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In-vitro Antihelmintic Activity of Condensed Tannins from *Rhus Glutinosa*, *Syzygium Guineensa* and *Alpizia gumifera* a Gainst Sheep *Hemonchus contortus*

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Abstract: Experimental study was conducted to investigate *in-vitro* anthelmintic activities of condensed tannins on egg hatch ability and larval development inhibition of sheep *Hemonchus contortus*. In view of that, three indigenous medicinal plants: *Rhus glutinosa*, *Syzygium guineensa* and *Alpizia gumifera* were selected based on their relatively high content of condensed tannins and their aqueous acetone extraction was used for egg hatchability and larval development inhibition assays. The results showed that various concentrations of the extracts of all three condensed tannins demonstrated statistically significant ($p<0.05$) dose dependent inhibition of both egg hatchability and larval development. According to ED_{50} and ED_{90} values, the condensed tannin inhibiting egg hatching and larval development most potently was *Rhus glutinosa* followed in descending order of activity by *Syzygium guineensa* and *Alpizia gumifera*. Finally, our work suggests that condensed tannins might be recommended as one of the options for the control of *Hemonchus contortus* of sheep.

INTRODUCTION

Helminth parasites play an important role in small ruminant's production leading to enormous economic losses through mortality, weight loss, reduced milk, meat and wool production (Githiori *et al*., 2003; Troell *et al*., 2005; Eguale *et al*., 2007). *Haemonchus contortus* (*H. contortus*) is singly the most important of all gastrointestinal helminths that constrain the survival and productivity of sheep owned by rural poor farmers in the developing world (Qadir *et al*., 2010).

The control of these parasites 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 widespread application of poor quality synthetic or semi synthetic anthelmintics and consequently the development of resistant nematode strains (Chartier *et al*., 2001; Bartley *et al*., 2003; Jabbar *et al*., 2007; Artho *et al*., 2007). *H. contortus* is prominent amongst the reports of anthelmintic resistance that has emerged in all countries of the world that produce small ruminants (Qadir *et al*.,

2010). 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 (Pessoa *et al*., 2002; Hordegen *et al*., 2003; Githiori *et al*., 2005; Bizimenyera *et al*., 2006; Athanasiadou *et al*., 2007). 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 (Alawa *et al*., 2003; Bizimenyera *et al*., 2006).

Condensed tannins are poly-phenolic compounds derived from plant's secondary metabolism (Olivera *et al*., 2009). Several species of medicinal plants are recognized for their high content of condensed tannins and the anthelmintic effect of some the species has been confirmed using *in vitro* tests (Paolini *et al*., 2003; Max *et al*., 2007; Alonso-Diaz *et al*., 2008a). Some authors have reported a relatively good effect of condensed tannins on worm burden of abomasum worms (Hordegen *et al*., 2003; Min and Hartt, 2003; Minho *et al*., 2008; Ibanez *et al*., 2009), after the use of condensed tannins in ruminant diet. It has also been reported that certain plants with high condensed tannin content are accepted by browsing sheep making them possible candidate for nematode management (Hernandez-Orduno *et al*., 2008).

Ethnoverterinary surveys conducted so far in Ethiopia indicate that several traditional healers use medicinal plants for treatment of various animal health problems including treatment of helminth infection (Tadesse *et al*., 2009; Eguale *et al*., 2006, 2007; Dereje *et al*., 2009). However, very few efforts have been made to scientifically screen and evaluate the anthelmintic effect of condensed tannins.

Therefore, the objective of this research work was to investigate the *in-vitro* anthelmintic activities of condensed tannins from *Rhus glutinosa* (*R. glutinosa*), *Syzygium guineensa* (*S. guineensa*) and *Alpizia gumifera* (*A. gumifera*) on the egg hatchability and larval development inhibition of sheep *H. contortus*.

