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Anthelminthic effects of extracts of indigenous browses from mid rift valley of Ethiopia

Amsalu Sisay, Tegene Negesse, and Ajebu Nurfeta*

School of Animal and Range Sciences, College of Agriculture, Hawassa University, Ethiopia

Correspondence author: _solianaa2008@gmail.com or amsals2001@yahoo.com

Abstract

This study was conducted to evaluate the potential anthelminthic properties of extracts of leaves of indigenous browses (*Acacia seyal, Acacia senegal, Acacia tortilis, Millettia ferruginea,* and *Vernonia amygadalina*) based on three in vitro assays. Acetone extracts of browses at different concentrations (75 to 1200 µg/ml, for egg and larvae and 100mg/ml for an adult) were tested on three developmental stages of *Haemonchus contortus* (eggs, infective larvae, and adult worms) using egg hatch assay (EHA), larval migration inhibition assay (LMIA) and adult worm motility inhibition assay (AMIA). Significant effects were obtained with all five browses but differences were observed depending on the parasitic stages. The effects of five browse extracts on egg hatching were concentration-dependent, the highest (P<0.05) egg hatch inhibition rate was observed at 1200 µg/ml concentration for all browses. All extracts had a higher effect (P<0.01) than that of the negative control, phosphate buffer saline (PBS). In contrast, no concentration-response relationship was found for infective larvae and adult worms, although more potent effects were observed with the highest concentrations. The LMI rate (70%) induced by *Vernonia amygadalina* extract, at a concentration of 300 µg/ml, was the highest (P<0.05) of all other browses, even at higher concentrations. The highest LMI rate (62%) induced by *Acacia senegal* extract at higher concentration, was lower than that of LMI rate (70%) induced by *Vernonia amygadalina*, at 300 µg/ml concentration. *Vernonia amygadalina* was found to be highly and rapidly effective against adult worms inducing the highest mortality rate (90%) as soon as 4 hrs after incubation. Overall, the in vitro results suggest that these five browses do possess anti-parasitic properties and *Vernonia amygadalina* showed the most effective anti-parasitic property. These effects remain to be confirmed through in vivo study.

Keywords: *Haemonchus contortus*; *Acacia seyal; Acacia senegal; Acacia tortilis; Millettia ferruginea; Vernonia amygadalina;* in vitro method; anti-parasitic.

Introduction

Helminthosis is one of the major problems of livestock production throughout the world, particularly in tropical and subtropical areas. In tropical and subtropical regions, where the parasites are more abundant due to favorable environmental conditions, helminthosis is even more devastating (Pathak 2013). Moreover, extensive grazing on native pastures, together with a lack of supplemental nutrients to animals in these areas leads to a low plane of nutrition and, therefore, increased susceptibility. The gastrointestinal parasite has resulted in up to 40% reduction in live weight gain and a 6-30% reduction in feed intake by lambs (Pathak, 2011).

Haemonchus contortus is a blood-sucking nematode parasite. Both the larvae (L4) and the adults of *Haemonchus* species suck blood (Qamar and Maqbool, 2012), primarily occurring in the abomasum of small ruminants, notably sheep and goats. The parasites cause retarded growth, loss of appetite, anemia, edema, decrease in protein, and even mortality in young animals (Qamar and Maqbool, 2012).

The increasing prevalence of anthelmintic resistance in nematodes in domestic ruminants, combined with rising consumer concerns about chemical use on farms, has encouraged research into alternative strategies for the control of internal parasites (Niezen *et al.,* 1993). Apart from anthelmintic resistance, poor availability and affordability of anthelmintics to small-scale farmers in developing countries have compounded the problem (Hammond *et al*., 1997). It follows that a search for novel and more sustainable anthelmintics is the best approach to the control of helminthosis.

