoholic extracts exhibited higher adulticidal activity than aqueous extracts, despite aqueous extracts showing better ovicidal effects. These findings indicate that optimization of extraction methods could enhance the therapeutic efficacy of traditional remedies.

Several limitations of *in vitro* studies must be acknowledged when interpreting these results. The controlled laboratory conditions do not account for metabolic biotransformation, interactions with feed materials, or absorption dynamics that occur *in vivo* [6] [15]. Consequently, the concentrations effective *in vitro* may differ substantially from those required in living animals. Furthermore, the use of adult helminths collected from abattoirs rather than experimentally maintained parasites introduces variability that, while reflecting field conditions, may affect reproducibility. Despite these limitations, *in vitro* assays provide valuable preliminary screening data that can guide subsequent *in vivo* investigations.

5. Conclusion

In conclusion, *C. macrostachyus* extracts demonstrated concentration-dependent *in vitro* anthelmintic activity against adult *H. contortus*, with the hexane fraction showing particularly promising activity. These results provide preliminary support for further investigation of this plant as a potential source of anthelmintic compounds. However, the findings do not yet establish the extract as a practical alternative to conventional anthelmintics. Future work should include bioassay-guided fractionation, compound identification, toxicity assessment and controlled *in vivo* studies.

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The traditional ethnoveterinary knowledge documented in this research originates from and remains the intellectual and cultural heritage of the Kuria and Luo communities of southwestern Kenya and any future therapeutic or commercial applications derived from this work should be pursued with equitable benefit-sharing, ethical engagement and full respect for indigenous intellectual property rights.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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