MATERIALS AND METHODS

Collection of plant materials and extraction protocol: Plant samples of *R. glutinosa*, *S. guineensa* and *A. gumifera* with known high content of tannins were collected from their natural habitat in and around Jimma area including Gibe river basin. The plant materials were dried in a well-aerated room protected from sun and dust. Then an aqueous acetone (70%) extraction of each plant was performed by decoction. Briefly, dried (finely ground) plant material (200 mg) was taken in a glass beaker of approximately 25 mL capacity. The 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 min at room temperature. The content of the beaker was then transferred to centrifuge tubes, cooled by keeping on ice and was subjected to centrifugation for 15 min at 2000 RPM. Then the extracts were stored at 4°C for biological tests.

Phytochemical analysis and total tannin quantification of the extract: The phytochemical test to detect the presence of tannins was performed following the method described by Matos. These tests are based on visual observation of color modification or precipitate formation after addition of specific reagents. The total tannin quantification was then performed by the Folin-Denis spectrophotometric method according to Pansera etc. For this test, 5 mg of the extract was diluted in 100 mL distilled water and 2 mL of this solution was added to 2 mL of Folin-Denis reagent. Subsequently, the mixture was vigorously shaken and left at rest for 3 min. Then, 2 mL of 8% sodium carbonate aqueous solution was added to the mixture which was shaken again and left at rest for 2 h. Solutions ranging from 2-24 mg mL^{-1} of tannic acid diluted in water were prepared to quantify total tannins. The absorbance was measured at 725 nm and a negative control was performed at each reading. The readings with three replicates per sample were performed in a spectrophotometer. An analytical calibration curve was plotted from the results.

Collection of adult parasites andegg recovery technique: To collect adult female parasites of *H. contortus*, the abomasums of naturally infected sheep from Jimma municipal abttoir were incised along the greater curvature and washed slowly under tap water several times. Then, adult worms were picked manually using forceps and put in a universal bottle containing phosphate buffered saline (PBS, pH: 7.2) and were transported in cold chain (4°C) to Jimma University college of agriculture and veterinary medicine, school of veterinary medicine, parasitology and pathology laboratory. The eggs recovery was performed according to the method described previously by Jabbar *et al*. (2006). 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.

Infecting donor sheep with *H. contortus* egg: About 2500 larvae were inoculated to two de-wormed sheep that were maintained in a partitioned animal house of the college of agriculture and veterinary medicine, Jimma University to be served as don or of *H. contortus* egg for the *in-vitro* tests.

Collection and counting of egg from donor sheep: Feces were collected from *H. contortus* egg donor sheep and were processed accordingly. The filtrate was centrifuged in test tubes for 1 min at 2000 RPM and supernatant was discarded. Tubes were then agitated on a vortex mixer to loosen the sediment and saturated sodium chloride solution was added until a meniscus formed above the tube. A cover slip was placed and was plucked offcarefully after 5 min from tubes and eggs were washed off into a conical glass centrifuge tube. The tube was filled with water and centrifuged for 1 min at 2000 RPM. The supernatant was decanted and eggs were re-suspended in saline solution. The concentration of recovered egg samples was determined using a modified McMaster technique where the sample solution was placed into one half of a McMaster slide. This was repeated to fill the other half of the slide. The number of eggs counted in both sides of the chamber was multiplied by 50 to estimate the total number of eggs in the sample. Results were reported as eggs per gram (epg).

Egg hatchability inhibition test: The egg hatchability inhibition test was conducted according to the procedure described by Coles *et al.* (2006) with little modifications. Condensed tannins extracts from the three plants were used as the test treatments. Albendazole dissolved in Dimethyl Sulfoxide (DMSO) and diluted in distilled water was used as positive control while untreated eggs in distilled water were used as negative control. The test was conducted in 5 mL test tubes. In the assay, approximately 150-250 eggs in 1.5 mL of water were placed in each test tube. Various serial concentrations (0.0156, 0.0313, 0.0625, 0.25, 0.5, 1 and 2) of each plant extract were added in total volume of 0.5 mL distilled water. The test tubes were covered and kept in an incubator at 27°C for 48 h. The experiment was repeated three times. Hatched larvae and unhatched eggs were then counted under dissecting microscope at 40× magnification.

