ARTICLE / INVESTIGACIÓN

Effect of aqueous extract of *Azadirachta indica* leaves on gastrointestinal nematodes in ruminants: an *in vitro* study

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Abstract: High rates of gastrointestinal parasite infestation lead to the frequent use of anthelmintics for their control, resulting in resistance in the main species of gastrointestinal nematodes. Natural plant extracts, particularly those from *Azadirachta indica* A. Juss leaves, have been described as having antiparasitic, antimicrobial, insecticidal, antioxidant, and anticorrosive activities, which are related to the diversity of their chemical composition. This research aimed to evaluate the effect of the aqueous extract of *A. indica* leaves on the control of gastrointestinal nematodes in ruminants. To achieve this, an in vitro test was performed using the larval development assay, with fecal samples obtained from Pelibuey sheep, Criolla goats in the development category, and Criolla calf cattle for the culture of gastrointestinal nematode larvae. Three concentrations (minimum, medium, and maximum) were evaluated for each animal species against *Haemonchus placei* and *Cooperia* sp larvae for cattle and *Haemonchus contortus*, *Trichostrongylus sp*, and *Trichuris* for sheep and goats. The survival percentage indicator was evaluated at 0, 24, 48, and 72 h. The research concluded that the minimum concentration was valid for sheep and goats but not for cattle, and the medium and maximum concentrations were effective against all three animal species, resulting in 100% larval mortality.

Key words: *Azadirachta*, drug effects, Ruminants, *Trichostrongyloidea*.

Introduction

Gastrointestinal strongylid infestations are a significant economic limitation to developing bovine husbandry in tropical conditions. Especially when *Haemonchus spp*. predominates, it can cause blood loss, resulting in a rapid drop in hematocrit values, low feed conversion, difficulties in weight gain, loss of appetite, growth retardation, and even death. Young animals are particularly susceptible, and these factors translate into economic losses for farmers[1,2]. Gastrointestinal helminths also affect farming systems worldwide[3]. These parasitic infections disrupt nutrient absorption in animals, resulting in a reduction in body weight and an increased susceptibility to secondary infections[4].

An additional promising alternative for parasite control is plant extracts, based on an ethnobotanical concept that exploits the accumulated knowledge of indigenous communities in tropical America[5]. Using plants with anthelmintic properties has become one of the most exciting alternatives in recent years[6,7]. Traditional medicinal plants have proven significant as an alternative to anthelmintics in developing and developed countries. Additionally, the use of medicinal plants is a tradition in many countries[8-11].

Previous findings related to traditional medicinal plants have shown that many plant species act as anthelmintics and are an alternative to conventional anthelmintics[12]. They can help reduce parasite influx in livestock[13,14], and are sustainable and ecologically acceptable15. Among the most commonly used species for this purpose is Neem (*Azadirachta indica* A. Juss. Meliaceae)[16,17]. Extracts from this tree have been widely used to promote health since ancient times due to its wide variety of therapeutic properties[18].

Different studies report the antiparasitic effect of *A. indica* leaves in sheep[19-21], with variable results. These could be due to different reasons, such as the type of extract, method of material collection used, as well as the phenological state of the plant, which could modify the type and concentration of active compounds[22]. Cruz *et al.*[21] demonstrated a significant reduction in the excretion of gastrointestinal nematode eggs after administering 0.8 g/kg of aqueous extract of *A. indica* leaves. On the other hand, previous studies have evaluated the anthelmintic effect of *A. indica* leaves in *Haemonchus contortus* in goats[23-26] and bovine strongylosis[27].

Due to the growing challenge of antihelmintic resistance against common drugs, there is a great need to explore natural resources that can replace these compounds due to their therapeutic action against gastrointestinal nematodes. In this sense, this research aimed to evaluate the effect of an aqueous extract of *A. indica* leaves on controlling gastrointestinal nematodes in ruminants.

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Materials and methods

This study was conducted at the microbiology laboratory and the Juan Tomás Roig Experimental Station of the University of Ciego de Ávila. The Department of Natural Products facilities at the Bioplants Center in Cuba were also used.

Preparation of the extract

To obtain the powdered extract from Neem (*Azadirachta indica* A. Juss) leaves, they were manually harvested in February 2022 from plants established at the Experimental Station. Immature fruits were removed, and the leaves were washed with running water and then sun-dried on racks for 24 hours, as recommended by Schmutterer[28], to prevent the decomposition of active principles. The dried leaves were collected in a nylon bag and placed in an oven at 60°C for 72 h until they were crispy and crumbled when touched[19].

