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RESEARCH

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In vitro assessment of deworming potential of *Guiera senegalensis* in Nigerian ethnoveterinary industry using *Caenorhabditis elegans*

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Abstract

Background: Although *Guiera senegalensis* is used as a dewormer in ethnoveterinary health care in Nigeria, its anthelmintic potential has not been validated. Hence, this work investigated the in vitro anthelmintic potential of *G. senegalensis* extracts on two *Caenorhabditis elegans* strains: Bristol N2 (wild type/ivermectin susceptible) and DA1316 (ivermectin resistant).

Results: Aqueous and methanol extracts of *G. senegalensis* were tested against the motility of the L4 larvae at two exposure periods of 24 and 48 h and found to be active against the *C. elegans* strains. Motility of *C. elegans* DA1316 was reduced to 18.6% and 8.3% by aqueous and methanol extracts, respectively, at 2.0 mg/ml after 48 h, whereas that of *C. elegans* DA1316 treated with ivermectin (0.02 µg/ml) remained above 95%. The motility of *C. elegans* Bristol N2 was reduced to 16.6% and 7.2% by aqueous and methanol extracts, respectively, at 2.0 mg/ml after 48 h and ≤ 2.7% by ivermectin (0.02 µg/ml). Activity of the plant extracts was concentration and time dependent.

Conclusions: This work confirms the anthelmintic activity of *G. senegalensis* and its effectiveness against ivermectin-resistant nematodes, thus validating its ethnoveterinary use as an animal dewormer in Nigeria and pharmacological potential as a source of anthelmintic compounds against ivermectin-resistant nematodes. There is, however, the need for in vivo studies to confirm the in vitro efficacy of the extracts.

Keywords: Anthelmintic, *Caenorhabditis elegans*, Ethnoveterinary, *Guiera senegalensis*, Larval motility, Nigeria

Background

Gastrointestinal parasitic nematode infection is one of the global pathological problems with severe consequences for small ruminant production (Zajac and Garza 2020; Arsenopoulos et al. 2021). The pathological effects of this infection on animal health include low intake of food, anaemia, impairment of digestion, poor absorption of nutrients, weight lost and diarrhoea (Hoste 2001). Chemotherapeutic agents are commonly used for the management of the parasites (Zajac and Garza 2020; Arsenopoulos et al. 2021). However, in several parts of the world, the parasites have developed resistance to synthetic drugs such as ivermectin and several others (Jackson et al. 2012; Arsenopoulos et al. 2021). For instance, Herrera-Manzanilla et al. (2017) reported of benzimidazole, levamisole and ivermectin resistance in gastrointestinal nematodes of sheep in Mexico. Resistance to anthelmintics has also been reported in livestock in India (Singh et al. 2017), the Netherlands (Ploeger and Everts 2018) and South Africa (Mphahlele et al. 2021). The development of resistance to modern chemotherapy, side effects of the

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drugs in treated animals, residual effects of anthelmintics in the environment and food as well as the cost of these commercial anthelmintics call for natural anthelmintics from plants as an alternative to synthetic drugs (Mazhangara et al. 2020). The search for natural anthelmintics involves in vitro screening of plants or plant products as the first line of action to determine their efficacy. In vitro screening of plants for anthelmintics faces several challenges such as high cost, time-consuming procedures, ethical constraints and difficulty in obtaining adult stage nematodes for efficacy evaluation. These challenges have been addressed to a minimum with the discovery of *Caenorhabditis elegans* and its subsequent uses as a laboratory model in evaluating anthelmintics (Katiki et al. 2011; Hahnel et al. 2021; Taki et al. 2021).

Sustainable control of worms has been threatened by the emergence of resistant parasitic species especially those of small ruminants because understanding the genetic basis of resistance in parasitic nematodes is extremely difficult. However, with its emergence as a research model, *C. elegans* has been central to defining the mechanism of action of drugs and serves as a model for understanding and managing anthelmintic resistance (Holden-Dye and Walker 2014). Although the extent of reproducing the effect of anthelmintics on *C. elegans* on other nematodes is variable (Keiser 2015), it is useful for identifying potential anthelmintics (Holden-Dye and Walker 2014; Burns et al. 2015; Taki et al. 2021).

