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Comparison of the *In vitro* Inhibitory Effects of Doses of Tannin Rich Plant Extracts and Ivermectin on Egg Hatchability, Larvae Development and Adult Mortality of *Haemonchus contortus*

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Abstract: The study report here was undertaken to quantify and compare the *in vitro* inhibitory effects of doses of tannin rich plant extracts, ivermectin and distil water on egg hatchability, larvae development and adult mortality of *Haemonchus contortus*. *Ficus sychomorus, Phyllanthus sepialis* and *Rhus glutinosa* species suspected with high contents of condensed tannins (CT) were collected from their natural habitat in Omo Nada district of Jimma zone, south western Ethiopia. The leaves of plant species were dried at 55°C to constant weight, ground to 1.5 mm sieve and subjected to CT analysis. Aqueous acetone (70%) was incorporated for the extraction of plant samples. The extracts were prepared to obtain 0.1ml, 0.3ml and 0.5 ml from 50 mg /ml stock solution and accompanied by controls: Ivermectin (positive control) and distill water (negative control). The data were subjected to a two-way analysis of variance following the general linear model procedure of SAS in a 5×3 factorial arrangement. The CT contents of the respective plants were 120, 110 and 188 g/kg DM, respectively. Interspecies variations were significant for CT concentration across the dosage levels (P<0.001). The mean egg inhibition percentage of plant extracts varied from the lowest inhibition effect for *P. sepialis* (46-57.33%) to the highest inhibition effect for *R. glutinosa* (59.67-76%) (P<0.001). The larval development inhibition of ivermectin was not found to be superior compared to tannin rich extracts (P>0.05).The fastest adult motility was observed for different doses of ivermectin and *R. glutinosa* as compared to other treatment groups (P<0.001). Among plants, the fastest and slowest adult motility rates were recorded for *R. glutinosa* (1:22h to 0:58h) and *P. sepialis* (3:00h to 2:50h) (P<0.001). Yet, the effects of negative control didn't vary in inhibiting egg hatchability, larval development and adult motility (P>0.05). In general, all plants showed anti-*H. contortus* activity in the three-life cycle of the parasite and it was also associated with the dosage levels and the concentration of CTs contained in the plants. Furthermore, since the anti-*Haemochus* activity of *R. glutinosa* was found to be feasible as that of ivermectin, therefore, this plant can be used as alternative anthelmentic for the treatment of *Haemonchosis.*

Key words: Tannin · *Haemonchus Contortus*; *In vitro* · Ivermectin

INTRODUCTION

Sheep husbandry is an important component of livestock sector and is critical sources of cash income, meat and fiber for smallholder keepers in different farming systems [1-3], yet, productivity of sheep under tropical settings is severely influenced by parasitic infestation [4-6]. *Haemonchus contortus,* helminths parasite, plays an important role in sheep production leading to enormous economic losses through mortality, weight loss, reduced milk, meat and wool production [7,8,9]. Sheep acquire little immunity to *H. contortus* and remain susceptible throughout their lives to diseases caused by this parasite [10]. Infections with *H. contortus* occur in many countries throughout the world; however, the highest prevalence is seen in warm, moist temperate and tropical regions [11,12].

The control of *H. contortus* in domestic animals is widely based on the use of pharmaceutically derived anthelmintic drugs. However, the current efficacy of these drugs has been reduced, because of the wrong use and/or

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widespread application of poor quality synthetic or semi-synthetic anthelmintics and consequently the development of resistant parasite strains [13-15]. Moreover, the high cost of these drugs, residual concern in food animals and environmental pollution have stirred up interest in medicinal plants as an alternative source of anthelmintic drugs [16-18]. Hence, the use of indigenous plant preparations as livestock dewormers is gaining ground as one of the options and sustainable methods readily adapted to rural farming communities [19, 20]. Tannin extractives from foliages of tropical fodder trees and shrubs can be the best strategy to use as alternative strategy for controlling helminthes [21].

Ethno-veterinary surveys conducted so far in Ethiopia indicate that several traditional healers use locally available medicinal plants for treatment of various animal health problems including treatment of helminth infection [8, 22]. However, very few efforts have been made to scientifically screen and evaluate the anthelmintic effect of condensed tannins (CT). Besides, the search for alternative treatment for chemical treatments is now a days by the emergence and widespread diffusion of resistance to anthelmintics in populations of gastrointestinal nematodes in ruminants specially sheep and goats. Therefore, the present study is intended to compare the efficacy of CT-rich extracts of leaves of *Ficus sychomorus*, *Phyllanthus sepialis* and *Rhus glutinosa* with positive and negative controls against *Haemonchus contortus* of local sheep.