The dried leaves were ground to obtain a light green powder with particles of 1 mm and a moisture content of 8 to 10%[19]. The aqueous extract was obtained using the method developed by Toledo *et al.*[29]. The aqueous extraction process was carried out in the afternoon before the day of the animal treatment to avoid fermentation of the sugars contained in Neem, which leads to the loss of its properties[19]. For the development of this method, the powdered extract was mixed with distilled water at a temperature of 45°C in an electric mixer until the mixture was homogeneous (Figure 1).

Determination of extraction yield

To determine the extraction yield, aliquots of 1 ml (in triplicate) were taken from the obtained extracts and concentrated to dryness at 60°C in a Speed Vac SC100 Savant, Russia. The resulting product was weighed, and the extraction yield was determined in mg of dry extract per ml. The three concentrations to be evaluated were obtained by diluting the extract in water based on the yield calculation.

In vitro test

An in vitro test was performed using the larval development inhibition test in a 96-well plate to evaluate the effect of the crude aqueous extract. Samples of fecal matter were obtained from 18 female animals, including 6 Pelibuey sheep, 6 Criollo goats, and 6 Criollo breed calves.

Larvae identification

The larvae identified in the experiment belong to the order Strongylida, of the superfamily Trichostrongylidae, for cattle (*Haemonchus placei* and *Cooperia sp.*) and sheep-goat (*Haemonchus contortus, Trichostrongylus sp*, and T*richuris*).

Experimental design and statistical analysis

The experimental design consisted of three treatments and two control groups: positive control (Ivermectin 100 mg/ml) and negative control (Distilled water). The treatments consisted of three concentrations (0.06 mg/ml, 0.07 mg/ml, and 0.08 mg/ml of crude extract of *A. indica* leaves) for each animal species, with 6 wells per triplicate for 100 larvae per well, divided into three assays. The survival percentage was determined at 0, 24, 48, and 72 h after adding the extract. The statistical analysis was performed using IBM SPSS Statistics version 23, and a two-factor ANOVA was conducted.