Traditionally, herbal practitioners especially from socio-economically disadvantaged countries within the tropical and subtropical regions have been relying on natural products from plants for the treatment of a wide range of infections including helminth parasites owing to their low cost, easy accessibility and little or absence of side effects (Yadav and Singh 2011; Mazhangara et al. 2020). Despite the long history of the application of traditional herbal medicine in the treatment of diverse ailments, there is scarcity of proper documentation and validation of its use (Mazhangara et al. 2020).

Guiera senegalensis (Myrtales: Combretaceae), a small tropical shrub, is used for human and veterinary health purposes in Africa (Adamu et al. 2005; Ademola 2016; Alshafei et al. 2016; Dirar and Devkota 2021). Although there is extensive utility of this plant in Nigerian ethnoveterinary industry as a dewormer by small ruminant breeders, its anthelmintic potential has not been validated. This study assessed the efficacy of extracts of *G. senegalensis* against the motility of larvae of *C. elegans* strains with contrasting ivermectin-resistance status to validate the ethnoveterinary use of the plant.

Methods

Plant material and extraction

Bark of *G. senegalensis* stem was collected from Azare in Bauchi State, Nigeria in December 2016 and authenticated (Voucher specimen No. 900103) and deposited as described in Gagman et al. (2018). The bark was peeled with a sterile blade, washed with distilled water, crushed into smaller fragments and shade-dried for 3 weeks before being pulverized into powder. Phytochemical extraction was carried out by maceration using distilled water and 80% methanol as solvents for the aqueous and methanol extracts, respectively (Lienou et al. 2015) at the School of Biological Sciences, Universiti Sains Malaysia. Briefly, distilled water (250 ml) was added to the powdered sample (50 g) and stored at room temperature (27.5 ± 2.5 °C) for 5 days. The infusion was filtered through a Whatman No. 1 filter paper. The filtrate was concentrated by drying at a temperature of 45 °C before storing the extract at 4 °C for further use (Lienou et al. 2015). The same procedure was followed for the methanol extraction using 80% methanol. The percentage yield of the extracts was calculated based on the formula used by Zhang et al. (2007) as follows:

$$\text{Extract yield } \% = \frac{\text{weight of the dry extract}}{\text{weight of the original sample}} \times 100.$$

Caenorhabditis elegans populations for bioassay

The strains (Bristol N2 (wild type/ivermectin susceptible) and DA1316 (ivermectin resistant) were sourced from the *Caenorhabditis elegans* Genetic Center, USA. Nematode growth medium (NGM) (mixture of 3 g NaCl, 17 g agar and 2.5 g of Bacto peptone in 1000 ml of distilled water and 1 ml of 1 M $CaCl_2$, 1 ml of 5 mg/ml cholesterol, 1 ml of 1 M $MgSO_4$ and 25 ml of 1 M KPO_4 buffer, all autoclaved except cholesterol) seeded with a lawn of *Escherichia coli* OP50 was used to maintain the *C. elegans* as described by Brenner (1974).

The synchronized population of *C. elegans* strains used in this experiment was obtained by adding 6 ml of distilled water to an old plate of the required strain and then agitated by hand shaking to stir eggs and gravid worms. The suspension containing eggs and gravid worms was transferred to 15 ml centrifuge tube and centrifuged at 1500 rpm for 1 min. The clear supernatant was carefully discarded followed by the addition of 5 ml of fresh alkaline bleaching solution (a mixture of 1 M NaOH and hypochlorite solution in the ratio of 1:2) to about 1 ml of the pelleted mixture. The content was vigorously shaken with occasional vortexing and monitored under a microscope until appreciable dissolution of bacteria and gravid worms was observed. Eight millilitres of Minimal salts (M9) buffer (3 g KH_2PO_4, 6 g Na_2HPO_2, 5 g NaCl and

1 M $MgSO_4$ in 1 l of distilled water) (Stiernagle 2006) was then added to end the bleaching process. The content was centrifuged at 1500 rpm, aspirated and re-suspended in M9 and the process of centrifuging was repeated until the bacteria and the dissolved worms were washed off. The pelleted eggs were re-suspended in 1 ml of M9 buffer and transferred to NGM plate and incubated overnight at 20 °C in a shaker. The L1 larvae were observed the next day (Baugh 2013). The newly hatched L1 larvae were incubated on *E. coli* OP50-seeded NGM plate at 20 °C for 40 h after which the L4 larvae were observed (Radman et al. 2013).