MATERIALS AND METHODS

Description of the Study Area: The study of tannin component and *in vitro* anti-*haemonchus contortus* activity of condensed tannins (CT) were carried out in animal nutrition and parasitology laboratories, respectively, of Jimma University, College of Agriculture and Veterinary Medicine (JUCAVM) campus which is located in Jimma city, south western Ethiopia. Jimma city is situated at 7°40'N and 36°50'E latitude and longitude, respectively [23].

Collection of Plant Leaves and Tannin Extraction: Three tannin-rich plant species namely *Ficus sychomorus, Phyllanthus sepialis* and *Rhus glutinosa* were collected from their natural habitat, Omo Nada district of Jimma zone southwestern Ethiopia. The browse species were selected based on their abundance in the area, preference and accessibility to browsing livestock as well as additional uses other than livestock feed [24]. The collected fresh leaves were dried at approximately 55°C for 72 hours to constant weight using air forced drying oven and ground to 1mm using Wiley mill. About 50 mg of ground materials were placed in a glass beaker of approximately 25 ml capacity. For each sample 10 ml of aqueous acetone (70%) was added and the beaker was suspended in an ultrasonic water bath and subjected to ultrasonic treatment for 20 minute at room temperature (average 25°C). The content of the beaker was then transferred to centrifuge tubes, cooled on ice and subjected to centrifugation for 15 minute at 3000 rpm [25].

Collection of Adult Parasites and Egg Recovery Techniques: The adult female parasites of *H. contortus* were collected from the abomasa of naturally infected sheep. The egg recovery test was performed according to the method described by Jabbar *et al.* [14] where female adult worms were crushed using pestle and mortar. After liberation, the eggs were cultured in a 250 ml jar filled with autoclaved sheep feces for eight days at room temperature.

Egg Hatchability Inhibition: The egg hatchability inhibition test was conducted according to the procedures described by Coles *et al*. [26]. Extracts of CTs from the three plant species were used as the test treatments. Ivermectin and distill water were used as a positive and negative controls, respectively. The trial was conducted in 10 ml test tubes. In the assay, approximately 170-270 eggs in 1.5 ml of distill water were placed in each test tube. The three doses (0.1 ml, 0.3 ml and 0.5 ml) of each plant extract was added in to test tubes separately and the test tubes were covered with aluminum foil making 15 to 20 holes for air circulation and kept in an incubator at 27°C for 48 hr. The experiment was repeated three times. Hatched larvae and unhatched eggs were then counted under dissecting microscope at 40 × magnification. Thereafter, the anti- *H. contortus* efficacy of CT was assessed by field controlled faecal egg count reduction test calculated according to Taylor *et al.* [27] and Coles *et al*. [26]. The percent fecal egg count reduction was calculated using the following formula:

% FECR = ((a-b)/a)*100; where, *a,* EPG pre-treatment and *b*, EPG post-treatment

Larval Development Inhibition Assay: The larval development inhibition test was conducted with a modification of the technique described by Costa *et al.* [27]. The CT extracts obtained from the three plant species

were used as the test treatments. Ivermectin was as positive control while untreated eggs either with ivermectin or CT extracts in distilled water were used as a negative control. After incubating the eggs at 27°C for 24 hours, an aliquot of 1.5ml, containing 100-150 first stage larvae (L1) of *H. contortus* was mixed with 10 gm of feces that collected from abomasa of natural infected sheep. Each of 0.1ml, 0.3ml and 0.5ml of CT extracts was added on a sheep feces which containing L_1. The test materials were incubated for 6 days at room temperature. At the end of the 6th day the wall of each cup containing the sample was thoroughly rinsed with 10 ml of distill water to collect the larvae. Then one drop of Lugol's iodine solution was added and all (L3) stage larvae were counted under stereomicroscope.

Adult Motility Test: Adult motility test was performed according to Petersen *et al*. [28]. Accordingly, 0.1ml, 0.3ml and 0.5ml of each of CT extracts was taken and diluted with distilled water. Iveremactin was used as a positive control while distilled water was used as negative control. For each treatment 30 adult parasites were used. The different concentrations of each plant extracts (0.1ml, 0.3ml and 0.5ml) were placed in petridish. The parasites were then immersed and the time for mortality was recorded for each respective concentration.

