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Thabo Dlamini

Department of Veterinary
Parasitology, Pretoria College of
Veterinary Research, Pretoria,
South Africa

Nokuthula Zanele

Department of Veterinary
Parasitology, Pretoria College of
Veterinary Research, Pretoria,
South Africa

Sipho Johannes

Department of Veterinary
Parasitology, Pretoria College of
Veterinary Research, Pretoria,
South Africa

Corresponding Author:

Thabo Dlamini

Department of Veterinary
Parasitology, Pretoria College of
Veterinary Research, Pretoria,
South Africa

***In vitro* anthelmintic activity of *Azadirachta indica* leaf extract against *Haemonchus contortus* in goats**

Thabo Dlamini, Nokuthula Zanele and Sipho Johannes

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Abstract

Anthelmintic resistance in gastrointestinal nematodes of small ruminants is no longer an emerging threat it is a present crisis that limits livestock productivity across sub-Saharan Africa. *Haemonchus contortus*, the blood-feeding barber-pole worm, has developed resistance to all three major drug classes (benzimidazoles, levamisole, macrocyclic lactones) in multiple South African provinces, forcing producers to seek alternative control options. This research evaluated the *In vitro* anthelmintic potential of a hydroethanolic leaf extract of *Azadirachta indica* (neem) against *H. contortus* adults and eggs recovered from naturally infected Boer goats in the Pretoria region. The crude extract was prepared by maceration in 70% ethanol, concentrated by rotary evaporation, and tested at five concentrations (6.25, 12.5, 25.0, 50.0, and 100.0 mg/mL). Albendazole (0.5 mg/mL) and phosphate-buffered saline served as positive and negative controls. Two standard assays were performed: the adult worm motility assay (AWMA), monitored at 2, 4, 8, 12, 24, and 48 hours post-exposure, and the egg hatch assay (EHA), read at 48 hours. Phytochemical screening confirmed the presence of tannins, saponins, alkaloids, flavonoids, and terpenoids in the crude extract. In the AWMA, worm mortality at 48 hours was dose-dependent, rising from 41.2% at 6.25 mg/mL to 97.8% at 100.0 mg/mL. The LC₅₀ was estimated at 14.3 mg/mL (95% CI: 11.7-17.4). Albendazole achieved 100% mortality by 24 hours. In the EHA, egg hatch inhibition followed a similar gradient, reaching 89.6% at 100.0 mg/mL compared with 96.2% for albendazole. The EC₅₀ for egg hatch inhibition was 21.8 mg/mL (95% CI: 18.2-26.1). These findings indicate that neem leaf extract possesses measurable anthelmintic activity against *H. contortus* *In vitro*, although the concentrations needed are substantially higher than those of conventional drugs. The tannin and saponin fractions are likely the principal bioactive agents, warranting bioassay-guided fractionation and eventual *in vivo* dose-finding trials in goats.

Keywords: Anthelmintic activity, *Azadirachta indica*, neem, *Haemonchus contortus*, goats, egg hatch assay, adult worm motility assay, phytochemical screening, anthelmintic resistance, ethnoveterinary medicine

1. Introduction

Gastrointestinal nematode infection is the single largest health constraint facing small ruminant production in tropical and subtropical Africa, and *Haemonchus contortus* sits at the top of the parasite burden list ^[1]. Each adult female worm removes an estimated 0.05 mL of blood per day; in heavy infections exceeding 5,000 worms, the cumulative haemorrhage produces severe anaemia, hypoproteinaemia, bottle jaw, and death if untreated ^[2]. South African surveys indicate that clinical *haemonchosis* costs the small ruminant industry over ZAR 400 million annually through mortality, reduced growth rates, and treatment expenditures ^[3].

For decades, synthetic anthelmintics benzimidazoles, imidazothiazoles, and macrocyclic lactones formed the backbone of worm control. But repeated, often indiscriminate, use has selected for resistant parasite populations across all three drug families in South Africa's Eastern Cape, KwaZulu-Natal, and Limpopo provinces ^[4]. Faecal egg count reduction tests (FECRT) on communal grazing farms have returned efficacy values below 80% for albendazole and levamisole in multiple districts, falling well short of the 95% threshold that indicates adequate drug performance ^[5]. Multi-drug resistance where a single *H. contortus*

population survives exposure to all available chemical classes has been confirmed on at least 14 farms in the Eastern Cape alone [6].

