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Full Length Research Paper

In vitro anthelmintic activity of Cotyledon *orbiculata, Hermannia depressa* and *Nicotiana glauca* extracts against parasitic gastrointestinal nematodes of livestock

N. I. Molefe[1], A. M. Tsotetsi[1,3], Anofi O. T. Ashafa[2] and O. M. M. Thekisoe[1]*

[1]Parasitology Research Program, Department of Zoology and Entomology, University of the Free State - Qwaqwa Campus, Private Bag X13, Phuthaditjhaba, 9866, South Africa.
[2]Department of Plant Sciences, University of the Free State – Qwaqwa Campus, Private Bag X13, Phuthaditjhaba, 9866, South Africa.
[3]Parasites, Vectors and Vector-borne Diseases, ARC-Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort 0110, South Africa.

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The anthelmintic activity of acetone and water extracts from the shoots of *Cotyledon orbiculata, Hermannia depressa* and *Nicotiana glauca* were investigated using the egg hatch, larval development and larval mortality assays. In all extracts tested, *C. orbiculata* water extracts at 7.5 mg/ml prevented nematode eggs from hatching with 82.63% success. Other extracts exhibited egg hatch inhibition in a degree of less than 50%. Similarly, *C. orbiculata* water extracts suppressed nematode larval development at 85.32% when the concentration of 2.5 mg/ml was used followed by 66.69% of *H. depressa* extract at 7.5 mg/ml concentration. However, *N. glauca* water and all acetone extracts induced the 100% larval development inhibition. The *in vitro* larval mortality rate revealed that the water extracts from all the plants were able to kill all larvae at 2.5 mg/ml within 48 to 96 h. The results from this study have shown that the extracts from the three plants have the potential to prevent and ameliorate diseases associated with gastrointestinal nematodes.

Key words: *Cotyledon orbiculata*, egg hatch assay, *Hermannia depressa*, larval development assay, larval mortality assay, *Nicotiana glauca*.

INTRODUCTION

Gastrointestinal nematodes are a major factor that limits small ruminant production worldwide (Cala et al., 2012) due to large economic losses resulting from reduced feed intake, impaired fertility, reduced immunity, damaged gastric function and high mortality rate (Carvalho et al., 2012). Nematosis is responsible for 28% of small stock mortality and between 3 and 8% of livestock weight loss in many countries, costing an affected country about US$2 billion per year; South Africa included (van Wyk et al., 1999; Zarlenga et al., 2001; Eguale et al., 2007).

Tembely et al. (1997) stated that in order to determine the degree of the nematode infection, several factors need to be taken into consideration such as the age of the host animal, breed type of the host, parasite species, and epidemiological patterns including husbandry practices, physiological status, as well as the environmental conditions including rainfall, temperature and humidity. Commercial drugs have been used effectively to control the infection by curing clinical symptoms and/or diseases and basically to reduce mortality rates (Molefe et al., 2012). However, repeated or indiscriminate administration of the drug on the host provides a suitable medium for nematodes to build up a wide range of resistance

*Corresponding author. E-mail: thekisoemmo@qwa.ufs.ac.za. Tel: +27-58-718-5331. Fax: +27-58-718-5444.

(Carvalho et al., 2012; Hernandez-Villegas et al., 2012). According to Carvalho et al. (2012), the first case of resistance against the commercial anthelmintic drugs was described accurately by Drudge et al. (1964) and afterwards many reports followed.

Commercial drugs are however, unaffordable and unavailable to farmers with poor socio-economic status in most of the developing countries since, in many cases, drugs are imported (Amin et al., 2009); hence, are expensive and inaccessible as they are sold at the agricultural offices for safety purposes (Tsotetsi and Mbati, 2003; Cala et al., 2012). Farmers tend to rely on the ethnoveterinary medicine, because of the un-affordability and unavailability (Eguale et al., 2011). It has already been documented that more than 80% of the population in developing countries depend on plants for medicinal needs (Fyhrquist et al., 2002). Numerous plants are capable of producing bioactive compounds such as the secondary metabolites, as a chemical defence mechanism against pathogens and predation (Sathiyamoorthy et al., 1997), and are friendlier to the environment than the synthetic chemicals used when producing anthelmintic drugs (Nwosu et al., 2006). The search is therefore ongoing at the direction of an alternative way of treating the infection using medicinal plants. The objective of this study was to determine the *in vitro* anthelmintic activity of *Cotyledon orbiculata* (Crassulaceae), *Hermannia depressa* (Malvaceae) and *Nicotiana glauca* (Solanaceae) extracts on gastro-intestinal nematodes using the egg hatch, larval development and larval mortality assays.