Larval development inhibition assay: The test was conducted with a modification of the technique described by Costa *et al*. (2008). Condensed tannins extracts from the three plants were used as the test treatments. Ivermectin 1% (10 mg mL^{-1}) dissolved in distilledwas used as positive control while untreated eggs in distilled water were used as negative control. After incubating the eggs at 27°C for 24 h, an aliquot of 1 mL, containing 95-125 first stage larvae (L_1) of *H. contortus* was mixed with 5 gm of feaces that was collected from a de-wormed sheep free of gastrointestinal nematodes. Various serial concentration of each condensed tannin extract (1.562, 3.125, 6.25, 12.5, 25 and 50 mg mL^{-1}) were prepared in distilled water to make total volume of 7 mL together with water containing L_1 and volume of egg free feces. The test materials were then incubated for 6 days at room temperature. At the end of 6th day the wall of each cup containing the sample was thoroughly rinsed with 10 mL of water to collect the larvae. Then one drop of Lugol's iodine solution was added and all L_3 stage larvae wee counted under dissecting microscope at 40× magnification.

Statistical analysis: All data from egg hatchability inhibition test and larval development inhibition assay were entered in to an Excel spreadsheet and was transferred to SPSS 16 for analysis. The results of the *in-vitro* tests were expressed as mean efficacy percentage of egg hatching or larval development inhibition±standard deviation. The concentrations of the extracts required to inhibit 50% (ED_{50}) and 90% (ED_{90}) of egg hatching as well as larval development and the relative median potency estimates of the condense tannin extracts on egg hatchability larval development inhibition as compared to the positive control were calculated by probit analysis. Comparison of the mean egg hatchability and larval development inhibition was carried out by a one-way ANOVA $p<0.05$ was considered statistically significant at 95% confidence level for all analysis.

RESULTS AND DISCUSSION

Phytochemical analysis and total tannin quantification of the extracts from the three plant samples were performed and the result indicated that *R. glutinosa* showed the highest tannin content whereas *A. gumifera* was the least (Table 1).

The results of mean inhibition percentage (±SD) of condensed tannin extracts (Table 2 and 3) showed that all three condensed tannin extracts demonstrated various degrees of dose dependent inhibition on both egg hatchability and larval development with *R. glutinosa* being the highest followed by *S. guineensa* whereas *A. gumifera* showed the lowest inhibition.

The ED_{50} and ED_{90} values of condensed the tannin extracts on egg hatchability and larval development inhibition is shown on Table 4 and 5. Accordingly, the highest ED_{50} and ED_{90} values for egg hatchability and larval development inhibition was recorded with *A. gumifera* followed by *S. guineensa* whereas the lowest value was recorded with *R. glutinosa*. Hence, the condensed tannin inhibiting egg hatching and larval development most potently was *R. glutinosa* followed in descending order of activity by *S. guineensa* and *A. gumifera*. The results suggest that all the 3 condensed the tannin extracts exhibited various potency to inhibit the egg hatch and larval development.

Probit analysis was used to compare egg hatchability and larval development inhibition of the condense tannin extracts by comparing their relative potency with that of

Table 1: Tannin contents of the extracts of plant samples

Plant samples	Tannin contents (%) of the extracts	Confidence interval (95 %)	
		Lower bound	Upper bound
A. gumifera	7.20	2.13	12.27
S. guineensa	17.20	9.80	24.60
R. glutinosa	18.80	11.14	26.46

Table 2: Mean inhibition percentage (±SD) of different concentrations of condensed tannin extracts on egg hatchability of sheep *H. contortus*

