In Vitro and In Vivo Anthelmintic Effects of Medicinal Plants Against Gastrointestinal Nematodes of Goats at Haramaya University Farm, Eastern Ethiopia

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Abstract: A study was conducted to investigate the *in-vitro* and *in vivo* anthelmintic effects of crude methanol extracts of *Azadirachta indica, Vernonia amygdalina, Nicotiana tabacum, Moringa oleifera, Croton macrostachyus,* and *Hagenia abyssinica* against gastrointestinal nematodes of goats. The plants were collected from East Hararghe Zone during November 2017 to March 2018. Leaves from each plant species were separately collected, dried, ground, and crude methanol soluble was extracted. Three graded concentration of crude extract of 100mg/ml, 50mg/ml and 25mg/ml were prepared and evaluated for *in vitro* anthelmintic effects using standard techniques of larval development assay, larvae inhibition and egg hatch tests. For the egg hatch test, the wells containing about 100 eggs/ml were incubated at 27°C for 48 hours and evaluated based on the characteristics such as dead, embryonated, or hatched egg to L_1. Similar egg concentrations were used for larval development assays and exposed to various concentrations of the plant extracts to evaluate the development of eggs to infective larvae (L_3) over a period of six to seven days. For the larvae inhibition test, approximately 100 larvae were placed into test tubes containing plant extracts. The results were evaluated by adding a drop of warm water and counting of active and dead larvae at an interval of three hours, for a total period of twelve hours. For the *in vivo* trial, 1g/kg of the crude extract of the plants were prepared and administered via oral drenching. High ($p<0.05$) percentage of *in vitro* egg hatchability inhibition was recorded for *Croton macrostachyus* as compared to the other plant species while the lowest inhibition was recorded for *Moringa oleifera* and *Hagenia abyssinica*. *Nicotiana tabacum* caused 100% larval (L_3) mortality within three hours at 100mg/ml and 50mg/ml while the other plants extract did not show substantial effect. The development of larvae from L_1 to L_3 was arrested following exposure to extracts of *Nicotiana tabacum, Vernonia amygdalina,* and *Croton macrostachyus*. *In vivo* test result showed 96.6% fecal egg count reduction in crude extract of *Nicotiana tabacum* drenching, which was better ($p<0.05$) than the other plants tested and the positive control. In general, the *in vitro* and *in vivo* results show that crude extracts of *Nicotiana tabacum, Vernonia amygdalina,* and *Croton macrostachyus* were promising for the control of gastrointestinal nematodes. Thus, further studies are required to evaluate toxicity, dose and active ingredients/chemicals determination of crude extracts of these plants for possible production of alternative natural anthelmintic.

Keywords: *Anthelmintic effects, Gastrointestinal nematodes, Goats, Medicinal plant extracts.*

Introduction

Helminthosis is among the most prevalent and important disease threat to livestock industry in the developing world. Studies indicated that infection by nematodes in small ruminants is highly prevalent and widespread in all agro-ecologies of Ethiopia (Bersissa, and Abebe 2006; Bersissa *et al.,* 2008). They cause direct effect by heavy production losses and the death of significant number of young ruminant animals. It is also associated with indirect economic losses due to high cost of anthelmintic drugs and considerable losses of parts or the entire carcasses during meat inspection (Pessoa *et al.,* 2002).

The most common control method of gastrointestinal (GI) nematodes is largely based on repeated use of chemical anthelmintic drugs. In recent years, the emergence of resistant parasites to the commonly used anthelmintic drugs (albendazole, levamisole, teramisole and ivermectin), has been limiting their use in different parts of the world increasing the threat to livestock industry (Mideo *et al*., 2013). Several studies from different parts of the world have confirmed the occurrence of anthelmintic resistance against the commonly used anthelmintic drugs (Chartier *et al.,* 2001; Howell *et al.,* 2008; Bersissa and Girma, 2009; Crook *et al.,* 2016). Moreover, there is an increasing global public health threat due to drug residues in meat and milk (WHO, 1996; Gasbarre *et al*., 2001) and the environmental impact of drug residues in animal feces (Herd and Wardhaugh, 1993). These growing threat demand researchers to focus on searching for new approaches of GI nematodes control including pharmacological screening of medicinal plants. Medicinal plants are considered as affluent resources of ingredients which can be used in drug development and synthesis (Rasool, 2012). Traditional medicine is also an accessible and affordable treatment option practiced in developing countries (Bussmann