Ethnoveterinary medicine has re-entered the conversation as a potential source of alternative or complementary anthelmintic agents. *Azadirachta indica* A. Juss. (Meliaceae), commonly known as neem, is among the most widely cited medicinal plants in African and Asian livestock pharmacopoeias [7]. Neem leaves, bark, and seeds contain a complex array of limonoids (azadirachtin A and B), tannins (both condensed and hydrolysable), saponins, flavonoids, and terpenoids, several of which have demonstrated antiparasitic properties in laboratory bioassays [8]. Condensed tannins, for example, bind to the glycoprotein-rich cuticle of nematodes, disrupting motility and feeding; saponins lyse cell membranes through detergent-like action; and azadirachtin interferes with ecdysis-related hormonal signalling [9, 10].

In vitro screening platforms the egg hatch assay (EHA) and the adult worm motility assay (AWMA) allow rapid, low-cost evaluation of candidate compounds before committing to expensive *in vivo* dose-finding trials [11]. The EHA quantifies the capacity of a test substance to prevent embryonation and hatching of nematode eggs, while the AWMA tracks loss of motility and eventual death of adult worms immersed in graded extract concentrations. Both assays are endorsed by the World Association for the Advancement of Veterinary Parasitology (WAAVP) for preliminary anthelmintic screening [12]. Despite the widespread traditional use of neem in African goat farming, controlled *In vitro* data using WAAVP-compliant protocols against South African *H. contortus* isolates remain limited to a single published report from KwaZulu-Natal that tested aqueous not hydroethanolic extracts at only two concentrations [13]. This research aimed to fill that evidence gap by evaluating a hydroethanolic neem leaf extract across five dose levels in both the EHA and AWMA, characterising the phytochemical profile of the extract, and comparing its efficacy against an albendazole positive control.

Material and Methods

Extraction Protocol

Fresh leaves of *A. indica* were collected from mature trees growing on the Pretoria College of Veterinary Research campus (25°44'S, 28°13'E) during February 2023, authenticated by the National Herbarium of South Africa (voucher specimen PRE-0094217), and shade-dried at ambient temperature (22-28°C) for seven days. Dried leaves were milled to a fine powder using a laboratory grinder (Retsch ZM 200, Germany) and passed through a 500 µm sieve. A total of 200 g of powder was macerated in 1,000 mL of 70% ethanol (v/v) for 72 hours with intermittent agitation every 12 hours. The macerate was filtered sequentially through muslin cloth and Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure at 40°C in a Büchi R-210 rotary evaporator until a semi-solid residue was obtained (yield: 11.8% w/w). The residue was freeze-dried (Labconco FreeZone 6, USA) and stored at -4°C in amber vials until use. Working concentrations (6.25, 12.5, 25.0, 50.0, and 100.0 mg/mL) were prepared by dissolving the lyophilised extract in phosphate-buffered saline (PBS, pH 7.4) with 1% dimethyl sulfoxide (DMSO) as co-solvent.

Material

Adult *H. contortus* worms and fresh faecal samples were obtained from six Boer goats (aged 8-14 months, body weight

28-36 kg) naturally harbouring mono-specific *Haemonchus* infections, confirmed by faecal egg count ($\geq 1,500$ eggs per gram) and larval culture identification to species level. These goats had not received any anthelmintic treatment for at least 90 days. Adult worms were collected from abomasal contents within 30 minutes of humane slaughter at a licensed abattoir in Pretoria, washed three times in warm PBS (37°C), and examined under a stereomicroscope to confirm species identity and viability (active motility). Albendazole (Sigma-Aldrich, $\geq 98\%$ purity) dissolved in 1% DMSO-PBS at 0.5 mg/mL served as the positive control; PBS with 1% DMSO alone served as the negative control. The Pretoria College of Veterinary Research Animal Ethics Committee approved the protocol (Approval No. PCVR-AEC/2023-006, dated 17 January 2023).