Concentrations (mg mL^{-1})	Condensed tannins and positive control			
	A. gumifera	*S. guineensa*	*R. glutinosa*	Albendazole
0.0156	1.64±1.75	2.07±2.01	3.26±2.50	3.82±2.54
0.0312	2.93±2.46	3.14±2.41	3.29±2.52	7.07±3.66
0.0625	3.35±2.50	6.15±3.43	7.23±3.61	15.99±4.83
0.125	10.9±4.01	15.68±5.01	22.87±5.76	81.46±4.99
0.25	25.96±5.82	30.33±6.20	59.47±6.55	100±0.00
0.5	52.07±6.65	57.33±6.41	87.34±4.31	100±0.00
1	78.41±5.35	88.62±4.35	99.08±1.39	100±0.00
2	78.80±5.49	89.06±4.42	99.28±0.86	100±0.00

Table 3: Mean inhibition percentage (±SD) of different concentrations of condensed tannin extracts on larval development of sheep *H. contortus*

Concentrations (mg mL^{-1})	Condensed tannins and positive control			
	A. gumifera	*S. guineensa*	*R. glutinosa*	Albendazole
1.562	29.72±8.66	36.62±9.09	43.52±9.35	46.89±9.24
3.125	38.51±9.22	48.10±8.87	55.59±9.29	58.41±9.43
6.25	50.00±9.75	61.95±8.95	66.77±8.8	80.52±8.23
12.5	61.67±9.53	70.81±8.61	78.70±7.58	82.12±7.16
25	73.27±8.23	78.89±7.52	86.25±6.56	89.81±5.81
50	84.16±6.92	89.41±5.67	91.07±5.28	93.91±4.37

Table 4: The ED_{50} and ED_{90} in mg mL^{-1} of condensed the tannin extracts on egg hatchability inhibition of sheep *H. contortus*

Condensed tannins	ED_{50} (mg mL^{-1})	Confidence interval (95%)		ED_{90} (mg mL^{-1})	Confidence interval (95%)	
		Lower bound	Upper bound		Lower bound	Upper bound
A. gumifera	0.50	0.36	0.68	1.65	1.15	2.76
S. guineensa	0.41	0.30	0.54	1.19	0.87	1.87
R. glutinosa	0.21	0.17	0.26	0.49	0.39	0.68
Albendazole	0.08	0.06	0.11	0.23	0.17	0.37

Table 5: The ED_{50} and ED_{90} in mg ml^{-1} of the condensed tannin extracts on larval development inhibition of sheep *H. contortus*

Condensed tannins	ED_{50} (mg mL^{-1})	Confidence interval (95%)		ED_{90} (mg mL^{-1})	Confidence interval (95%)	
		Lower bound	Upper bound		Lower bound	Upper bound
A. gumifera	5.89	4.08	8.11	106.41	69.59	184.47
S. guineensa	3.45	2.36	4.73	62.22	42.58	100.88
R. glutinosa	2.27	1.51	3.16	39.34	27.76	60.90
Ivermectin	0.66	0.38	1.03	11.85	8.68	16.73

the standard counterparts (positive controls) thus, *R. glutinosa* was 3.7 and 5.9 times more potent in inhibiting egg hatchability than *S. guineensa* and *A. gumifera* respectively. Similarly, *R. glutinosa* was 2.5 and 7.3 times more potent in inhibiting larval development than *S. guineensa* and *A. gumifera* respectively (Table 6 and 7).

The values $F(2, 24) = 6.33$, $p<0.006$ and $F(2, 18) = 14.83$, $p<0.000$ in Table 8 indicate a one-way ANOVA for mean efficacy on egg hatchability and larval development inhibition of *A. gumifera* as compared to albendazole and a negative control. Accordingly, there was a statistically significant difference in the mean egg hatchability and larval development inhibition respectively across the three groups. However, Tukey's HSD post-hoc test revealed that the observed difference in the mean egg hatchability and larval development inhibition between *A. gumifera* (Mean = 62.93, SD = 72.50) and Albendazole (Mean = 118.56, SD = 98.71) was not statistically significant ($p = 0.24$). Similar results with their corresponding descriptive and ANOVA values were observed in Table 9 and 10 pertaining to *S. guineensa* and *R. glutinosa*.