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and Glenn, 2010). Attempts are being made by different scholars to find naturally occurring plants that have anthelmintic activity (Bachaya *et al.,* 2009).

Uses of medicinal plants for the treatment and/or control of human and livestock ailments has been studied by several scholars in different parts of Ethiopia (Mirutse *et al*., 2003; Teferi and Hahn, 2003; Tigist *et al*., 2007; Ermias *et al*., 2008; Fisseha *et al.,* 2009; Mirutse *et al.,* 2009; Kalayu *et al.,* 2013; Scantlebury *et al.,* 2013; Asmare *et al.,* 2014). Moreover, a surveillance study conducted by Anteneh *et al.* (2012) in eastern part of Ethiopia revealed that *Aloe pirottae, Azadirachta indica* and *Hydnora johannis* were the most preferred species for the treatments of different diseases. Novel approaches to use plants for control of gastrointestinal (GI) nematodes in small ruminants have been studied particularly by *in vitro* tests and the efficacy of several natural products eliminating helminthes were also reported by traditional system of medicine (Temjenmongla and Yadav, 2005). In spite of all these valuable documentations and huge diversity of medicinal plants found in Ethiopia, only scarce information is available about the efficacy of these plants. Very few efforts have also been made to scientifically evaluate these plants for their medicinal properties. Based on the relevant ethno-botanical literature, easy availability in the study area and environmental friendly properties, six plant species including *Azadirachta indica, Vernonia amygdalina, Croton macrostachyus*, *Hagenia abyssinica, Nicotiana tabacum* and *Moringa oleifera* were selected for evaluation of their *in vitro* and *in vivo* anthelmintic activity against gastrointestinal nematodes of goats kept in Haramaya University goat farm.

Materials and Methods

Plant Parts Collection

The experiments were conducted from November 2017 to March 2018 to evaluate the *in vitro* and *in vivo* anthelmintic activity of methanol crude extract of leaves of *Azadirachta indica, Vernonia amygdalina, Croton macrostachyus*, *Hagenia abyssinica, Nicotiana tabacum,* and *Moringa oleifera* against gastrointestinal nematodes of goats. The leaves parts of the plants were collected separately from different parts of east Ethiopia and authenticated by botanist at Haramaya University.

Plant Parts Preparation

Approximately 1kg fresh leaves of the plants were collected from branches of each plant at leafy stage and washed thoroughly 2-3 times with clean tap water. The leaves were spread out on paper sheets, dried in shaded area at room temperature for two weeks and finely ground with a machine grinder (mixer). The resulting powder was sieved through a 25μm pore size mesh to get fine powder. The containers containing the powders were labeled and kept at Plant Science Laboratory until the extraction was conducted. For extraction, the powder was soaked in methanol solvent at one to five (1:5) ratio in a separate flask and shaken for 24hrs in a shaker at 80 rpm at 37^0C (Magano *et al.,* 2011). The mixture was later strained using a muslin cloth and filtered using a Whatman® filter paper (No. 1) and the filtrate was concentrated in a vacuüm rotary evaporator at 40^oC followed by drying in an air oven at 37°C. After complete solvent evaporation, the filtrates were stored in capped labeled bottles and kept in refrigerator at 4°C until use (Bagavan *et al.,* 2009).