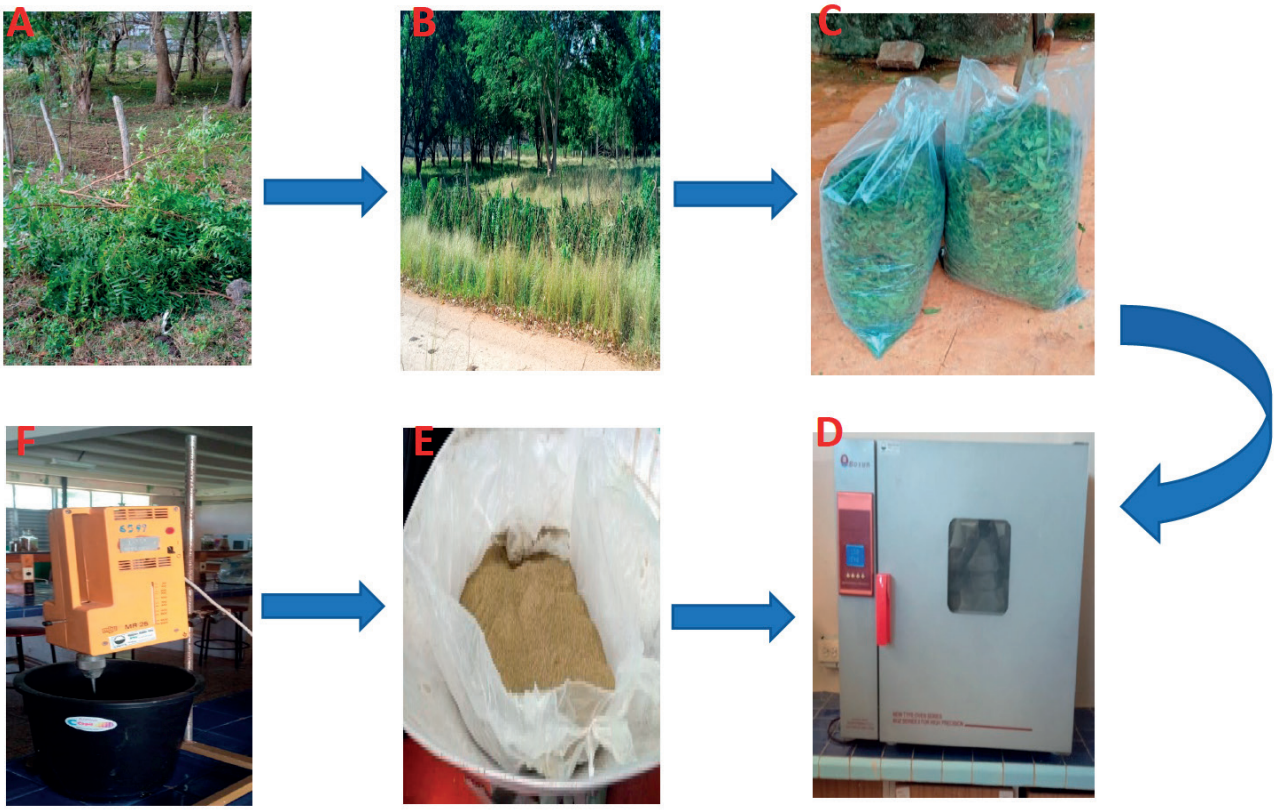

Figure 1. Scheme for obtaining the aqueous extract from Neem (*Azadirachta indica* A. Juss) leaves. A (Leaf harvesting), B (Sun-drying), C (Leaf collection), D (Oven-drying), E (Grinding), F (Mixture).

Results

Trial 1 (Sheep)

Table 1 shows the larval survival percentages for sheep. It was demonstrated that there are statistically significant differences in the effect of exposure time and concentration of the aqueous extract of *A. indica* leaves on larval survival.

The highest concentration (0.08 mg/ml) demonstrated better effectiveness at 24 and 48 h, with the lowest percentages of larval survival, below that of Ivermectin but higher than the other concentrations. All concentrations tested showed effectiveness at 72 h, with 0% larval survival (100% larval mortality), showing a similar behavior to Ivermectin.

Trial 2 (Goat)

Table 2 displays the percentage of larval survival for all concentrations tested at each time point for goat species. The results showed that at 24 and 48 h, the highest concentration of the crude aqueous extract from *A. indica* leaves (0.08 mg/ml) demonstrated the most excellent effectiveness, with the lowest percentages of larval survival among all the concentrations tested. However, the larval mortality rate was still slightly below that of Ivermectin.

For the goat species, the crude aqueous extract from *A. indica* leaves was effective for all three concentrations, showing the lowest larval survival percentages (0%) at 72 hours, with no differences between the treatments and Ivermectin. All extract concentrations showed effectiveness at 72 hours, with 0% larval survival, indicating 100% larval mortality. This result is similar to Ivermectin, commonly used as a parasiticide in veterinary medicine.

The results of the second experiment (goat trial) showed that the behavior of the concentrations of the aqueous extract from *A. indica* leaves on larval survival during the experimental times was similar to the first experiment (sheep trial). This is because the larvae used in both experiments belonged to the same taxonomic family and genus for both sheep and goat species. As a result, the aqueous extract from *A. indica* leaves was effective for all three concentrations tested in the goat species, with the lowest larval sur-

Treatment	Survival (%)			
	0 h	24 h	48 h	72 h
CAE 0.06 mg/ml	100 ±0 ^{i}	78.55 ±1.04 ^{h}	40.88 ±1.26 ^{e}	0 ±0 ^{a}
CAE 0.07 mg/ml	100 ±0 ^{i}	73.16 ±1.09 ^{g}	33.66 ±0.98 ^{d}	0 ±0 ^{a}
CAE 0.08 mg/ml	100 ±0 ^{i}	66.44±0.92 ^{f}	25.83 ±0.88 ^{c}	0 ±0 ^{a}
Ivermectin 100 mg/ml	100 ±0 ^{i}	14.94 ±0.89 ^{b}	0 ±0 ^{a}	0 ±0 ^{a}
Distilled water	100 ±0 ^{i}	100 ±0 ^{i}	100 ±0 ^{i}	100 ±0 ^{i}

The means with different letters differ significantly for the data transformed according to the formula: y′=2arcsin (sqrt(y/100)). (Two-way ANOVA and Tukey's test for $p \leq 0.001$, n=18).

Table 1. In vitro larvicidal effect of crude aqueous extract (CAE) from Azadirachta indica leaves on sheep gastrointestinal nematodes: impact of exposure time and concentration.