Bioassay

Synchronized populations of L4 larvae were used for the assay. Stock solutions of 2.0 mg/ml of *G. senegalensis* extracts were prepared by dissolving 200 mg of the extracts in 5 ml of 1% Tween 80 and 95 ml of M9 buffer. These were diluted further with M9 to obtain the test concentrations (Table 1) for the assay. A 0.02 µg/ml of ivermectin was also prepared and the assay was carried out in a 24-well culture plate as described in Gagman et al. (2020). A suspension (50 µl) containing about 100 L4 larvae of the *C. elegans* strain to be tested was added to each of the 24 wells. Exactly 1 ml of each of the concentrations (0.2, 0.6, 0.8, 1.0 and 2.0 mg/ml) of the desired extract (methanol or aqueous) was added to the larvae in 3 wells. Larvae in another set of 3 wells were treated with 0.02 µg/ml of ivermectin and served as positive control while larvae in 3 wells treated with M9 solution only served as negative control. The setup was incubated at 20 °C until observation and counting of worms was carried out after 24 and 48 h using an inverted microscope. The larvae were considered immotile in the absence of tail, head or pharyngeal movement especially when pricked with nickel wire for at least 5 s. Each experiment was repeated independently for 3 times. Percentage worm motility inhibition (WMI%) within each well was calculated based on the formula (Tariq et al. 2009) below.

$$\text{WMI\%} = \frac{\text{number of worms in negative control well} - \text{number of mobile worms in treatment well}}{\text{number of worms in negative control well}} \times 100.$$

Table 1 Motility of *C. elegans* L4 larvae after exposure to *G. senegalensis* extracts

Treatment	Concentration (mg/ml)	*C. elegans* Bristol N2		*C. elegans* DA1316	
		% motility ± SE			
		24 h	48 h	24 h	48 h
Aqueous extract	0.2	85.2 ± 0.6^{a}	72.2 ± 0.6^{a}	88.8 ± 0.6^{a}	73.2 ± 0.6^{a}
	0.4	82.9 ± 0.9^{a}	69.2 ± 0.6^{b}	84.2 ± 0.6^{b}	69.5 ± 0.7^{b}
	0.6	78.9 ± 0.7^{b}	64.2 ± 0.6^{c}	80.2 ± 0.6^{c}	63.2 ± 0.6^{c}
	0.8	72.9 ± 0.6^{c}	57.2 ± 0.6^{d}	76.6 ± 0.6^{d}	58.8 ± 0.6^{d}
	1.0	67.6 ± 0.7^{d}	47.2 ± 0.6^{e}	68.9 ± 0.7^{e}	52.5 ± 0.7^{e}
	2.0	58.9 ± 0.7^{e}	16.6 ± 0.6^{f}	63.9 ± 0.7^{f}	18.6 ± 0.7^{f}
Ivermectin (0.02 µg/ml)		38.6 ± 1.0^{f}	2.3 ± 0.4^{g}	98.5 ± 0.2^{g}	95.7 ± 0.9^{g}
Negative control		98.6 ± 0.7^{g}	97.4 ± 0.7^{h}	98.0 ± 0.5^{g}	96.1 ± 0.4^{g}
		$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
Methanol extract	0.2	77.9 ± 0.6^{a}	63.2 ± 0.6^{a}	80.2 ± 0.6^{a}	67.5 ± 0.6^{a}
	0.4	74.3 ± 0.5^{a}	60.3 ± 0.6^{a}	78.5 ± 0.7^{a}	62.2 ± 0.6^{b}
	0.6	69.6 ± 0.7^{b}	56.3 ± 0.6^{b}	74.2 ± 0.6^{a}	59.5 ± 0.7^{b}
	0.8	65.9 ± 0.7^{c}	52.3 ± 0.6^{c}	72.9 ± 4.2^{a}	55.3 ± 0.6^{c}
	1.0	57.3 ± 0.6^{d}	35.9 ± 0.7^{d}	61.2 ± 0.6^{b}	39.3 ± 0.6^{d}
	2.0	51.8 ± 0.6^{e}	7.2 ± 0.6^{e}	54.2 ± 0.6^{b}	8.3 ± 0.7^{e}
Ivermectin (0.02 µg/ml)		36.5 ± 0.9^{f}	2.7 ± 0.8^{f}	98.0 ± 0.2^{c}	96.7 ± 0.3^{f}
Negative control		98.5 ± 0.7^{g}	97.3 ± 0.4^{g}	98.1 ± 0.7^{c}	97.4 ± 0.7^{f}
		$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Motility values with the same alphabet in the same column for the same extract treatments are not significantly different at $p < 0.05$ (Tukey's HSD test)