Working Concentrations

The stock solutions of the crude extracts obtained from test plants were prepared by dissolving the crude extract in distilled water. For *in vitro* anthelmintic efficacy test, the working concentrations were prepared to contain 100, 50 and 25 mg/ml weights of crude extracts and tetramisole 0.55 mg/ml (positive control) and mixed properly with magnetic stirrer. For *in vivo* anthelmintic test, 1g/kg dose of crude extract was prepared for each animal. For each experiment, the untreated group was used as negative and tetramisole treated group as positive control.

Collection of Helminth Eggs

For the assays, the eggs were obtained from naturally infected goats at Haramaya University goat farm. Fecal pellets were collected from the rectum of goats and placed in small bucket. Water was slowly added to the feces and the pellets stirred until a relatively uniform homogenate liquid suspension was obtained. The suspensions were filtered through sieve and centrifuged for 2 min at 2000rpm and the supernatant decanted. Saturated sodium chloride was added to the test tube until the meniscus and then cover slip was placed. After 3 to 5 min, the cover slip was carefully removed and eggs washed into test tubes. Sodium chloride from the eggs was removed with excess tap water. The recovered eggs were adjusted to 100 eggs in 1mL of distilled water before diluting it to the required concentration for use in the intended test (Soulsby, 1982).

In Vitro Anthelmintic Test

In vitro anthelmintic activity of the plant extracts were evaluated using egg hatch test (EHT), larval inhibition test (LIT) and larva development assay (LDA). The majority of nematodes eggs used for the test were strongyle type.

Egg Hatch Test (EHT)

The egg hatch assay was performed and interpreted as outlined in the World Association for the Advancement of Veterinary Parasitology (WAAVP) recommendations (Coles *et al.,* 1992). About 100 eggs/ml added per well (in a 96-well polystyrene plate) were mixed with various concentrations of the plant extracts. Simultaneously, egg suspension was added to wells containing phosphate buffered saline (PBS) and anthelmintic drugs separately to the control wells. The wells were incubated at 27°C for 48 hours. After 48 hours, a drop of Lugol's iodine was used to stop further hatching. Then all eggs and larvae at each

concentration were counted as dead, embryonated, or hatched to L1 for each plant (Coles *et al.,* 1992). Egg hatchability inhibition percent was calculated for each extract concentration using the following modified formula (Coles *et al.,* 1992): Inhibition (%) = $100(1-X_1/X_2)$, where X_1 is the number of eggs hatched in test extracts and X_2 is the respective number in negative control.

Larval Development Assay (LDA)

Around 100 nematode eggs per ml were added per well and mixed with various concentrations of the plant extract and allowed to develop to infective L_3 larvae over six to seven days. Eggs in wells were assumed to hatch and develop through L_1 and L_2 stages depending on the concentration and type of the plant extract. Thus, isolates not responding to plant extracts develop in wells containing higher concentrations than susceptible isolates (Lyndal-Murphy, 1993).

Larval Inhibition Test (LIT)

Pooled feces collected from goats were cultured for two weeks at room temperature (about 22–25°C) for 14 days. At the end of 14 days, the cultured feces were subjected to modified Baermann technique (Hansen and Perry, 1990). Then L_3 were counted and identified according to the morphological keys given by van Wyk *et al.* (2004) and Zajac and Conboy (2012). The proportion of L_3 identified were *Trichostrongylus* spp (54.6%), *Teladorsagia* spp (17.6%), *Haemonchus* spp (14.4%), *Muellerius capillaris* (7.6%), *Oesophagostomum* spp. (1.7%), *Nematodirus* spp (1.7%), *Strongyloides papillosus* and *Cooperia* (0.9%), *Chabertia* (0.5%) and *Trichuris* (0.1%). One ml of the liquid containing about 100 larvae is placed in each test tube containing test concentrations of the plant extract, a commercial anthelmintic drug and distilled water. Observations were recorded after examination of the test sample microscopically starting after an hour by adding a drop of warm water and counting of active and dead larvae at an interval of three hours, for a total of twelve hours.