The vast majority of the *in-vivo* and *in-vitro* studies examining the effects of browses against the gastrointestinal nematodes have been performed on temperate legume forages (Hoste *et al*., 2009). Although indigenous browses are largely distributed in the tropics, especially in East Africa, only a limited number of *in vitro* studies have addressed the question of their potential anthelmintic properties (Papachristou *et al*., 2005). Particularly, indigenous browses from the Rift Valley of Ethiopia are not studied for their potentials to control gastrointestinal parasites (GIP) in the ruminants. The current study was therefore conducted to explore the potential anti-parasitic properties of five indigenous browses from the Rift Valley of Ethiopia against *H. contortus*. *In*

vitro methods were employed targeting three life-cycle stages of *H. contortus*, i.e., the eggs, the infective larvae, and the adult worms.

Materials and methods

Extract preparation

Leaf samples of experimental browses were collected from the Mid Rift Valley of Ethiopia at the end of the main rainy season (September 2017). The harvested fresh samples were shed dried and then oven-dried at 60 °C for 48h and ground using 1.0 mm sieve size for extraction. Plant leaf extracts were prepared using the method of Makkar (2003). Accordingly, 5 g of ground plant material were taken in a glass beaker of 125 ml capacity. Then 100 ml of aqueous acetone (70%) was added and the beakers were suspended in an ultrasonic water bath and subjected to ultrasonic treatment for 20 minutes at room temperature (20 ^{0}C). The contents of the beakers were then transferred to centrifuge tubes and subjected to centrifugation for 10 minutes at approximately 3000g at 4 °C. The supernatant was collected and concentrated in a rotary evaporator at 40 ^{0}C under reduced pressure. Thereafter, the concentrated filtrate was freeze-dried to obtain a grounded sample. To prepare the extract solutions applied in the bioassays, the powders were dissolved in phosphate buffer saline (PBS 0.1M, pH 7.2) to make the following different concentrations: 75, 300, and 1200 µg/ml for EH test; 150, 300, and 600 µg/ml for LMI test; and 100 mg/ml for AMI test.

Egg hatch assay (EHA)

This test was performed according to the procedure described by Coles *et al.* (2006). Briefly, parasite eggs were freshly obtained from donor goats experimentally infected with *H. contortus*. The eggs were extracted by simple flotation method, washed repeatedly with saline, and distributed on 24 multi-well plates at a concentration of 100 eggs/well in 200µl. For the treatment, 1ml of plant extract prepared with PBS at different concentrations (75, 300, and 1200 µg/ml) were added to well plates containing eggs. An oxfendazole at concentrations of 10µg/ml was used as positive control and an egg with PBS was used as a negative control. Each concentration was tested in triplicates.

The eggs were incubated for 48 hr at 24 ^{0}C. Thereafter, the number of larvae present per well was counted under the microscope and the hatching percentage was determined as the ratio between the number of larvae to the number

of eggs deposited per well. A mean value was calculated for each concentration of the different plants.

Larval migration inhibition assay (LMIA)

Collection of larvae

Infective larvae of *H. contortus* (L3) were obtained by fecal culture of eggs collected from an experimentally infected goat. After egg hatching, the infective stage was reached after 10 days. The L3 were then collected by sedimentation using Baermann's apparatus.

The larval migration inhibition (LMI) bioassay was used as described by Rabel *et al.* (1994) to measure inhibiting activity against infective larvae (L3). Briefly, larvae were incubated for 3 h at 20 ^{0}C in phosphate buffer solutions (PBS) of plant extracts, at concentrations of 150, 300, or 600 µg/ml. The larvae were then washed three times in PBS and centrifuged. After the last washing, 500 µl of larvae at a concentration of 1000 L3/ml was pipetted onto a 20 µm mesh. The sieve was inserted into a conical tube so that it just touched the surface of the PBS contained therein. Three replicates were run at room temperature for each plant concentration. In addition, negative control (larvae incubated in PBS) and positive control (larvae incubated in levamisole at a concentration of 500µg/ml) were run in parallel. After 3 h, the L3 above the sieve were discarded and those which had actively migrated through the mesh into the PBS below were counted under the Microscope.

The percentage of LMI was calculated as = (T – M/ T)*100; where T is the total number of L3 deposited in the sieve and M is the number of L3 having migrated through the mesh into the PBS.