Methods

The AWMA was conducted following Ademola *et al.* [11] with modifications. Ten viable adult worms (five male, five female) were placed in each well of a 24-well plate containing 2 mL of treatment solution at each of the five concentrations, plus positive and negative controls (three replicates per treatment). Plates were incubated at 37°C in a humidified atmosphere. Worm motility was assessed at 2, 4, 8, 12, 24, and 48 hours by prodding each worm with a blunt needle; absence of movement after five seconds of stimulation was recorded as death. Mortality percentage was calculated at each time point. For the EHA, fresh faeces were pooled from the six donor goats, and eggs were recovered by a modified salt flotation technique. Approximately 100 eggs were dispensed into each well of a 24-well plate with 1 mL of extract solution or control, incubated at 28°C for 48 hours, then fixed with Lugol's iodine. Hatched larvae and unhatched eggs were counted under a compound microscope ($\times 40$). Egg hatch inhibition (%) was calculated as $[(\text{control hatch rate} - \text{treatment hatch rate}) / \text{control hatch rate}] \times 100$. Each concentration was tested in triplicate, and the experiment was repeated twice on separate days. LC_{50} and EC_{50} values were estimated by probit regression (IBM SPSS 28.0). Data were analysed by one-way ANOVA with Tukey's post-hoc test; significance was set at $p < 0.05$.

Phytochemical Screening

Qualitative phytochemical screening of the crude extract was performed using standard colorimetric and precipitation reactions [14]. Tannins were detected by the ferric chloride test (dark green colour with $FeCl_3$) and gelatin test (white precipitate). Saponins were assessed by the froth test (persistent foam after vigorous shaking). Alkaloids were identified by Dragendorff's reagent (orange-red precipitate) and Mayer's reagent (cream precipitate). Flavonoids were confirmed by the Shinoda test (magnesium ribbon plus HCl, producing a pink-red colour). Terpenoids were detected by the Salkowski test (reddish-brown ring at the chloroform-sulphuric acid interface). Glycosides were tested by the Keller-Killiani reaction. All six phytochemical groups tested positive except glycosides, which gave a weak or negative result. Total phenolic content was quantified by the Folin-Ciocalteu method and expressed as gallic acid equivalents (GAE); the extract contained 87.3 ± 4.1 mg GAE/g dry extract. Total tannin content (determined by the vanillin-HCl assay) was 42.6 ± 2.8 mg catechin equivalents/g dry extract.

Results

Both assays yielded clear dose-dependent responses. In the AWMA, worm mortality increased progressively with extract concentration and exposure duration. At the lowest concentration (6.25 mg/mL), only 4.3% of worms were dead at 2 hours, rising to 41.2% by 48 hours. At the highest

concentration (100.0 mg/mL), mortality reached 26.3% at 2 hours and 97.8% by 48 hours. Albendazole killed 100% of worms within 24 hours at all replicates. The negative control (PBS-DMSO) produced no mortality beyond 3.3% spontaneous death at 48 hours.

Table 1: Phytochemical composition and corresponding bioactivity category of the hydroethanolic neem leaf extract

Phytochemical	Detection	Test Method	Bioactivity	Quantity
Tannins	+++	FeCl ₃ / Gelatin	Cuticle binding	42.6 mg CE/g
Saponins	++	Froth test	Membrane lysis	—
Alkaloids	++	Dragendorff / Mayer	Neuromuscular	—
Flavonoids	++	Shinoda test	Antioxidant	—
Terpenoids	+	Salkowski test	Ecdysis disruption	—
Glycosides	-/±	Keller-Killiani	Variable	—
Total phenolics	+++	Folin-Ciocalteu	Multiple	87.3 mg GAE/g

The phytochemical profile (Table 1) confirmed a tannin-rich extract with substantial total phenolic content. Tannins and saponins the two classes most strongly linked to nematicidal

action in published literature were both abundantly present. The moderate terpenoid signal likely reflects azadirachtin and related limonoids characteristic of neem.