In Vivo Anthelmintic Activity

A total of 40 goats of both sexes naturally infected with gastrointestinal (GI) nematodes, were used for the experiment. On day 0, fecal samples were collected from each goat enrolled in to the study and those with an EPG (eggs per gram of feces) greater than 150 were included. The body weight of each animal was also measured in order to administer the correct dose of the anthelmintic drug and crude extracts. The amount of crude extract calculated was based on the weight of each goat and considering 1g/kg dose. Administration of crude extracts was done through oral drenching. The goats (n=40) were randomly divided into 6 treatment groups each with five goats on the basis of fecal egg counts (mean ± S.E. of eggs per gram of feces) and assigned to different treatments. The goats were allowed pasture grazing at Haramaya University separately during the day time and supplemented with hay and concentrate during the night. Approximately 10 to 20 goats are bedding in group for night shade and house cleaning is done every day during morning. Sick goats and goats with newborn were separated for special treatments. Fecal samples were collected at 10 days post treatment to determine the EPG. The treatments were Group I: untreated control; Group II: Tetramisole at 7.5 mg/kg; Group III: *Azadirachta indica* at 1g/kg body weight (bw); Group IV: *Vernonia amygdalina* at 1g/kg bw; Group V: *Nicotiana tabacum* at 1g/kg bw; Group VI: *Moringa oleifera* at 1g/kg bw; Group VII: *Croton macrostachyus* at 1g/kg bw; Group VIII: *Hagenia abyssinica* at 1g/kg bw.

Anthelmintic efficacy was assessed as per the guidelines of WAAVP (Wood *et al.,* 1995). The fecal egg count reductions of GI nematodes in goats was determined after fecal samples of each animal in the treatment groups were collected directly from the rectum in the morning, on day 0 and day 10 post-treatment. The fecal samples were homogenized so that the eggs were uniformly distributed throughout the fecal suspension prior to counting. The total numbers of nematode eggs (fecal egg counts) were determined using MacMaster egg counting technique (Soulsby, 1982). Fecal egg count percent reduction (FECR %) was calculated using the formula described by Lone *et al.* (2012) as follows:

$$\text{FECR\%} = \frac{\text{Pretreatment egg count/gram - Post treatment egg count/gram}}{\text{Pretreatment egg count/gram}} \times 100$$

Statistical Analysis

SPSS software for Windows (SPSS Version 20, 2011) was used for data analysis. *In vitro* anthelmintic effects of different concentration of plants extract were analyzed with one-way analysis of variance (ANOVA) with multiple comparison tests (Post Hoc/Tukey's test/HSD) to compare parameter between groups for egg hatchability inhibition, larvae inhibition and larvicidal activity. The results were expressed as mean and the difference between the means were considered significant at $p<0.05$. Fecal egg count percent reduction (FECR %) was calculated using the formula described by Lone *et al.* (2012).

Results

Egg Hatch Test (EHT)

Significantly high percentage inhibition of egg hatchability was observed at all concentration of *Croton macrostachyus* while low inhibition percentage was recorded by *Moringa oleifera* and *Hagenia abyssinica.* There was no difference ($p \geq 0.05$) in inhibitory effects of egg hatchability between the other four plants at 100g/ml concentration and the standard drug (Table 1).

Table 1. Mean egg hatchability inhibition percentage of six plants crude extract recorded after 48 hours of incubation

Plants	Concentrations		
	100 mg ml-1	**50 mg ml-1**	**25 mg ml-1**
Azadirachta indica	79.3±3.5[a]	51.7±3.5[b]	27.6±1.5[b]
Vernonia amygdalina	99±1[a]	48.3±5.5[b]	24.2±0[b]
Nicotiana tabacum	90±2[a]	60±2.7[a]	20±1.5[b]
Moringa oleifera	10.3±2[b]	37.9±5[b]	24.2±3[b]
Croton macrostachyus	96.5±1[a]	96.5±1.5[a]	93.1±1.5[a]
Hagenia abyssinica	41.4±4.5[b]	31±4.5[b]	10.3±4.5[b]
Tetramisole	73±5[a]	-	-
Untreated control	10±0[b]	-	-

Values with similar letter superscripts ([a–b]) in a column do not differ ($P \geq 0.05$); the values represent mean ± SEM of egg hatchability inhibition percentage.