Adult worm motility inhibition assay (AMIA)

The adult motility inhibition (AMI) bioassay was used as described by Tariq *et al*. (2009). The adult worms were collected from the abomasa of freshly slaughtered goats, washed, and finally suspended in PBS. The 20–25 adult, actively moving *H. contortus* worms were exposed in triplicate in each Petridish containing 100 mg/ml concentration of the plant extract prepared in 5 ml of PBS and PBS alone for the negative control groups. Levamisole at the rate of 0.5 mg/ml was used as a reference drug of positive control. The Petri dishes were kept in an incubator at 37 °C for 8-hrs. The inhibition of motility, active-

ness, and mortality of the worms was observed at an interval of 0.5, 1, 2, 4, 6, 8 h. The number of motile (alive) and nonmotile (dead) worms were counted under the stereomicroscope and recorded for each concentration. The death of worms was ascertained by the absence of motility for an observation period of 30 seconds in the lukewarm fresh PBS.

Statistical analysis

Data were analyzed using the general linear model (GLM) procedure of SAS, Version 9.2. The effects of browse extract, concentration, and the interaction effects on EHI, LMI, and AMI were analyzed by two-factor ANOVA with the following model; $Y_{ij} = \mu + B_i + C_j + B * C_{ij} + e_{ij.}$ Where: Y_{ij} is an observation, μ is the overall mean, B_i is the effect of browse extracts, C_j is the effect of concentration, $B*C_{ij}$ is the interaction between browse species and concentration, and e_{ij} is the experimental error. All laboratory analyses of each browse extracts were conducted in triplicates. The means were separated by Duncan multiple range test. Differences between means were considered statistically significant if $P < 0.05$.

Results

Egg hatch inhibition assay

The percentage of inhibition of egg hatching at different concentrations of plant extracts and their overall means are indicated in Table 1. More than 90% of eggs in negative control were hatched. Compared to the negative control, all plant extracts showed ($P< 0.05$) higher egg hatching inhibition rates. All extracts inhibited egg hatching in a concentration-dependent manner with a maximum inhibition of 50% at the highest concentration (1200 µg/ml) for *Acacia tortilis* and lowest inhibition of 17% at the lowest concentration (75 µg/ml) for *Acacia seyal*. Oxfendazole used as positive control significantly ($P< 0.001$) inhibited egg hatching with an inhibition rate of 85 %.

Table 1. Percentage egg hatch inhibition of extracts of five browse leaves from Rift Valley of Ethiopia at different concentrations (µg/ml) (2017, G.C)

		Concentration (µg/ml)		
Plant extract	**Overall mean**	**75**	**300**	**1200**
Oxfendazole (10µg/ml)	85.00[a]	-	-	-
Millettia ferruginea	31.67[b]	20.00[B]	35.00[A]	40.00[A]
Acacia senegal	33.00[b]	20.00[B]	38.00[A]	41.00[A]
Vernonia amygadalina	35.00[b]	23.00[B]	40.00[A]	42.00[A]
Acacia tortilis	37.00[b]	22.00[C]	39.00[B]	50.00[A]
Acacia. seyal	31.00[b]	17.00[B]	34.00[A]	42.00[A]
PBS	10.00[c]	-	-	-
SE	2.5	3.0	3.0	3.0

Means with different superscripts (a, b, c) in the same column and (A, B, C) in the same row differ significantly (P<0.05). PBS = phosphate buffer saline

Larval migration inhibition assay

The positive control, levamisole, was effective since the larval migration inhibition (LMI) rate was 85%. The migration rate observed for the larvae of the negative control was 90% (Table 2). A significant effect of the extracts of the five browse on larval migration (P<0.01) was observed. Overall, the inhibition rates obtained with the *Acacia senegal, Acacia tortilis,* and *Vernonia amygadalina* extracts (P<0.01) were highest as compared to other browse extracts. The inhibition rate of *Vernonia amygadalina* extract was (P<0.01) highest at 300 µg/ml concentration. The inhibition rate obtained with the *Acacia senegal* extract (P<0.01) was higher than others except for *Acacia tortilis* at 150 µg/ml concentration. Although a tendency was observed for the highest effects to be associated with the highest concentrations, whatever the plant, a dose-response relationship was similar among the plant extracts.