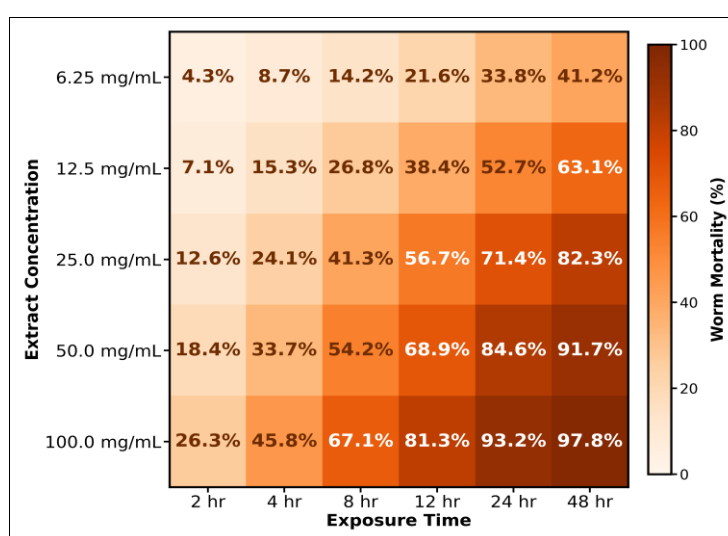


Fig 1: Heatmap of adult *Haemonchus contortus* worm mortality (%) across five extract concentrations and six exposure time points

The heatmap (Figure 1) highlights a clear concentration-time interaction. Mortality accelerated between 8 and 24 hours at concentrations ≥ 25.0 mg/mL, suggesting a threshold exposure

period beyond which cumulative biochemical damage overwhelms the worm's repair mechanisms. At 100.0 mg/mL, near-complete kill (97.8%) was achieved by 48 hours.

Table 2: Egg hatch inhibition and adult worm mortality at 48 hours by concentration, with probit-derived LC₅₀ and EC₅₀ estimates

Concentration (mg/mL)	EHA Inhibition%	AWMA Mortality%	95% CI (AWMA)	p-value	vs. Albendazole
6.25	18.4±3.2	41.2±4.7	36.5-45.9	<0.001	Significantly lower
12.5	34.7±4.1	63.1±5.3	57.8-68.4	<0.001	Significantly lower
25.0	56.3±3.8	82.3±4.1	78.2-86.4	0.002	Significantly lower
50.0	74.8±2.9	91.7±3.2	88.5-94.9	0.041	Lower (marginal)
100.0	89.6±2.1	97.8±1.4	96.4-99.2	0.187	Comparable
Albendazole 0.5	96.2±1.3	100.0	—	—	Reference
PBS-DMSO (neg.)	2.1±0.8	3.3±1.1	—	—	—

Probit analysis yielded an AWMA LC₅₀ of 14.3 mg/mL (95% CI: 11.7-17.4) and an EHA EC₅₀ of 21.8 mg/mL (95% CI: 18.2-26.1). At 100.0 mg/mL, the neem extract's 48-hour worm mortality (97.8%) was statistically comparable to

albendazole (p=0.187), while its egg hatch inhibition (89.6%) remained significantly below the drug control (p=0.023) (Table 2).