Statistical Analysis: A two-way analysis of variance was followed using the general linear model (GLM) procedures of statistical analysis system [29]. Differences were deemed significant when $P<0.05$, whereas $0.05<P<0.10$ was considered to show a statistical tendency for difference. Levene's test was carried out to detect the homogeneity of experimental variances. Based on this test, there was no evidence that the residuals (i.e., errors) were not normally distributed.

RESULTS

Egg Hatchability Inhibition: Egg hatchability inhibition percentages of plant extracts were associated with the doses of CT extracts (Table 1). The highest values of % egg hatchability inhibition was recorded for the largest doses of plant extracts ($P<0.001$). The mean inhibition percentage obtained for each plant extract was significantly different with differences in doses of application ($P<0.001$). Across the treatments except distill water, % egg hatchability inhibition was linked to doses as well as tannin concentration ($P<0.001$). Even though ivermectin, a positive control, inhibited egg hatchability by 74% for 0.5ml dose, it had clearly observed in the present study that tannin extracts from various tannin rich plants had ability to inhibit egg hatchability to maximum 76% for 0.5 ml dose.

The egg inhibition capacity of doses of tannin rich plants were comparable with ivermectin, the commercial anthelmintics drug (Figure 1). For 0.1ml CT extract, the highest and lowest percentage egg hatchability inhibition was recorded for *R. glutinosa* and distill water, respectively ($P<0.001$).

Larval Development Inhibition: The experiment was done with exposure of *H. contortus* L_1 through L_3 (Table 2). Similar to percent % egg hatchability inhibition, the larval development inhibition effects of the doses of plant extracts was clearly associated with either tannin concentration or doses of the plant extracts, meaning, larger volumes (doses) had the greatest larval development inhibition ability ($P<0.001$). The larval development inhibition was ranged from 51.33% at 0.1ml dose, 62% at 0.3ml dose, 73.33% at 0.5ml dose for *R. glutinosa* as well as 54.67% at 0.1ml dose, 56.33% at 0.3ml dose, 66.33% at 0.5ml dose for *P. sepialis*. Anti-*Haemonchus contortus* activity of ivermectin was not found to be superior compared to tannin rich extracts (Figure 2).

The larval development inhibition (LDI) percentages of 0.1mm of tannin rich extracts were higher than ivermectin and distil H_2O. However, the LDI was not significantly different for 0.3ml except for *P. sepialis* (Figure 2). The 0.5ml of *R. glutinosa* was superior in inhibiting larval development compared to all treatment groups ($P<0.001$).

Adult Motility Test: Table 3 presents adult parasite mortalities in hours; where the larger number of hours indicated the slower mortality rate of the parasite. In other words, the lower time indicates the fastest motility rate ($P<0.001$). It was observed for all treatment groups that the adult motility inhibition was highly associated with doses of the plant extracts, i. e, shorter times were recorded for smaller doses ($P<0.001$).

The fastest adult motility was observed for different doses of ivermectin and *R. glutinosa* as compared to other treatment groups ($P<0.001$, Figure 3). Among plants, the fastest adult motility rate was *R. glutinosa* for dose (0.1ml for1:22h, 0.3ml for 1:15h, 0.5ml for 0:58h) whereas the slowest adult motility rate was *P. sepialis* at dose (0.1ml for 3:00h, 0.3ml for 2:53h, 0.5ml for 2:50h) ($P<0.001$).

Table 1: Effects of doses of tannin rich plant extracts ivermectin and distill water on percent egg hatchability inhibition

Treatments (T)	Dose (ml, D)	Mean	SEM	*P*-value (T × D)
Ficus sychomorus	0.1	48.00[c]	5.292	
	0.3	57.33[b]	3.667	
	0.5	63.33[a]	3.667	<0.001
Rhus glutinosa	0.1	59.67[c]	3.667	
	0.3	64.67[b]	3.667	
	0.5	76.00[a]	2.000	<0.001
Phyllanthus sepialis	0.1	46.00[c]	8.145	
	0.3	54.00[b]	8.145	
	0.5	57.33[a]	6.888	<0.001
Ivermectin	0.1	50.67[c]	7.965	
	0.3	68.67[b]	1.667	
	0.5	74.00[a]	4.726	<0.001
Distill water	0.1	15.00	2.000	
	0.3	15.00	2.000	
	0.5	14.00	1.320	<0.175

[a,b,c] means with different superscripts in the column are significantly different (P<0.001); T, treatment; D, doses; T × D, treatment dose interaction