Our *in vitro* study was aimed at investigating the direct effects of condensed tannins on the egg hatchability and larval development inhibition of *H. contortus* of

Table 6: Relative median potency estimates of the condense tannin extracts on egg hatchability inhibition of sheep *H. contortus* as compared to the positive control

		Confidence interval (95%)	
Condensed tannins and control	Estimates	Lower bound	Upper bound
A. gumifera	0.16	0.04	0.39
Albendazole	6.12	2.58	25.04
S. guineensa	0.20	0.05	0.45
Albendazole	4.94	2.23	18.60
R. glutinosa	0.39	0.19	0.63
Albendazole	2.54	1.58	5.42

Table 7: Relative median potency estimates of the condense tannin extracts on larval development inhibition of sheep *H. contortus* as compared to the positive control

		Confidence interval (95%)	
Condensed tannins and control	Estimates	Lower bound	Upper bound
A. gumifera	0.11	0.06	0.19
Ivermectin	8.95	5.22	8.09
S. guineensa	0.19	0.11	0.30
Ivermectin	5.23	3.31	9.30
R. glutinosa	0.30	0.18	0.45
Ivermectin	3.35	2.21	5.52

Table 8: One-way ANOVA for egg hatchability and larval development inhibition test of *A. gumifera* against sheep *H. contortus*

				Confidence interval for mean (95%)	
Descriptive/treatments	N*	Mean egg hatchability inhibition	SD	Lower bound bound	Upper bound
Egg hatchability inhibition assay					
A. gumifera	9	62.93	72.50	7.20	118.66
Albendazole	9	118.56	98.71	42.68	194.43
Distilled water	9	0.00	0.00	0.00	0.00
ANOVA	Sum of squares	df	Mean square	F-values	p-values
Between groups	63329.24	2	31664.62	6.33	0.006
Within groups	119996.17	24	4999.84		

Table 9: Larval development inhibition test

				Confidence interval for mean (95%)	
Descriptive/treatments	N*	Mean egg hatchability inhibition	SD	Lower bound bound	Upper bound
A. gumifera	7	51.90	29.03	25.06	78.75
Ivermectin	7	76.86	36.53	43.07	110.64
Distilled water	7	0.00	0.00	0.00	0.00
ANOVA	Sum of squares	df	Mean square	F-values	p-values
Between groups	21522.07	2	10761.04	14.83	0.000
Within groups	13061.68	18	725.65		

N* = Number of serial dilutions

Table 10: One-way ANOVA for egg hatchability and larval development inhibition test of *S. guineensa* against sheep *H. contortus*

				Confidence interval for mean (95%)	
Descriptive/treatments	N*	Mean egg hatchability inhibition	SD	Lower bound bound	Upper bound
Egg hatchability inhibition assay					
S. guineensa	9	66.41	74.51	9.13	123.68
Albendazole	9	118.55	98.71	42.68	194.43
Distilled water	9	0.00	0.00	0.00	0.00
ANOVA	Sum of squares	df	Mean square	F-values	p-values
Between groups	63554.38	2	31777.19	6.23	0.007
Within groups	122360.62	24	5098.36		

sheep. In view of that three indigenous medicinal plants were selected for this study based on their relatively high content of condensed tannins. The effect of condensed tannin extracts which was demonstrated in our study is in accord with a series of *in vitro* studies that supported the anthelmintic property of condensed tannins (Athanasiadou *et al*., 2001; Niezen *et al*., 2002; Molan *et al*., 2003; Paolini *et al*., 2003; Min and Hart, 2003; Ademola and Idowu, 2010; Max *et al*., 2011; Alonso-Diaz *et al*., 2008a, b; Minho *et al*., 2008;

Table 11: Larval development inhibition test

				Confidence interval for mean (95%)	
Descriptive/treatments	N*	Mean egg hatchability inhibition	SD	Lower bound bound	Upper bound
S. guineensa	7	63.05	32.26	33.21	92.89
Ivermectin	7	76.86	36.53	43.07	110.64
Distilled water	7	0.00	0.000	0.00	0.00
ANOVA	Sum of squares	df	Mean square	F-value	p-value
Between groups	23503.03	2	11751.51	14.84	0.000
Within groups	14252.95	18	791.83		