Data analysis

The percentage means and standard error of the motility inhibition of the various concentrations of aqueous and methanol extracts of the plant against the larvae were calculated. Motility comparison between treatments was done using a one-way analysis of variance (ANOVA) for each treatment time for each extract for the different *C. elegans* strains. Post hoc test was performed using Tukey's honestly significant difference (HSD) test. Two-way ANOVA was conducted for the effect of treatment and treatment time on larval motility and their interaction for each extract on the nematode strains. Probit analysis was used in the computation of extract concentration required to cause 50% motility inhibition (IC_{50}). Statistical analysis was carried out using IBM SPSS® version 23 (IBM Corp. 2015).

Results

The percentage dry weight of 9.90 and 10.80% was recorded for aqueous and methanol extracts, respectively. Generally, motility inhibitory activity of the extracts against the larvae increased with increasing concentration of the extracts and the incubation time of the bioassay. Both the aqueous and methanol extracts exhibited good inhibitory activity against motility of the two *C. elegans* strains. The lowest performance for aqueous extract was recorded at 0.2 mg/ml after 24 h as the worm's motility was only reduced to 85.2% against *C. elegans* Bristol N2 and 88.8% against *C. elegans* DA1316 by the aqueous extract (Table 1). As concentration of the aqueous extract increased to 2.0 mg/ml at 24 h, the worm's motility was further reduced to 58.9% against *C. elegans* Bristol N2 and 63.9% against *C. elegans* DA1316. The methanolic extract had a higher activity than the aqueous extract with a reduction in worm motility to 77.9% and 80.2% for *C. elegans* Bristol N2 and DA1316, respectively, at 0.2 mg/ml after 24 h and to 51.8 and 54.2%, respectively, at 2 mg/ml after 24 h of methanolic extract exposure.

The highest efficacy of the extracts was observed at the highest concentration of 2.0 mg/ml after 48 h as aqueous extract reduced larval motility to 16.6% whereas methanol extract reduced the motility to 7.2% against *C. elegans* Bristol N2. On the other hand, aqueous extract reduced the motility of *C elegans* DA1316 to 18.6% while methanol extract reduced the motility to 8.3% (Table 1). The results indicated a significant difference ($p < 0.001$) between the efficacy exhibited by concentrations of the plant extracts, ivermectin and the negative control in both strains. The efficacy of ivermectin was higher and significantly different from that of the extracts and the negative control on *C. elegans* Bristol N2. However, efficacy of plant extracts was significantly higher than both the negative control and ivermectin throughout the various concentrations at 24 h as well as 48 h against *C. elegans* DA1316. Larval motility for the treatments was significantly different at 24 ($p < 0.001$) and 48 h ($p < 0.001$) for both aqueous and methanol extracts for the two *C elegans* strains. There was also a significant difference in motility under the different treatments ($p < 0.001$), treatment time ($p < 0.001$) and treatment–treatment time interaction ($p < 0.001$) for the aqueous and methanol extracts of *G. senegalensis* on *C elegans* Bristol N2 strain. Similarly, larval motility was significantly different for the treatments ($p < 0.001$), treatment time ($p < 0.001$) and their interaction ($p < 0.001$) for the two extracts on *C elegans* DA1316 strain.

Data in Table 2 indicated higher efficacy of methanol extract of *G. senegalensis* than aqueous extract and this was proven by the lower IC_{50} of 0.679 mg/ml for methanol extract compared to the IC_{50} of 0.947 exhibited by the aqueous extract against *C. elegans* Bristol N2. Similarly, methanol extract exhibited a lower IC_{50} of 0.766 mg/ml compared to the IC_{50} of 1.004 mg/ml recorded by the aqueous extract against *C. elegans* DA1316.