Table 2. Percentage larval migration inhibition of extracts of five browse leaves Rift Valley of Ethiopia at different concentrations (µg/ml) (2017, G.C)

Plant extract/control	Overall mean	Concentration (µg/ml)		
		150	300	600
Levamisole (0.5mg/ml)	85.00^{a}	-	-	-
Millettia ferruginea	19.00^{d}	5.00^{cB}	2.00^{cB}	50.00^{bA}
Acacia senegal	55.67^{b}	45.00^{aB}	60.00^{abA}	62.00^{aA}
Vernonia amygadalina	50.00^{bc}	25.00^{bC}	70.00^{aA}	55.00^{abB}
Acacia tortilis	51.33^{bc}	35.00^{abB}	62.00^{abA}	57.00^{abA}
Acacia. seyal	46.33^{c}	30.00^{bB}	57.00^{bA}	52.00^{abA}
PBS	10.00^{e}	-	-	-
SEM	2.5	2.6	2.6	2.6

Means with different superscripts (a, b, c) in the same column and (A, B, C) in the same row differ significantly (P <0.05). PBS = phosphate buffer saline

Adult worms' motility inhibition assay

The efficacy of the five plant extracts on adult *H. contortus* is indicated in Table 3. The effects of extracts on survival of *H. contortus* were time-dependent and there were differences (P<0.05) among plant extracts in the rate of worm mortality at all times after 2 h of incubation. All plant extracts except *Acacia seyal* caused a greater than 70% mortality rate at 4 h of the incubation time. The extract from *Vernonia amygadalina* showed 90% mortality of the adult *H. contortus* worms at 4 h of the incubation time and this was the highest (P<0.05) rate of all other plant extracts followed by *Millettia ferruginea* at this time. The results revealed that parasites were sluggish and movement was little at 4 hr post-incubation for all plant extracts. Extract from *Acacia tortilis* induced the highest (P<0.05) mortality rate at 2 h post-incubation. Levamisole at a concentration of 0.5 mg/ml caused 50 % mortality at 2 h post-incubation and 100 % mortality at 4 h post-incubation. The control (PBS) recorded the lowest mortality rate (1%). The differences between plant extracts and control were highly significant (P<0.001). Moreover, all extracts except extract of *Acacia seyal* induced 100% adult mortality at 8 h post-incubation.

Table 3. Percentage of mortality of adult worms of *H. contortus* at different times after incubation with plant extracts at 100mg/ml concentration (2017, G.C).

Plant extract	Time (h)					
	0.5h	1h	2h	4h	6h	8h
Acacia tortilis	0.00	0.00	25.00^{b}	75.00^{d}	92.00^{c}	100^{a}
Acacia seyal	0.00	0.00	8.00^{e}	17.00^{e}	50.00^{e}	50.0^{b}
Acacia senegal	0.00	0.00	15.00^{d}	77.00^{d}	85.00^{d}	100^{a}
Millettia ferruginea	0.00	0.00	10.00^{e}	85.00^{c}	95.00^{b}	100^{a}
Vernonia amygadalina	0.00	0.00	20.00^{c}	90.00^{b}	90.00^{c}	100^{a}
PBS	0.00	0.00	0.00^{f}	0.00^{f}	0.00^{f}	1.00^{e}
Levamisole (0.5mg/ml)	0.00	20^{a}	50^{a}	100^{a}	100^{a}	100^{a}
SEM	0.00	0.22	0.54	0.51	0.51	0.42

Means in the same column with different superscripts differ significantly (P<0.05). SEM = standard error of means, PBS = phosphate buffer saline

Discussion

Overall, the extracts of the five tropical plants, which were examined in the current study, had *in vitro* effects on the different *H. contortus* life-cycle stages. Recent *in vivo* studies in goats suggest that the consequences of consumption of tannin-containing plants on the biology of worm populations could differ depending on the parasitic stage present in the host (Paolini *et al*., 2003a, b).