Larval Inhibition Test (LIT)

The larvae (L_3) exposed to crude extract of *Nicotiana tabacum* showed 100% mortality within three hours at 100 mg/ml and 50 mg/ml concentrations and 87.5% mortality was recorded at 25 mg/ml. Other tested plants, positive and negative control groups did not cause larval morality and the survival of the larvae continued for several hours to days (Table 2).

Table 2. Larvicidal activity of crude extracts of six plants against gastrointestinal nematode larvae (L_3) at 6 and 12 hours of exposure

Concentrations	Mean Mortality % and SEM											
	At 6hrs						**At 12hrs**					
	AI	VA	NT	MO	CM	HA	AI	VA	NT	MO	CM	HA
100 mg ml-1	-	-	100±0	-	-	-	-	-	-	-	-	-
50 mg ml-1	-	-	100±0	-	-	-	-	-	-	-	-	-
25 mg ml-1	-	-	87.5±0	-	-	-	-	-	100±0	-	-	-
PC	-						-					
NC	-						-					

- =no death is recorded; values are expressed as mean of mortality percentage ± SEM; Mortality parentage values in the same row for each time exposure are significantly different ($P < 0.05$); AI= Azadirachta indica; VA= Vernonia amygdalina; NT= Nicotiana tabacum; MO= Moringa oleifera; CM= Croton macrostachyus; HA= Hagenia abyssinica; PC= Positive control; NC= Negative control; SEM= Standard error of mean.

Larval Development Assay (LDA)

All the three concentrations of *Nicotiana tabacum, Vernonia amygdalina* and *Croton macrostachyus* crude extracts tested for their effect against the development of larvae from L_1 to L_3 stage produced full arrest of larvae development comparable with positive control (Table 3). The lowest inhibitory effect was recorded in *Azadirachta indica* treated group. There was no larval development at 100 mg/ml and 50 mg/ml of *Moringa oleifera* and *Hagenia abyssinica,* but exposure to lower dose (25 mg/ml) showed mean percentage larval development of 3 and 17, respectively.