N* = Number of serial dilutions

Table 12: One-way ANOVA for egg hatchability and larval development inhibition test of *R. glutinosa* against sheep *H. contortus*

				Confidence interval for mean (95%)	
Descriptive/treatments	N*	Mean egg hatchability inhibition	SD	Lower bound bound	Upper bound
R. glutinosa	9	85.74	86.16	19.52	151.97
Albendazole	9	118.55	98.71	42.68	194.43
Distilled water	9	0.00	0.00	0.00	0.00
ANOVA	Sum of squares	df	Mean square	F-value	p-value
Between groups	67451.12	2	33725.56	5.89	0.008
Within groups	137325.06	24	5721.88		

Table 13: Larval development inhibition test

				Confidence interval for mean (95%)	
Descriptive/treatments	N*	Mean egg hatchability inhibition	SD	Lower bound bound	Upper bound
R. glutinosa	7	66.43	34.90	34.16	98.70
Ivermectin	7	76.86	36.53	43.07	110.64
Distilled water	7	0.00	0.00	0.00	0.00
ANOVA	Sum of squares	df	Mean square	F-values	p-values
Between groups	24333.238	2	12166.62	14.30	0.000
Within groups	15313.016	18	850.72		

N* = Number of serial dilutions

Calderon-Quintala *et al*., 2010). Demonstration of various degrees of dose dependent inhibition on both egg hatchability and larval development by all condensed tannin extracts is in agreement with the previous studies by different authors (Ademola *et al*., 2006; Ademola and Idowu, 2007; Bahuaud *et al*., 2006; Hoste *et al*., 2006; Iqbal *et al*., 2006; Al-Shaibani *et al*., 2009; Adama *et al*., 2009). There are two hypothesis proposed to elucidate the anthelmintic effects of condensed tannins. Primarily, the direct hypothesis that is the ability of these compounds to interact with proteins of the cuticle, oral cavity, esophagus, cloaca and vulva of nematodes, changing their chemical and physical properties.

Secondly, the indirect hypothesis that is the capacity of condensed tannins to bind of dietary proteins and protect them from rumen degradation increasing protein flow to and amino acid absorption by, the small intestine improving host immune response against worms (Hoste *et al*., 2006; Al-Shaibani *et al*., 2009).

The effective dose (ED_{50} and ED_{90}) is defined as the concentration of drug or extract producing 50 and 90%, respectively inhibition on egg hatching or larval development (Varady *et al*., 2005). Consequently, the three condensed tannin extracts in this study revealed a range of efficacies to inhibit the egg hatch and larval development. The observed differences in potencies among the extracts might be associated with the corresponding variation in their tannin contents. Related work with *in vitro* inhibitory effect of condensed tannins on egg hatchability and larval development of *H. contortus* was reported by Minho *et al.* (2008) (Table 11-13).

It has been stated that controls of *H. contortus* could not be resolved mere by the use of conventional anthelmintic drugs (Minho *et al*., 2008) as there is worldwide problem regarding the development of anthelmintic-resistant worm populations. The three species of plants were chosen for our trial based on their relatively high tannin contents and their wide availability in the study area. The promising result of the present study that all the three plants exhibited various dose dependent *in vitro* efficacies on egg hatchability and larval development of sheep *H. contortus* could provide baseline information on the possibility of considering condensed tannins as one of the alternatives in the packages towards the control of hemonchosis in sheep. Thus, the findings of such work need to be supported by further *in vivo* studies.

CONCLUSION

All three condensed tannin extracts demonstrated various degrees, yet very close dose dependent inhibition

of both egg hatchability and larval development. According to ED_{50} and ED_{90} values, the condensed tannin inhibiting egg hatching and larval development most potently was *R. glutinosa* followed in descending order of activity by *S. guineensa* and *A. gumifera*. Finally, our work suggests that condensed tannins might be recommended as one of the options for the control of *H. contortus* of sheep.

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