MATERIALS AND METHODS

Plant and preparation of extracts

Plant materials were collected in May, 2012 from multiple populations of *C. orbiculata* and *H. depressa* around Qwaqwa area in Maluti-A-Phofung Municipality of the Eastern Free State province (28°32'0"S and 28°49'0" E; altitude 1,673 m). *N. glauca* was also collected in May, 2012 from Wolmaranstad in Maquassi Hills Local Municipality of the North West province (27°12'0"S and 25°58'0"E; altitude of 1170 m) of South Africa (Wolfram, 2012). The mean annual rainfall of the Maluti-A-Phofung Municipality is about 653 mm per annum with temperatures ranging from as low as 0.1°C during July nights, 14.2°C during the day in June and maximum of 24.7°C during the day in January. The mean annual rainfall of the Maquassi Hills Local Municipality is about 391 mm per annum with temperatures ranging from as low as 0°C during July nights, 17.6°C during the day in June, and maximum of 30°C during the day in January (Wolfram, 2012). The plants were identified by Dr. A. O. T. Ashafa of the Department of Plant Sciences, University of the Free State (UFS) - Qwaqwa Campus and voucher specimens (MolMed/02/2012) were prepared and deposited at the UFS Qwaqwa Campus herbarium.

The shoots of the plants were separated and dried in an Ecortherm oven (Laboratory Consumables Pty, South Africa) at a temperature of 42°C to a constant weight before it was pulverized. Ten grams each of powdered material was extracted in acetone and distilled water. Acetone was of high analytical grade (Merck Chemicals Pty, Wadeville, South Africa). All extracts were filtered using Whatman No-1 filter paper (Whatman, United Kingdom). The filtrates from acetone were concentrated under reduced pressure at 40°C using rotary evaporator (Cole-Parmer, Laboratory Consumables and Chemical Supplies Co. Ltd, China). The water extract was freeze dried using freeze dryer (Virtis SP Scientific, United States of America). Individual extracts were reconstituted in their respective solvents to give a stock solution of 50 mg/ml (Ashafa and Afolayan, 2009). This was diluted to the required concentrations of 2.5, 5.0 and 7.5 mg/ml for the bioassay analysis.

Diagnostic methods

The McMaster technique (Soulsby, 1982; Reinecke, 1983) was used to determine nematode egg presence in this study. Briefly, 2 g of pooled faecal samples collected from sheep were mixed with 58 ml of 40% sugar solution. Samples were thoroughly crushed and mixed using a blender. Two chambers of a McMaster slide were filled with a Pasteur pipette. The slides were allowed to stand for about 4 min so that the eggs can float on the surface of the flotation medium and lie in contact with the upper glass of the chamber. A dissecting microscope (Nikon Eclipse E100 Company, Japan) was used for egg detection. Indistinguishable eggs coming from different genera such as the *Haemonchus, Trichostrongylus, Oesophagostomum* and *Chabertia* were grouped together, and recorded separately from those that were easily distinguished such as the *Nematodirus, Strongyloides* and *Trichuris. Nematodirus* eggs were distinguished by a thin and colorless shells with a length of 150 µm and a width of 75 µm, *Strongyloides* eggs are broad eclipsed, slightly flattened and embryonated with the presence of L_1 larvae and *Trichuris* eggs have typical polar plugs on both ends.

In vitro assays

Egg recovery assay

Egg recovery was conducted as according to Maphosa et al. (2010) protocol with some modifications. Four grams of collected faecal sheep pellets were weighed, then water was slowly added to them and the pellets were smashed until a relatively liquid suspension (slurry) was obtained. The slurry was then filtered through sieves of 117, 70 and 25 µm. The contents of 25 µm sieve were backwashed with distilled water and transferred into 60 ml centrifuge tubes. The suspension was allowed to stand for 30 min and the supernatant was decanted while the sediments were suspended in 40% sugar solution. The suspension was transferred into another set of tubes, allowed to stand for another 30 min and the supernatant was washed through a 25 µm pore mesh sieve using distilled water. The eggs were then washed off from the 25 µm sieve with distilled water into a 1 L conical flask where they were allowed to sediment for 2 h. The concentration of the eggs was estimated by counting the number of eggs in 2 aliquots of 0.5 ml of the suspension in a microscope slide repeatedly, and the mean number of eggs per 0.5 ml was determined.