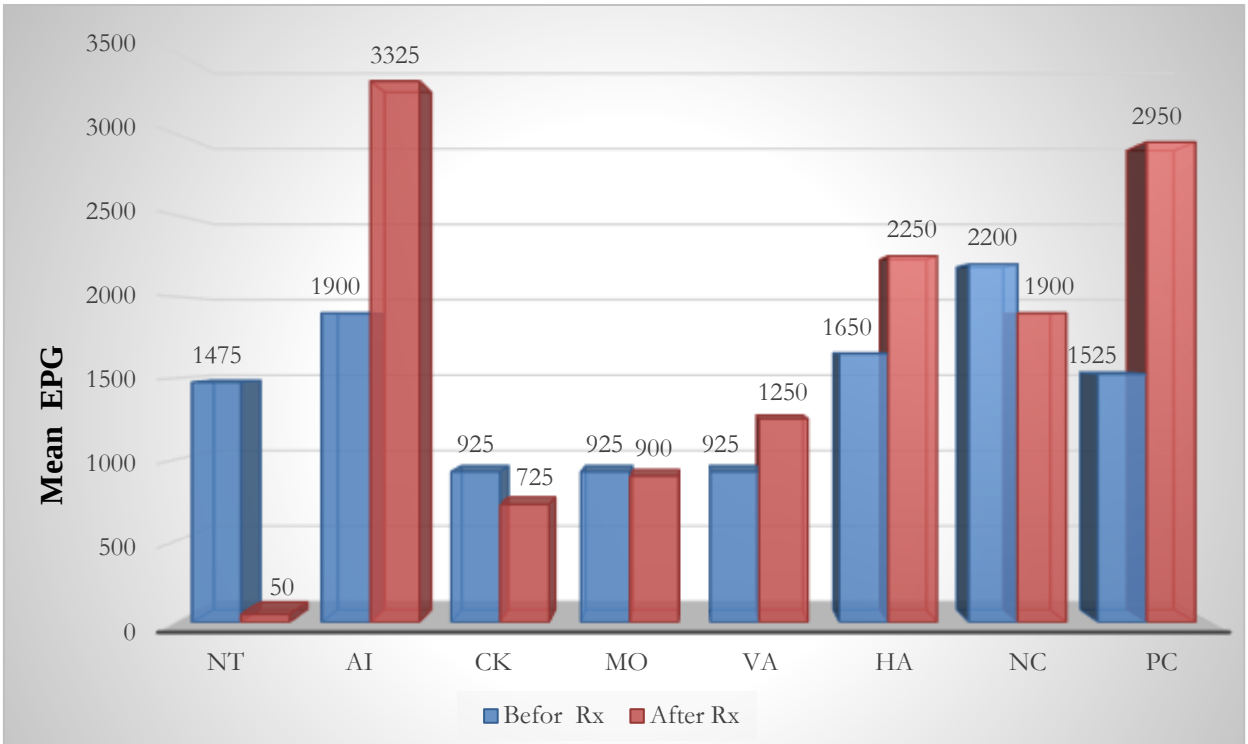
Table 3. Larval development percentage of six medicinal plants crude extract after six to seven days of incubation

Plants	Concentrations		
	100 mg ml-1	**50 mg ml-1**	**25 mg ml-1**
Azadirachta indica	5± 0.2	20±1.5	26±3.5
Vernonia amygdalina	**-**	**-**	**-**
Nicotiana tabacum	**-**	**-**	**-**
Moringa oleifera	**-**	**-**	3±0.1
Croton macrostachyus	**-**	**-**	**-**
Hagenia abyssinica	**-**	**-**	17±2.3
Tetramisole	**-**	**-**	**-**
Untreated control	75± 3	75± 3	75± 3

***-** =No larvae development seen; values are expressed as mean of inhibitory percentage ± SEM.*

In Vivo Anthelmintic Activity Evaluation

The result for *in vivo* anthelmintic activity of the plants showed a relatively high (96.6%) reduction in mean EPG count in goats treated with *Nicotiana tabacum* than in any of the other plants tested as well as the positive control group (Figure 1).



AI= *Azadirachta indica*; VA= *Vernonia amygdalina*; NT= *Nicotiana tabacum*; MO= *Moringa oleifera*; CM= *Croton macrostachyus*; HA= *Hagenia abyssinica*; NC= Negative control; PC= Postive control; Rx= Treatement.

Figure 1. *In vivo* anthelmintic activity based on EPG count before and after treatments of six medicinal plants crude extract.

Discussion

In the present study, the crude extract of *Nicotiana tabacum, Vernonia amygdalina, Croton macrostachyus* and *Azadirachta indica* produced a better *in vitro* anthelmintic activity. Similar earlier studies carried out in different parts of Ethiopia reported that *C. macrostachyus* had potential *in vitro* anthelmintic effect against *Haemonchus contortus* (Tadesse *et al.,* 2006). Study done by different scholars noted that the leaf extract of *V. amygdalina* has different phytochemicals that contribute to the potential sources for novel *in vitro* anthelmintic activities (Ayoola *et al.,* 2008; Ademola and Eloff, 2011; Nalule *et al.,* 2013; Adediran *et al.,* 2014). *Nicotiana tabacum* own significant *in vitro* anthelmintic activity, particularly at the highest concentrations (Nouri *et al.,* 2016). Study conducted by Salas *et al.* (2013) also reported that *N. tabacum* extracts is a promising alternative for controlling nematodes in ruminants. In addition, Iqbal *et al.* (2006) revealed that the aqueous and methanol extracts of *Nicotiana tabacum* show dose dependent anthelmintic activity both *in vitro* and *in vivo* against gastrointestinal nematodes of sheep.