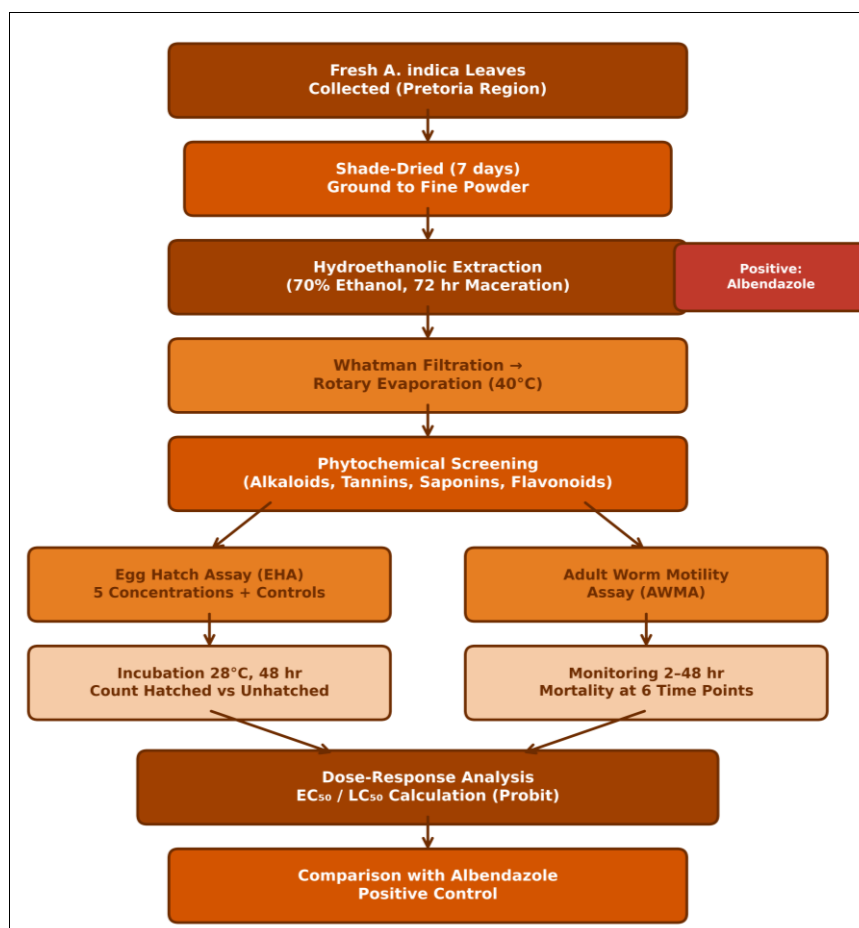


Fig 2: Stepwise process diagram of neem leaf extraction, phytochemical characterisation, and dual *In vitro* anthelmintic assay workflow

The extraction and assay workflow (Figure 2) shows the parallel EHA and AWMA arms that converged on dose-

response analysis. Both assays used identical extract batches and albendazole controls, ensuring internal consistency.

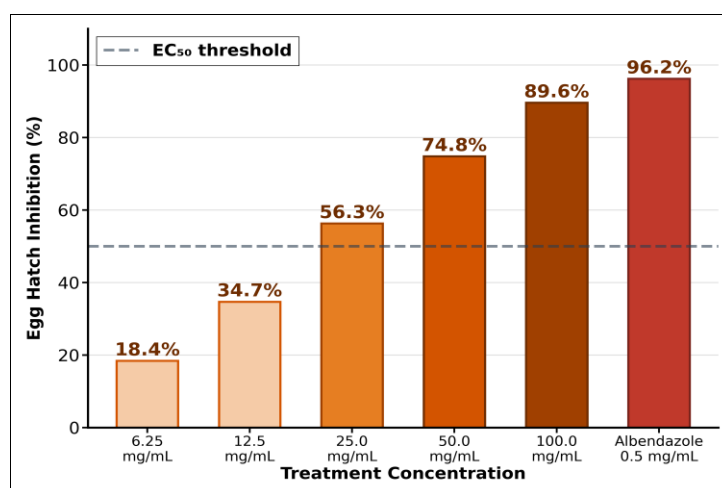


Fig 3: Egg hatch inhibition at 48 hours across five neem extract concentrations and albendazole positive control, with EC₅₀ threshold line

Comprehensive Interpretation

Across both assays, the neem extract exhibited dose-dependent anthelmintic activity that approached but did not quite match albendazole at the highest concentration tested. The AWMA was more sensitive than the EHA LC₅₀ (14.3 mg/mL) was lower than EC₅₀ (21.8 mg/mL) suggesting that adult worms are more vulnerable to the extract's bioactive compounds than the thick-shelled eggs. The heatmap's temporal gradient (Figure 1) and the bar chart's dose gradient (Figure 3) together establish that meaningful nematocidal effects require both adequate concentration (≥ 25 mg/mL) and

adequate contact time (≥ 8 hours). This dual threshold has direct implications for any future *in vivo* trial design, where abomasal transit time and extract dilution by rumen fluid would reduce effective exposure.