Egg hatch assay

The egg hatch assay was conducted as published by McGaw et al. (2007) and Bizimenyera et al. (2006). The number of eggs that were contained in the egg suspension of 0.5 ml was counted and afterwards, they were pipetted into a 96-well microtitre plate. The numbered wells from 1 to 3 were used for the *C. orbiculata* experiments, from 5 to 7 for the *H. depressa* experiments and from 9 to 12 for the *N. glauca* ones. In addition, 0.5 ml of each plant extract at different concentrations of 7.5, 5 and 2.5 mg/ml were

Table 1. Inhibition percentages of *C. orbiculata, H. depressa* and *Nicotiana glauca* on egg hatch of gastrointestinal nematodes of livestock.

Concentration (mg/ml)	Water extract			Acetone extract			Control	
	C.o	H.d	N.g	C.o	H.d	N.g	+ve	-ve
2.5	38.96	39.17	15.46	7.60	22.47	15.91	100.00	0.00
5.0	19.84	22.73	10.68	17.84	7.06	4.11	100.00	0.00
7.5	82.62	40.14	19.12	10.00	7.40	7.03	100.00	0.00

C.o: *Cotyledon orbiculata*; H.d: *Hermannia depressa*; N.g: *Nicotiana glauca*; +ve: Positive control; -ve: Negative control.

added. A commercial anthelmintic drug Tramisol® (Afrivet, South Africa) was used as the positive control at the same concentrations, while distilled water was used as a negative control. All tests were repeated 3 times. The plate was incubated at the temperature of 25°C for 48 h, thereafter a drop of Lugol's iodine solution was added to each well so as to stop further hatching. All unhatched eggs and first-stage larvae (L_1) were then counted. Inhibition percentages were calculated using a formula by Cala et al. (2012):

$$E = \frac{(Eggs + L_1) - L_1}{Eggs + L_1} \times 100$$

Larval development assay

The larval development assay was conducted as described by Bizimenyera et al. (2006). The counted number of eggs in a 0.5 ml of the egg suspension was put into each well in a 96-microtitre plate with a 100 µl of lyophilized penicillin-streptomycin in order to combat fungal growth. The contents of the wells were then mixed, and the plates were placed in an incubator under humidified conditions at ambient temperature for 48 h. By this step the eggs were helped to incubate. Forty eight hours later, 0.5 ml of the water and acetone extracts of *C. orbiculata, H. depressa* and *N. glauca* as well as Tramisol® (Afrivet, South Africa) as a positive control at 7.5, 5 and 2.5 mg/ml were added to respective plates. The negative control plates received 0.5 ml of distilled water. All experiments were repeated 3 times. Incubation of the plates was continued for 5 days, after which all the plates were examined to determine the survival of larvae at different concentrations. All the third-stage larvae (L_3) in each well were counted and a percentage inhibition of larval development was calculated using the formula described by Cala et al. (2012):

$$E = \frac{(L_1 + L_2 + L_3) - L_3}{L_1 + L_2 + L_3} \times 100$$

Larval mortality assay

The larval mortality assay was conducted according to the method described by McGaw et al. (2000) and Zafar et al. (2006) and modified by Molefe et al. (2012). In specific, *in vitro* cultures from nematode eggs were prepared after collection from microscopically positive sheep's faecal samples. After seven days of incubation, the eggs hatched into larvae. L_3 larvae were harvested from the *in vitro* cultures prepared and were transferred into a single petri dish. The 150 µl of L_3 larvae solution were placed in microtitre plate and crude extracts of the same volume were added at different concentrations, Tramisol® (Afrivet, South Africa) was used as positive control and distilled water as a negative control. After the addition of extracts, larval counts were conducted on daily basis and the larval mortality rates were recorded until all the larvae had died. All tests were repeated three times. All live and motile L_3 stage larvae in each well were counted and a percentage inhibition of larval development was calculated using the formula described by Coles et al. (1992) and Bizimenyera et al. (2006) with slight changes:

Inhibition percentage (%) = $100(1-X_1/X_2)$

where X_1 is the initial number of larvae in test extracts pre-treatment, and X_2 is the number of larvae that obtained post-treatment

Statistical analysis

Kruskal-Wallis test was used to analyse the egg hatch and larval mortality assays in order to determine if there was significant difference in the hatched eggs and larval mortality from different treatments with different concentrations (Kuskal and Wallis, 1952).