Table 2: Effects of doses of treatments on larval development inhibition

Treatments	Dose (m1)	Mean	SEM	*P*-Value (T×D)
Ficus sycomorus	0.1	57.67[c]	7.424	
	0.3	59.00[b]	11.269	<0.001
	0.5	67.33[a]	10.138	
Rhus glutinosa	0.1	51.33[c]	4.410	
	0.3	62.00[b]	7.000	<0.001
	0.5	73.33[a]	9.667	
Phyllanthus sepialis	0.1	54.67[c]	6.119	
	0.3	56.33[b]	8.007	<0.001
	0.5	66.33[a]	8.667	
Ivermectin	0.1	43.67[c]	4.410	
	0.3	55.67[b]	3.180	<0.001
	0.5	63.33[a]	6.360	
Distil H_2O	0.1	27.00	3.215	
	0.3	24.33	5.812	0.455
	0.5	21.73	8.891	

[a,b,c] means with different superscripts in the column are significantly different (P<0.001); T, treatment; D, doses; T Í D, treatment dose interaction

Table 3: Adult motility inhibitions of tannin rich plant extracts, ivermectin and distill water

Treatments	Dose (ml)	Mean (time)	SEM	*P*-value (T×D)
Ficus sycomorus	0.1	2:59[a]	0.219	
	0.3	2:49[b]	0.259	<0.001
	0.5	2:41[c]	0.295	
Rhus glutinosa	0.1	1:22[a]	0.094	
	0.3	1:15[b]	0.079	<0.001
	0.5	0:58[c]	0.172	
Phyllanthus sepialis	0.1	3:00[a]	0.245	
	0.3	2:53[b]	0.2489	<0.001
	0.5	2:50[c]	0.269	
Ivermectin	0.1	0:57[a]	0.219	
	0.3	0:52[b]	0.235	<0.001
	0.5	0:40[c]	0.250	
Water	0.1	7:00	0.306	
	0.3	7:00	0.306	0.784
	0.5	7:00	0.306	

[a,b,c] means with different superscripts in the column are significantly different (P<0.001); T, treatment; D, doses; T Í D, treatment dose interaction

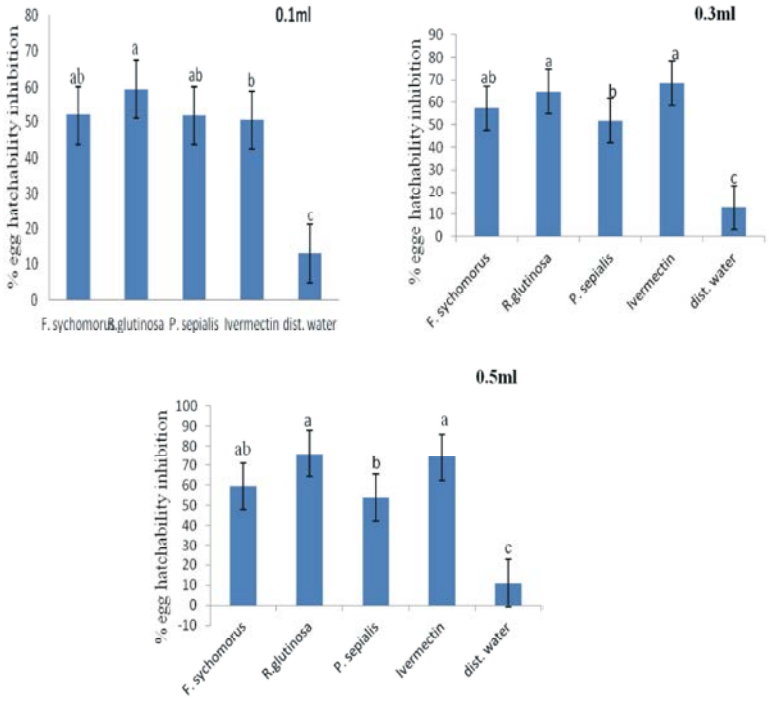

Fig. 1: Comparisons of doses of tannin rich plant extracts, ivermectin and distill water against % egg hatchability inhibition ([a,b,c] different superscripts for % egg hatchability inhibition, P<0.001)