Treatment	Survival (%)			
	0 h	24 h	48 h	72 h
CAE 0.06 mg/ml	100 ±0 ^{h}	74.00 ±1.13 ^{g}	32.55 ±1.04 ^{d}	0±0 ^{a}
CAE 0.07 mg/ml	100 ±0 ^{h}	68.16 ±0.92 ^{f}	27.66 ±1.15 ^{c}	0 ±0 ^{a}
CAE 0.08 mg/ml	100 ±0 ^{h}	56.38±0.92 ^{e}	16.33 ±0.91 ^{b}	0 ±0 ^{a}
Ivermectin 100 mg/ml	100 ±0 ^{h}	7.38 ±1.06 ^{a}	0 ±0 ^{a}	0 ±0 ^{a}
Distilled water	100 ±0 ^{h}	100 ±0 ^{h}	100 ±0 ^{h}	100 ±0 ^{h}

The means with different letters differ significantly for the data transformed according to the formula: y′=2arcsin (sqrt(y/100)). (Two-way ANOVA and Tukey's test for $p \leq 0.001$, n=18).

Table 2. In vitro larvicidal effect of crude aqueous extract (CAE) from A. indica leaves on goat gastrointestinal nematodes: impact of exposure time and concentration.

vival percentages (0%) at 72 hours, similar to the results observed in the sheep species.

These findings demonstrate that the aqueous extract from *A. indica* leaves has a larvicidal effect on gastrointestinal nematodes in both sheep and goat species, reducing the number of larvae and their subsequent death during the experimental times.

Trial 3 (Bovine)

The results of the third experiment on the effect of exposure time of concentrations of the aqueous extract from *A. indica* leaves on the survival of larvae from the bovine species showed differences between the concentrations tested, as indicated in Table 3. However, there were no significant differences between the minimum concentration (0.06 mg/ml) and the control group (distilled water). These findings suggest that for the *Haemonchus placei* and *Cooperia sp larvae* of the bovine species evaluated in this experiment, the concentration of 0.06 mg/ml is not adequate. Therefore, for in vivo tests in this animal species to validate this *in vitro* experiment, concentrations starting from 0.07 mg/ml or higher should be used.

In the third experiment, only the medium (0.07 mg/ml) and the highest (0.08 mg/ml) concentrations of the crude aqueous extract from *A. indica* leaves showed effectiveness at 72 h, with 0% larval survival (100% larval mortality), which is similar to the results observed for Ivermectin. However, at the start of the experiment (0 h), all larvae showed the highest percentage of survival (100%), indicating active motility.

The highest concentration (0.08 mg/ml) demonstrated better effectiveness at 24 and 48 h, with the lowest percentages of larval survival, although the results were still below those observed with Ivermectin. These findings are consistent with the results observed in the previous experiments. These results suggest that the aqueous extract from *A. indica* leaves has potential as a larvicidal agent against gastrointestinal nematodes in bovine species. However, further research is needed to evaluate the safety and efficacy of this extract *in vivo*.

Overall, the findings of this study demonstrate that the crude aqueous extract from *A. indica* leaves has a significant anthelmintic effect against gastrointestinal nematodes in ruminants. The extract could be a natural alternative to synthetic anthelmintics for veterinary clinical treatments in animal production systems. However, further research is needed to evaluate the safety and efficacy of the extract in vivo.

Discussion

The study demonstrated that the aqueous extract of *A. indica* leaves has anthelmintic properties against sheep, goats, and cattle nematodes, suppressing larval development. Neem extract may interfere with the development and molting of parasitic nematodes. By disrupting the formation of new cuticles, Neem can disrupt the life cycle and decrease their ability to multiply. Jabbar *et al.*[31] reported that *A. indica* leaves have been used in popular veterinary medicine as an anthelmintic for ruminants. However, Vieira *et al.*[32] did not observe any anthelmintic effect of Neem at a dose of 30 g of dried leaves per goat/day for 5 days.

In another study by Rafique *et al.*[15], the efficacy of aqueous, methanolic, and ethanolic extracts of dried leaves of medicinal plants Moringa oleifera and *A. indica* was tested for ovicidal and larvicidal activities *in vitro* against *Haemonchus, Trichuris, Coccidia*, and *Trichostrongylus* of wild sheep. The study evaluated six concentrations of these plant extracts (ranging from 1.56 to 50 mg/ml), which were different from those evaluated in the present experiment, and used the egg hatch assay (EHA) and larval development assay (LDA) in three replicates. The inhibitory effect on larval development was found in aqueous, methanolic, and ethanolic extracts of *M. oleifera*, with lethal concentration values (LC50) of 4.15 mg/ml, 2.75 mg/ml, and 1.9 mg/ml, respectively. Aqueous, methanolic, and ethanolic extracts of *A. indica* also showed inhibitory effects with LC50 values of 3.35 mg/ml, 1.89 mg/ml, and 2.85 mg/ml, respectively. The overall findings of this study demonstrate that the leaf extracts of *M. oleifera* and *A. indica* possess significant anthelmintic efficacy against sheep GIN, and could be a natural alternative to synthetic anthelmintics to treat worm infections in animals.