RESULTS

In this study, the nematode species specification was made according to the McMaster technique which included *Haemonchus*, *Oesophagostomum*, *Trichostrongylus* or *Teladorsagia*. At the concentration of 2.5 mg/ml, the water extracts inhibited 38.96, 39.17 and 15.46% egg hatch, whereas the acetone extracts exhibited 7.60, 22.47 and 15.91% for *C. orbiculata, H, depressa* and *N. glauca,* respectively. Similarly, at 5.0 mg/ml, the water extracts inhibited 19.84, 22.73 and 10.68%, while the acetone extracts showed 17.84, 7.06 and 4.11% egg hatch inhibition. Moreover, at 7.5 mg/ml, the water extract displayed 82.62, 40.14 and 19.12% inhibition, whereas the acetone extracts exhibited 10.00, 7.40 and 7.03 egg hatch inhibition (Table 1). The controls gave 100% of egg inhibition for the positive and 0% for the negative.The water extracts of *C. orbiculata* inhibited 85.32, 81.80 and 78.88% inhibition of larval development and for the same kind of extracts, *H. depressa*, there was an inhibition of 44.12, 58.16 and 66.69% for the larval development at 2.5, 5.0 and 7.5 mg/ml, respectively.

Table 2. Inhibition percentages of *C. orbiculata, H. depressa* and *Nicotiana glauca* on larval development of gastrointestinal neamtodes of livestock.

Concentration (mg/ml)	Water extract			Acetone extract			Control	
	C.o	H.d	N.g	C.o	H.d	N.g	+ve	-ve
2.5	85.32	44.12	100.00	100.00	100.00	100.00	100.00	0.00
5.0	81.80	58.16	100.00	100.00	100.00	100.00	100.00	0.00
7.5	78.88	66.69	100.00	100.00	100.00	100.00	100.00	0.00

C.o: *Cotyledon orbiculata*; H.d: *Hermannia depressa*; N.g: *Nicotiana glauca*; +ve: Positive control; -ve: Negative control.

Table 3. Effects of *C. orbiculata* extracts on larval mortality of gastrointestinal nematode of livestock.

Time (h)	Water extract (mg/ml)			Acetone extract (mg/ml)			Control	
	2.5	5.0	7.5	2.5	5.0	7.5	Positive	Negative
0	0	0	0	0	0	0	0	0
6	42.29	29.76	48.88	56.39	64.36	55.63	100	0
24	98.6	96.13	98.75	81.869	80.1896	87.39	100	0
48	100	100	100	92.3	83.02	95.04	100	0
72	*	*	*	95.599	90.56	99.55	100	0
96	*	*	*	100	96.22	100	100	0
120	*	*	*	**	100	**	100	0

*All larvae died within 48 h. **All larvae died within 96 h.

Moreover, *N. glauca* water and acetone extracts as well as acetone extracts of *C. orbiculata* and *H. depressa* induced a 100% larval development inhibition (Table 2). Positive control induced a 100% larval development inhibition whereas the negative control inhibited 0% of pre-infective larvae from developing to the infective stage.

Generally, the water extract of *C. orbiculata* displayed the highest egg hatch inhibition percentage of 82.62% at 7.5 mg/ml followed by water extract of *H. depressa* with 40.12% also at 7.5 mg/ml. The egg hatch lowest inhibition percentage was observed from *N. glauca* acetone extracts at 5.0 mg/ml with 4.11%. In all cases, larval development inhibition percentages were higher than 50%, except for *H. depressa* water extracts which exhibited 44.12% at 2.5 mg/ml.