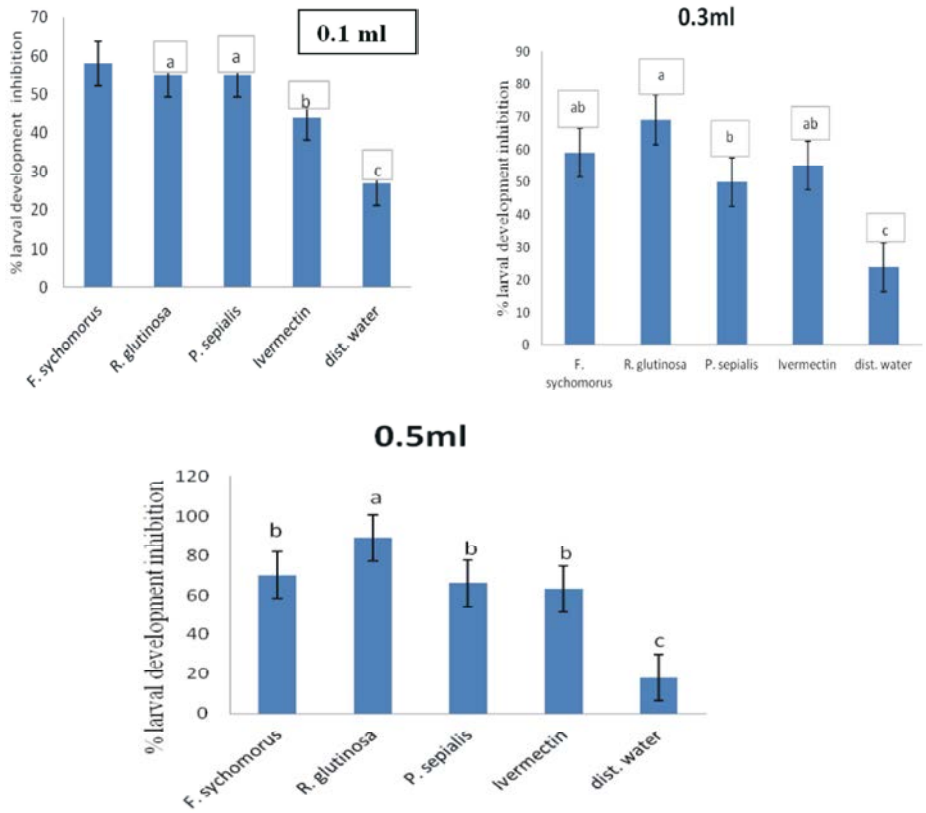

Fig. 2: Larval development inhibition activities of treatment groups ([a,b,c] different superscripts for % larval development inhibition, P<0.001)

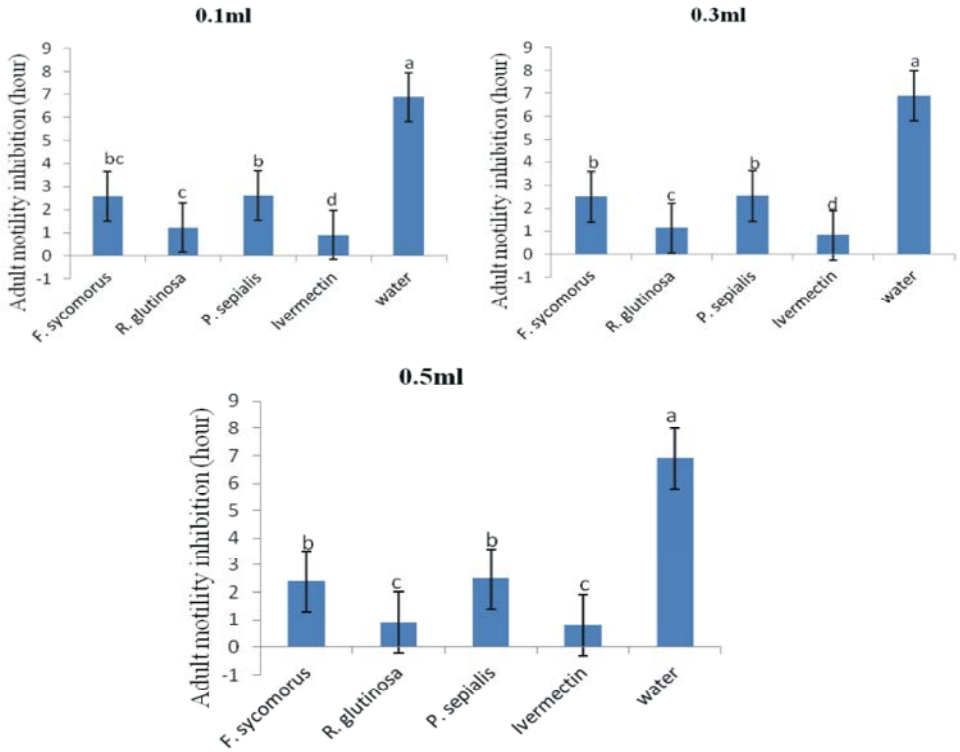

Fig. 3: Effects of doses of plant extracts, ivermectin and distill water on adult motility inhibition (hour, [a,b,c,d] different superscripts for % adult motility inhibition, P<0.001)