Discussion

Anthelmintic potential of *G. senegalensis* on larval motility of *C. elegans* was assessed in vitro as an alternative to commercial anthelmintics and in managing ivermectin-resistant parasitic nematodes. This study was done because despite the usage of *G. senegalensis* as a traditional anthelmintic in Nigeria (Akuodor et al. 2013; Ademola 2016), its activity against nematodes is unknown. This work is a corollary to Gagman et al. (2018) which evaluated the ability of *G. senegalensis* to inhibit the hatching of *C. elegans* eggs and the development of its larvae. Based on the motility inhibition thresholds (Powers et al. 1982), the aqueous extract (2 mg/ml at 48 h of treatment) of *G. senegalensis* was moderately effective

Table 2 IC_{50} of *G. senegalensis* extracts against *C. elegans* L4 larvae after 48 h of exposure

Extract	*C. elegans* Bristol N2			*C. elegans* DA1316		
	IC_{50} (mg/ml)	LB	UB	IC_{50} (mg/ml)	LB	UB
Aqueous	0.947[a]	0.879	1.021	1.004[a]	0.931	1.083
Methanol	0.679[b]	0.617	0.740	0.766[b]	0.707	0.827

IC_{50}, concentration that inhibits the motility of the larvae by 50%; UB, upper boundary; LB, lower boundary. IC_{50} values with different alphabets in the same column are significantly different based on their overlapping 95% confidence limits

(80–90% motility inhibition) against both strains of *C. elegans* while the methanol extract (2 mg/ml at 48 h of treatment) was highly effective (>90% motility inhibition). The *G. senegalensis* extracts in this study generally exhibited greater inhibition activity compared to aqueous and methanol extracts of *Cassia siamea* from Nigeria assayed on these *C. elegans* strains (Gagman et al. 2020).

Plant constituents such as secondary metabolites contribute to the biological efficacy of plant extracts. Secondary metabolites like tannins, alkaloids and flavonoids among others have been detected in extracts of *G. senegalensis* (Akuodor et al. 2013; Besier et al. 2016; Gagman et al. 2018). According to Azas et al. (2002), an alkaloid from *G. senegalensis* demonstrated the strongest antiplasmodial activity among several plants tested. Tannins also adversely affect the physiology of nematodes thereby marring developmental processes and abilities in eggs and larvae and culminating in mortality (Alonso-Díaz et al. 2011; Williams et al. 2014; Debiage et al. 2016). Work by Athanasiadou et al. (2000) also indicated the anthelmintic effects of condensed tannins on *Trichostrongylus colubriformis*. The effects include a reduction in hatching of eggs and development of larvae as well as inducing mortality (Athanasiadou et al. 2000; Min and Hart 2003). Saponins also contribute to anthelmintic activity of extracts by enhancing cell membrane permeability leading to nematode mortality (Wang et al. 2010). Alkaloids, tannins, saponins and phenols have been identified in *G. senegalensis* extracts sourced from the study location (Gagman et al. 2018; Adebayo et al. 2019), hence, the efficacy of *G. senegalensis* could be attributed to the secondary metabolites in the extracts.

The inhibitory efficacy of methanol and aqueous extracts increased with increasing concentration and treatment time, similar to the finding of Iqbal et al. (2012) where it was reported that the ovicidal and larvicidal efficacy of aqueous and methanol extracts of *Azadirachta indica* was time and concentration dependent. Also, Tariq et al. (2009) reported that the anthelmintic potency of *Artemisia absinthium* against ovine nematodes increased with increasing time and concentration. Recently, Hassan et al. (2021) also observed a concentration-dependent in vitro activity of crude ethanolic extracts of *Artemisia herba-alba*, *Allium sativa* and *Balanites aegyptiaca* on *Haemonchus contortus*. The IC_{50} of the *G. senegalensis* extracts indicates varying inhibition abilities on the *C. elegans* strains. A significant difference was observed between the IC_{50} of the aqueous and methanol extracts of *G. senegalensis* against motility of L4 larvae of *C. elegans* Bristol N2 and DA1316 ($p < 0.05$). The IC_{50} value of the aqueous extract (0.947 mg/ml) was higher than that of the methanol extract (0.679 mg/ml) against *C. elegans* Bristol N2. Similarly, the aqueous extract exhibited a higher IC_{50} value of 1.004 mg/ml compared to the methanol extract ($IC_{50} = 0.766$ mg/ml) against *C. elegans* DA1316. This relates to the findings of Elandalousi et al. (2013) and Lone et al. (2013) regarding the differential activities of plant extracts obtained with different solvents. The low performance of the aqueous extract in relation to the methanol extract could be attributed to the low diversity of metabolites in the aqueous extract compared to methanol extract (Gagman et al. 2018). While saponins, tannins and phenols were present in both extracts, alkaloids, terpenoids, flavonoids and several others were only present in the methanol extract (Gagman et al. 2018). The high diversity of metabolites in the methanol extract could be due to the ability of methanol to extract diverse compounds compared to water, which only extracted polar compounds (Tiwari et al. 2011; Iloki-Assanga et al. 2015; Nguyen et al. 2015).