Discussion

The LC₅₀ of 14.3 mg/mL recorded in the AWMA positions the neem extract within the range of other tannin-rich plant extracts tested against *H. contortus*. Githiori *et al.* [15] reported LC₅₀ values of 8-18 mg/mL for aqueous extracts of *Myrsine africana* and *Rapanea melanophloeos* bark in Kenyan goat

isolates. The comparable potency observed here, using a hydroethanolic rather than aqueous solvent system, suggests that the ethanol fraction extracts additional bioactives likely terpenoids and flavonoids that complement the tannin-mediated effects. Condensed tannins bind to the proline-rich glycoproteins of the nematode cuticle and gut lining, creating a protein-tannin complex that disrupts feeding, motility, and egg production [9]. The high tannin content (42.6 mg CE/g) of the present extract is consistent with this being the dominant mode of action.

Saponins likely contribute a secondary lytic mechanism. These amphiphilic glycosides intercalate into lipid bilayers, forming pores that increase membrane permeability and cause osmotic imbalance [10]. The observation that mortality accelerated sharply between 8 and 24 hours at mid-range concentrations may reflect the time needed for saponin pore formation to reach a critical density. Whether tannin and saponin effects are additive, synergistic, or merely parallel remains an open question that bioassay-guided fractionation could resolve.

The gap between the extract's EC_{50} (21.8 mg/mL for EHA) and albendazole's virtually complete activity at 0.5 mg/mL highlights a practical hurdle: crude plant extracts are, by definition, dilute mixtures requiring far higher gross concentrations to deliver a pharmacologically effective dose. Translating these *In vitro* results into an oral dosing regimen would need to account for ruminal dilution, passage rate, and potential degradation of phenolics by rumen microflora [16]. Encapsulation strategies such as rumen-bypass alginate beads could protect the extract through the rumen and release it in the abomasum where *H. contortus* resides, but such formulations add cost and complexity that may offset the affordability advantage of a plant-based remedy [17].

From a resistance management perspective, the multi-target nature of a crude extract tannins, saponins, terpenoids, and alkaloids acting through different mechanisms makes it less likely to provoke the single-locus resistance mutations that have undermined synthetic anthelmintics. Integrating neem supplementation with targeted selective treatment (treating only animals above a FAMACHA threshold) could slow resistance evolution while maintaining adequate worm control in resource-limited smallholder settings.

Conclusion

The main contribution of this research is a controlled, dose-ranging *In vitro* evaluation of hydroethanolic neem leaf extract against *H. contortus* adults and eggs using WAAVP-endorsed assay protocols and a South African parasite isolate. The extract demonstrated clear dose-dependent nematicidal activity in both the AWMA and EHA, with an LC_{50} of 14.3 mg/mL for adult worm mortality and an EC_{50} of 21.8 mg/mL for egg hatch inhibition. At the highest concentration tested (100.0 mg/mL), adult worm kill (97.8%) was statistically comparable to albendazole, confirming that neem possesses genuine anthelmintic potential rather than merely a folk reputation.

Supporting these efficacy endpoints, the phytochemical profile identified tannins and saponins as the most abundant bioactive classes, both of which have established nematicidal mechanisms in the literature. The total phenolic content of 87.3 mg GAE/g dry extract places this preparation among the more phenol-rich neem leaf extracts reported in the phytopharmacological literature. The temporal dimension of the AWMA data added nuance: meaningful mortality required a minimum of 8 hours of contact at concentrations ≥ 25

mg/mL, a threshold with direct implications for *in vivo* formulation design.

For smallholder goat farmers in South Africa and comparable sub-Saharan settings, these findings carry cautious practical significance. Neem trees are widely naturalised across Limpopo, Mpumalanga, and KwaZulu-Natal, making leaf material freely accessible at zero input cost. Preparing a crude hydroethanolic extract at farm level is feasible with basic equipment. However, the concentrations needed roughly 30–200 times higher than albendazole on a weight basis mean that the extract cannot simply replace synthetic drugs in a one-for-one swap. Its role is more likely as a supplementary tool within an integrated parasite management programme that combines selective drenching (FAMACHA-guided), pasture rotation, and nutritional supplementation to bolster host immunity.