DISCUSSION

Different values of egg hatching inhibition (Table 1) in relation to condensed tannin sources could be attributed to variation in genetic factors and biochemical activity of the plants. This could be also associated with the concentration of condensed tannin in plant species. Molan *et al.* [4] confirmed the effectiveness of different plant source tannins against egg hatching inhibition capacity. Condensed tannins can complex with the sheath proteins of nematodes, which have high proline content that prevent exsheathment and intercalate with DNA synthesis of parasites [30]. However, the exact mechanisms of action of these metabolites remain obscure and could differ depending on the parasite, its stage of development and possibly, the biochemical characteristics of the plant species [31] while some studies have shown the effects of tannins due to their ability to interact and protect degradation of ruminal proteins [32], others have demonstrated direct toxic effects of tannins on nematodes [33]. Findings from similar studies can therefore vary widely depending on the predominant mode of action. It has also been suggested that chemical structure of condensed tannins far as biological responses are concerned [35].

Results from the *in vitro* trials can be influenced and confounded by the presence, in the plants, of other unknown bioactive substances together with differences in nutritional values in addition to the different extraction solvents. Even if ivermectin (positive control) inhibited egg hatchability by 50.67-74%, condensed tannin extracted from *Rhus glutinosa* had more efficient (59.67-76%) in egg inhibition. This is also in line with the findings of Min *et al*. [35) who reported the effect of tannins in egg hatching inhibition of *H. contortus*.

The significant variations in larval development inhibition of CTs (Table 2) might be in concurrence with the concentration of tannins in each plants species. This could be also attributed to tannin's capacity to bind proteins and operate through several mechanisms. Condensed tannins may bind to the cuticle of larvae which is high in glycoproteins and cause their death [36]. The larval inhibition effect of tannins, third stage of larvae (L3), on *H. contortus* was reported by Barrau *et al.* [37].

The result of the current study on larval inhibition capacity was also supported with the work of Molan *et al.* [4] who reported a significant reduction of larval development (L_1 to L_3) in CT treated *H. contortus*. The results of present study also agree with findings of

Hoste *et al.* [30] who pointed out the most consistent anti-larval effect of tannins extracted from plants with highest tannin content. Hoste *et al*. [30], Ademola *et al*. [36] and Min *et al.* [35] had also reported a positive reduction effect of tannin in larval development of *H. contortus* in their studies.

Tannins may react directly with adult worms by attaching to their "skin", causing them distress, or in directly by improving protein nutrition of the host and decreasing the immune system [35]. The adult motility rate was associated with CT concentration as well as plant species (Table 3). Tannin rich plants perhaps represent an alternative option to control nematodes like *H. contotus* and the result of this study is in close conformity to Max *et al*. [32], Mihreteab *et al.* [6] and Verma *et al.* [37] who reported on the anthelmintic activity of CTs in parasitic reductions. Several authors [38-42] were also disclosed the improvement of the productive and reproductive performance of smallholders' herbivore livestock through indigenous plant preparations as livestock dewormers.

In conclusion, these findings confirmed the effectiveness of anti-*H. contortus* action of condensed tannins as ivermectin, a commercial synthetic drug. However, more investigation is required by using a serial dilution test in further to allocate the effective and efficient dose.

The main advantages of using *in vitro* tests/assays to screen the anti-parasitic properties of the plants and plant extracts are low costs and rapid turnover, which allow screening large number of plants. In spite of the advantages of *in vitro* tests on evaluation of anthelmintic activity of plants, considerations should be also taken in mind that potential bioactive substances used *in vitro* may not always correspond to *in vivo* bioavailability. Therefore, *in vitro* assays should always be accompanied by *in vivo* trials when used to validate anthelmintic activity of plant remedies. Thus, it would be necessary to achieve some *in vivo* toxicological and parasitological studies using condensed tannins. As a final point, because the anti-*Haemochus* activity of *Rhus glutinosa* was found to be feasible as that of ivermectin, therefore, this plant can be used as alternative anthelmentic for the treatment of *Haemonchosis.*

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