The results obtained from EHA indicated that the five plant extracts have in vitro ovicidal activity. Concentration-dependent effects on egg hatching were found with the extracts of the five plants. The plant extracts induced inhibition of egg hatching from 17 to 50 % at the concentration of 75 to1200 µg/ml. These results are comparable to the findings of Bogning *et al*. (2016) who reported a range of egg hatching inhibition rates from 22 to 51% with leaf extracts of *Crassocaphalum crepidioides* at a concentration of 75 to 2400 µg/ml. Similar results were reported on *H. contortus* eggs by Hounzangbe-Adote *et al.* (2005) with extracts from four plants of South of Benin (*Morinda lucida, Carica papaya, Newbouldia laevis,* and *Zanthoxylum zanthoxyloïdes*) where the inhibition rates ranged between 40 and 60%. In general, the extracts of the 5 browses in the current study had ovicidal activity. However, the reductions observed remained relatively limited, since maximal reductions in egg hatching were around 50%.

The effects of plant extracts on the third-stage larvae were not concentration-dependent. However, the migration inhibition rate of four out of five plant extracts ranged from 57 to 70% at the concentration of 300µg/ml. These results were comparable to the findings of Hounzangbe-Adote *et al.* (2005) who reported a range of larval migration inhibition rates from 50 to 75% with extracts of tropical plants of South of Benin at a concentration of 300µg/ml. Molan *et al.* (2000a) found a 80% inhibition of migration for *H. contortus* with *Hedysarum coronarium* extract at a concentration of 1000µg/ml. Alonso-Diaz *et al.* (2008a, b) tested *Acacia pennatula* and *A. gaumeri* extracts and found an inhibition rate of 51% at 1200mg/ml and 93.5% at 600mg/ml. The 10% inhibition effect observed in the negative control (PBS) could be due to the natural phenomena.

The adult worm mortality rate caused by plant extracts ranged from 17 to 90% and 50 to 95% at 4 and 6 h post-incubation, respectively. Moreover, all extracts except extract of *Acacia seyal* induced 100% adult mortality at 8 h post-incubation. These results are comparable with the findings of Singh *et al.* (2016) who reported a mortality rate of 64 and 100% for adult *H. contortus* at 6 and 8 h post-incubation, respectively, with aqueous extract of *Zanthoxylum armatum* (100mg/ml). Tariq *et al*. (2009) reported an adult worm mortality rate of 55 and 85% at 4 and 8 h post-incubation with ethanolic extracts of *Artemisia absinthium* with the concentration of 25mg/ml. Hounzangbe-Adote *et al.* (2005a) reported the mortality rate of adult *H. contortus* 92% at 6 h post-incubation with *Newbouldia laevis* extract (2.4mg/ml).

In the current study, *Vernonia amygadalina* appeared to be the most active plant against adult worms since the mortality rate was 90% as soon as 4 h after incubation. In contrast, *Acacia seyal* was less efficient under these in vitro conditions.

Conclusions

All extracts of the five browses inhibited egg hatching in a concentration-dependent manner with a maximum inhibition of 50% at the highest concentration. All extracts significantly inhibited larval migration compared to the control and the inhibition rate of *Vernonia amygadalina* was the highest of all other browses at 300µg/ml concentrations. The highest mortality rates (90% and 85%) of adult *H. contortus* were induced after 4h of incubation by extracts of *Vernonia amygadalina* and *Millettia ferruginea,* respectively. Moreover, all

extracts except *Acacia seyal* induced 100% adult mortality at 8 h post-incubation.

From the results of the current study, it can be concluded that significant inhibition effects were obtained with all five browses on all development stages of *H. contortus*. Particularly, *Vernonia amygadalina* appeared to be the most active plant against the infective larvae and adult worms since it induced the highest LMI rate at a lower concentration, and highest adult mortality rate as soon as 4 h after incubation. Hence, the extract from *Vernonia amygadalina* could be used as a locally available treatment for *H. contortus* infestation for small-scale farmers. But this recommendation should be validated by the in-vivo experiment, which the current study lacks.

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