In a study by Wondimu & Bayu[26], the *in vitro* and *in vivo* anthelmintic effects of crude methanolic extracts of leaves

Treatment	Survival (%)			
	0 h	24 h	48 h	72 h
CAE 0.06 mg/ml	100 ±0 ^{g}	100 ±0 ^{g}	100 ±0 ^{g}	100 ±0 ^{g}
CAE 0.07 mg/ml	100 ±0 ^{g}	76.9 ±0.89 ^{f}	30.7 ±1.12 ^{d}	0 ±0 ^{a}
CAE 0.08 mg/ml	100 ±0 ^{g}	66 ±0.87 ^{e}	25.9 ±0.89 ^{c}	0 ±0 ^{a}
Ivermectin 100 mg/ml	100 ±0 ^{g}	18 ±1.20 ^{b}	4.1±0.91 ^{a}	0 ±0 ^{a}
Distilled water	100 ±0 ^{g}	100 ±0 ^{g}	100 ±0 ^{g}	100 ±0 ^{g}

The means with different letters differ significantly for the data transformed according to the formula: y´=2arcsin (sqrt(y/100)). (Two-way ANOVA and Tukey's test for $p \leq 0.001$, n=18).

Table 3. In vitro larvicidal effect of crude aqueous extract (CAE) from A. indica leaves on bovine gastrointestinal nematodes: impact of exposure time and concentration.

of *A. indica*, *Vernonia amygdalina, Nicotiana tabacum, M. oleifera, Croton macrostachyus*, and *Hagenia abyssinica* were investigated against gastrointestinal nematodes of the strongylus type in goats. The researchers used three graduated concentrations of crude extract (100 mg/ml, 50 mg/ml, and 25 mg/ml). They evaluated the *in vitro* anthelmintic effects using standard larval development assay, larval inhibition, and egg hatch tests. The results showed that crude extracts of *N. tabacum*, *V. amygdalina*, and *C. macrostachyus* were promising for the control of gastrointestinal nematodes, with a high ($p<0.05$) inhibition of egg hatch *in vitro* for *C. macrostachyus* compared to other plant species. The lowest inhibition was observed for *M. oleifera* and *H. abyssinica*, while *N. tabacum* caused 100% larvae (L3) mortality in three hours at 100 mg/ml and 50 mg/ml, unlike other plant extracts that did not show a substantial effect. The development of L1 to L3 larvae was stopped after exposure to extracts of *N. tabacum, V. amygdalina*, and *C. macrostachyus*. This study infers that crude methanolic extracts of *A. indica* leaves do not have biological activity against eggs and larvae of gastrointestinal nematodes of goats. However, in the present experiment, the crude aqueous extract of *A. indica* leaves showed in vitro biological activity with 100% larval mortality 72 h after applying the plant extract.

There are no published studies on the in vitro anthelmintic effect of *A. indica* on gastrointestinal nematode larvae of cattle. This medicinal plant is known to be rich in compounds that have antiparasitic, anti-inflammatory, and antimicrobial activities, such as azadirachtin, salannin, terpenoids, meliantriol, nimbin, saponins, alkaloids, tannins, acids, and steroids[33,34]. These agents target parasites and may have protective properties on organs in hosts infected by parasites[35,36].

Conclusions

In conclusion, the current study demonstrated that the crude aqueous extract of *A. indica* leaves has *in vitro* anthelmintic activity against sheep, goats, and cattle gastrointestinal nematodes. The effective concentration of the extract was 0.06 mg/ml for sheep and goats but not for cattle. Concentrations of 0.07 mg/ml and 0.08 mg/ml were adequate for all three animal species, resulting in 100% larval mortality. These results suggest that *A. indica* leaf extract could be a natural alternative to synthetic anthelmintics for treating animal worm infections. However, more studies are needed to determine its efficacy and safety in live animals before widespread use.

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Conflicts of Interest

The authors declare no conflict of interest.

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