The difference in effectiveness of the extracts could also be due to the different concentrations of phenolic and tannic compounds in the extracts as reported by Badar et al. (2011). Gagman et al. (2018) recorded total phenolic and tannin contents of 326.37 GAE/mg and 2.78 TAE/mg for aqueous extract of *G. senegalensis* and 288.67 GAE/mg and 3.85 TAE/mg for methanol extract, respectively. However, sometimes the difference in potency between aqueous and methanol extracts may not be due to the quantity of tannins and other phenolic compounds, but rather due to the enzyme polyphenol oxidase which is very active in aqueous extracts and causes phenol degradation rendering them inactive irrespective of their quantity. The enzyme is however inactive in methanol extracts, thereby not inhibiting the activity of polyphenols in the extracts and invariably anthelmintic activity (Tiwari et al. 2011).

The difference between the positive control (larvae treated with 0.02 µg/ml ivermectin) and treatments with the various extract concentrations for both methanolic and aqueous extracts against *C. elegans* DA1316, which is ivermectin resistant, was statistically significant. The aqueous and methanol extracts of *G. senegalensis* were effective against the motility of *C. elegans* DA1316, but the organism was resistant to 0.02 µg/ml ivermectin even after exposure for 48 h. Susceptibility of *C. elegans* DA1316 to the extracts of *G. senegalensis* may be attributed to the fact that the extracts may exhibit different modes of action against the nematode compared to ivermectin. A similar assumption was made by Kumarasingha et al. (2014) who found that extracts from *Picria felterrae* induced a stress response in *C. elegans* wild type and stress reporter (GFP-tagged) strains in a manner different from those of doramectin and levamisole. Work by Ndjonka et al. (2014) and Piña-Vázquez et al. (2017) indicated that some plant extracts and their individual

constituents were effective against various drug-resistant *C. elegans* strains, providing an alternative source of effective chemotherapeutic agents. Susceptibility of the ivermectin-resistant strain of *C. elegans* to the extracts of *G. senegalensis* in this study indicates the potential of using these extracts against drug-resistant nematodes and as a source of anthelmintic drugs against nematodes which are resistant to conventional anthelmintics. These extracts could be explored for application in diverse fields including pest and disease control (Dirar and Devkota 2021). For instance, in Adebayo et al. (2019), *G. senegalensis* was suggested as a potential source of chemotherapeutic agents against breast cancer due to its antioxidant and antiproliferative effects and constituent compounds.

Conclusions

In this study, the anthelmintic use of *G. senegalensis* as a dewormer for small ruminants in Nigerian was validated in vitro using methanol and aqueous extracts of the plant against the motility of L4 larvae of two *C. elegans* strains: Bristol N2 and DA1316. The efficacy of *G. senegalensis* on both strains indicates its utility as a natural source of anthelmintic compounds for controlling ivermectin-resistant parasitic nematodes. Further research is required to establish the active compound(s) responsible for the activity of the extracts. There is also the need to conduct other in vitro and in vivo tests using *G. senegalensis* extracts on ivermectin-resistant parasitic nematodes.

Abbreviations

ANOVA: Analysis of variance; HSD: Honestly significant difference; IC_{50}: 50% Motility inhibition concentration; M9: Minimal salts; NGM: Nematode growth medium; WMI: Worm motility inhibition.

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Authors' contributions

HAG, HA, NAIINH and SWA designed various aspects of the experiments. HA and NAIINH supervised the work. HAG conducted the experiments and wrote the first draft. HAG and SWA performed statistical analysis. All authors revised and approved the final article. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are included in this published article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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