The aqueous extracts proved to be much effective on the infective stage larvae than on the pre-infective stage larvae. All experimental larvae mortality was observed within 48, 96 and 72 h of experimentation in water extracts of *C. orbiculata, H. depressa and N. glauca*, respectively. Acetone extracts of all plants were less effective, as more time than expected was necessary to totally kill the experimental larvae. The *C. orbiculata* acetone extracts duration differed with concentrations, however, the results were not concentration dependent whereby all the larvae died within 96, 120 and 96 h at 2.5, 5.0 and 7.5 mg/ml, respectively (Table 3). The *H. depressa* acetone extracts duration was constant for all concentrations with all larval mortality to have been observed within 120 h (Table 4). About 192 h was needed to result in a total larval mortality from *N. glauca* acetone extract with 5.0 mg/ml concentration which was the longest experimental time. The 2.5 mg/ml and 5.0 mg/ml took 120 and 144 h for total mortality of experimental larvae, however, it took longer with the 5.0 mg/ml concentration (Table 5).

DISCUSSION

In this study, microscopic diagnosis from sheep faecal samples revealed the presence of *Haemonchus, Oesophagostomum, Trichostrongylus* or *Teladorsagia* which have been documented as medically important species of gastrointestinal nematodes worldwide (Coles et al., 2006). The anthelmintic activity of three medicinal plants, namely, *C. orbiculata, H. depressa* and *N. glauca* was therefore determined on the eggs and the larvae of these gastrointestinal nematodes of livestock.

The major finding on this study was the high effectiveness of aqueous extracts as compared to acetone extracts on the egg hatch and larval mortality assay. According to Maphosa et al. (2010), anthelmintic activity of medicinal plants in most cases result in unexpected manner, because of different conditions in gastrointestinal tract and the *in vitro* provided conditions. In the egg hatch assay, water extracts proved to be much more ovicidal than the acetone ones, in all concentrations of all tested plants. Similar results have been validated by

Table 4. Effects of *H. depressa* extracts on larval mortality of gastrointestinal nematode of livestock.

Time (h)	Water extract (mg/ml)			Acetone extract (mg/ml)			Control	
	2.5	5.0	7.5	2.5	5.0	7.5	Positive	Negative
0	0	0	0	0	0	0	0	0
6	51.59	54.51	49.64	36.21	40.69	25.44	100	0
24	98.05	99.76	100	53.61	53.6818	31.8438	100	0
48	97.08	100	*	92.7797	87.8766	86.9063	100	0
72	98.3	**	*	90.4985	98.487	91.9643	100	0
96	100	**	*	99.2358	99.7857	99.4063	100	0
120	***	**	*	100	100	100	100	0

*All larvae died within 24 h. **All larvae died within 48 h. ***All larvae died within 96 h.

Table 5. Effects of *N. glauca* extracts on larval mortality of gastrointestinal nematode of livestock.

Time (h)	Water extracts (mg/ml)			Acetone extracts (mg/ml)			Controls	
	2.5	5.0	7.5	2.5	5.0	7.5	Positive	Negative
0	0	0	0	0	0	0	0	0
6	41.09	31.45	66.06	30.56	36.97	42.13	100	0
24	80.3	84.03	76.08	46.25	53.0359	62.9252	100	0
48	98.74	91.89	99.32	56.6688	64.6955	62.6168	100	0
72	100	100	100	62.9188	82.4268	78.8131	100	0
96	*	*	*	86.25	91.2158	97.1963	100	0
120	*	*	*	100	98.5623	96.1963	100	0
144	*	*	*	**	99.52	100	100	0
168	*	*	*	**	99.86	***	100	5.00
192	*	*	*	**	100	***	100	5.63

*All larvae died within 96 h. **All larvae died within 120 h. ***All larvae died within 144 h.

Maphosa et al. (2010) where water extracts of *Leonotis leonurus, Aloe ferox* and *Elephantorrhiza elephantine* were effective on the known problematic helminths, such as *Haemonchus contortus* and inhibited 11.5% of eggs from hatching.

From the larval development assay, *N. glauca* extracts induced 100% larval development inhibition in both water and acetone extracts. The *C. orbiculata* and *H. depressa* plants resulted in 85.32 and 44.12% larval development inhibition at 2.5 mg/ml concentration. However, acetone extracts of these two plants induced a 100% larval development inhibition. The inhibition of larval development was most probably due to the mortality of the pre-infective larvae.