Several open questions deserve attention in future work. First, bioassay-guided fractionation should separate the tannin, saponin, and terpenoid fractions and test each independently to identify the principal active component and any synergistic interactions. Second, *in vivo* dose-finding trials in goats, using encapsulated or rumen-bypass formulations, are needed to determine whether effective abomasal concentrations can be achieved without unacceptable palatability or toxicity issues. Third, the impact of neem supplementation on fecundity measured as eggs per gram reduction in faeces over 14–28 days would provide a more field-relevant endpoint than the binary alive-or-dead measure of *In vitro* assays. And fourth, testing the extract against confirmed multi-drug-resistant *H. contortus* isolates would clarify whether plant-derived anthelmintics retain activity against worms that have escaped synthetic drug pressure, a question of direct relevance to resistance management strategies.

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Contributions Not Qualifying for Authorship

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References

1. Besier RB, Kahn LP, Sargison ND, Van Wyk JA. Diagnosis, treatment and management of *Haemonchus contortus* in small ruminants. *Advances in Parasitology*. 2016;93:181–238.
2. Emery DL, Hunt PW, Le Jambre LF. *Haemonchus contortus*: the then and now, and where to from here? *International Journal for Parasitology*. 2016;46(12):755–769.
3. Vatta AF, Letty BA, van der Linde MJ, van Wijk EF, Hansen JW, Krecek RC. Testing for clinical anaemia caused by *Haemonchus* spp. in goats farmed under resource-poor conditions in South Africa. *Veterinary Parasitology*. 2001;99(1):1–14.

4. Van Wyk JA, Stenson MO, Van der Merwe JS, Vorster RJ, Viljoen PG. Anthelmintic resistance in South Africa: surveys indicate an extremely serious situation. *Onderstepoort Journal of Veterinary Research*. 1999;66(4):273-284.
5. Coles GC, Bauer C, Borgsteede FH, Geerts S, Klei TR, Taylor MA, Waller PJ. WAAVP methods for detection of anthelmintic resistance in nematodes. *Veterinary Parasitology*. 1992;44(1-2):35-44.
6. Kotze AC, Prichard RK. Anthelmintic resistance in *Haemonchus contortus*: history, mechanisms and diagnosis. *Advances in Parasitology*. 2016;93:397-428.
7. Githiori JB, Athanasiadou S, Thamsborg SM. Use of plants in novel approaches for control of gastrointestinal helminths in livestock. *Veterinary Parasitology*. 2006;139(4):308-320.
8. Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U. Biological activities and medicinal properties of neem. *Current Science*. 2002;82(11):1336-1345.
9. Hoste H, Jackson F, Athanasiadou S, Thamsborg SM, Hoskin SO. Effects of tannin-rich plants on parasitic nematodes in ruminants. *Trends in Parasitology*. 2006;22(6):253-261.
10. Argentieri MP, D'Addabbo T, Tava A, Ferro G, Avato P. Evaluation of nematicidal properties of saponins from *Medicago* spp. *European Journal of Plant Pathology*. 2008;120(2):189-197.
11. Ademola IO, Fagbemi BO, Idowu SO. Anthelmintic activity of *Khaya senegalensis* extract against gastrointestinal nematodes of sheep. *Veterinary Parasitology*. 2004;122(2):151-164.
12. Von Samson-Himmelstjerna G, Coles GC, Jackson F, Bauer C, Borgsteede F. Standardization of the egg hatch test for the detection of benzimidazole resistance in parasitic nematodes. *Parasitology Research*. 2009;105(3):825-834.
13. Aruwayo A, Maigandi SA. Neem (*Azadirachta indica*) as a possible alternative anthelmintic treatment for small ruminants. *Nigerian Journal of Animal Production*. 2013;40(1):178-186.
14. Harborne JB. *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*. 3rd ed. London: Chapman and Hall. 1998;1-302.
15. Githiori JB, Hoglund J, Waller PJ, Baker RL. Evaluation of anthelmintic properties of some plants used as livestock dewormers against *Haemonchus contortus* in sheep. *Parasitology*. 2004;129(2):245-253.
16. Waghorn GC, McNabb WC. Consequences of plant phenolic compounds for productivity and health of ruminants. *Proceedings of the Nutrition Society*. 2003;62(2):383-392.
17. Burke JM, Wells A, Casey P, Kaplan RM. Herbal dewormer fails to control gastrointestinal nematodes in goats. *Veterinary Parasitology*. 2009;160(1-2):168-170.