The egg hatchability inhibition effect of *Azadirachta indica* observed in the present study was consistent with the study conducted in Ethiopia by Abdi *et al.* (2013) who stated that *A. indica* showed *in vitro* anthelmintic activity against *Haemonchus contortus* egg hatchability. Different research output have also confirmed that *A. indica* has potent anthelmintic activity (Iqbal *et al.,* 2010; Al-Rofaai *et al.,* 2012; Salas *et al.,* 2013; Nawaz *et al.,* 2014; Jamra *et al.,* 2015).

In the present study, *Moringa oleifera* showed less anthelmintic effect both *in vitro* and *in vivo*. Contrary to this result the finding of Tayo *et al.* (2014) indicated that *M. oleifera* possessed potential ovicidal and larvicidal activities against *H. contortus*. In addition, methanol extract of *M. oleifera* leaves inhibited hatchability of eggs and the growth of L_3 (Soetan *et al.,* 2014; Cabardo and Portugaliza, 2017). These variations in efficacy might be due to differences in extraction methods, geographical areas of the plant growth and stage of development of the plant leaf used for extraction that may affect the concentration and the type of phytochemicals (Cowan, 1999).

In vivo anthelmintic activity of *Nicotiana tabacum* revealed higher FECR% (96.6%) indicating hope that this plant contains active ingredients that can be used for the control of gastrointestinal nematode. Previous work also showed that *N. tabacum* extracts is a promising alternative plant for controlling nematodes in ruminants (Salas *et al.,* 2013). The *in vivo* anthelmintic activity of crude extract of *Vernonia amygdalina* did not produced reduction of EPG count, which is not in accordance with the results obtained by Leonidas *et al.* (2013) who noted potent effect of *Vernonia amygdalina* in the reduction of EPG of strongyle type nematodes. The difference might be due to the methods used

including extraction, difference in the geographical areas where the plants grew and stage of development of the plant leaf used for extraction that may affect the concentration and the type of phytochemicals (Cowan, 1999).

Both *in vitro* and *in vivo* results showed weak response of nematode parasites to tetramisole, indicating some degree of anthelmintic drug resistance. Anthelmintic resistance (AR) has been a global issue in small ruminant and many parasites of veterinary importance have genetic feature that favor the development of AR to all major groups of anthelmintic drugs (Kaplan, 2004; Papadopoulos, 2008; Makvana and Veer 2009), suggesting careful use and frequent evaluation of commercial anthelmintic.

Conclusion

The current study confirms that the tested medicinal plants show variations in anthelmintic activities against gastrointestinal nematode of goats. *In vitro* treatment with crude extract of *Croton macrostachyus* inhibited egg hatchability, while larvae survival assays showed 100% mortality of larvae (L_3) when samples were treated with extracts of *Nicotiana tabacum.* In addition, the development of larvae from L_1 to L_3 stage was inhibited by crude extracts of *Nicotiana tabacum, Vernonia amygdalina* and *Croton macrostachyus* at all three tested concentrations. Only *in vivo* treatment of *Nicotiana tabacum* provided a promising result against gastrointestinal nematodes of goats. In general, the crude extracts of *Nicotiana tabacum* leaves have shown promising activity in both *in vitro* and *in vivo* applications. Therefore, further study is recommended for determining its toxicity to the host, dose-response measurements and isolation of the level of sensitivity of different genera and species of nematodes before producing and recommending the crude extracts of *Nicotiana tabacum* as alternative natural anthelmintic.

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Conflict of Interests

The authors declare that they have no competing interests.

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