All larvae died in the larval mortality assay and the only difference was found in the duration it took for the larvae to die. Water extracts proved to be more effective than the acetone extracts. Despite the concentration, water extracts of all the plants resulted in a total death of all the larvae within 48, 96 and 72 h for *C. orbiculata, H. depressa* and *N. glauca*. However, acetone extracts' total larval mortality was observed within 120 (*C. orbiculata*), 120 (*H. depressa*) and 192 (*N. glauca*) h of experimentation. The first larval mortality check was performed within 6 h post-treatment, within which all the larvae in the positive control had died.

Maphosa et al. (2010) stated that the efficacy of any plant extracts at the lowest concentrations against the gastrointestinal nematodes proves the anthelmintic activity of that plant. It is therefore concluded that *C. orbiculata, H. depressa* and *N. glauca* have an anthelmintic activity. Overall, the study revealed that the aqueous extracts of the shoots of all tested plants were more effective than acetone extracts concerning the egg hatch and larval mortality assays.

The ability of eggs and larvae of the gastrointestinal nematodes to withstand at unfavourable environmental conditions depend on the species and developmental stage of the parasite as well as the geo-ecological regions (Tembley, 1998).

Egg inhibition was observed in the current study, however, not all eggs were inhibited from hatching and most managed to hatch, this might be because the egg is the stage disseminated into the environment and protected with a thick wall making it resistant to various environmental conditions (Hounzangbe-Adote et al., 2005). According to Cheah and Rajamanickan (1997), the most favourable condition for the development of L_1

larvae to L_3 larvae are fairly high temperature in order to facilitate a rapid development rate and adequate humidity. The most susceptible stage of the larvae is the pre-infective one, the feeding stage in order to develop to the infective stage, however, it is the much exposed to medicinal components than the eggs, hence 100% larval development inhibition rate (Molan et al., 2003). The plants that took part in the current study led to a total larval mortality at different durations and concentrations. Significant anthelmintic effects of both acetone and water extracts of the three plants on the L_3 larvae were observed in terms of paralysis and death in different hours post treatment with greater activity in crude acetone and crude aqueous extracts as confirmed previously by Tariq et al. (2009). The *N. glauca* at 5.0 mg/ml killed larvae with a reduction of larvae in the negative control, which might be due to lack of nutrients or exhaustion in the negative control (Adamu et al., 2010). Chemical constituents can vary considerably between individual plant species due to genetic or environmental differences, age or developmental stage at harvesting, method of plant material drying, the storage technique and the type of solvent (Hördegen et al., 2003; Ononuju and Nzenwa, 2011). In this study, acetone and aqueous extracts were selected to be used, because not every medicinal component is water soluble. Acetone extract has been documented as suitable extractant in most cases, because it has the ability of extracting compounds from a wide variety of polarity range (Bizimenyera et al., 2006). Chemical constituents play a major role in the plant activities such as the anthelmintic activity, antimicrobial, etc. The *C. orbiculata* has been documented to carry saponins, cardiac glycosides, tannins, reducing sugars and triterpene steroids (Amabeoku et al., 2007), whereas *H. depressa* contains tannins and about less than 1% of cardiac glycosides (Reid et al., 2005) and *N. glauca* with nicotine and anabasine (Mhinana et al., 2010). Each of the chemical components has its own function. For example, the presence of tannins in plants is characterized with the connection to free proteins or to the larval cuticle, reducing the availability of nutrients which in turn results in the death of the larvae due to starvation; this is because tannins are rich in glycoproteins (Cala et al., 2012).

The *N. glauca* activity might be due to the presence of nicotine, the piperidine alkaloid from tobacco plant which can be toxic in high doses to both animals and humans. It is also soluble in water therefore explaining the high effectivity of water extracts (Webb and Dalzell, 1997), and anabasine as a highly toxic piperidine like alkaloid constituting about 70% of the plant as a whole (Mizrachi et al., 2000). *C. orbiculata, H. depressa* and *N. glauca* are therefore good candidates for treatment of gastro-intestinal larval infections. However, the mechanisms of their effectiveness still remain to be tested *in vivo.* Furthermore, safety and toxicity studies must be conducted *in vivo* to determine the minimum non-lethal concentrations needed for the treatment of nematode infections.

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