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Direct and indirect anthelmintic effects of condensed tannins in sheep

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Abstract

Anthelmintic activity of condensed tannins (CT) was evaluated both in vitro and in vivo. In vitro tests included egg hatch test and paralysis/mortality assay on adult *Haemonchus contortus*. In vivo anthelmintic effect was determined by faecal egg count reduction test in lambs. To this end, 18 lambs were divided into three groups (low tannin, high tannin and control). The lambs of low and high tannin groups were fed diets containing 2 and 3% CT while the control group was fed on diets without CT. In vitro trials showed a dose-dependent inhibition of nematode egg hatching; whereas, there was no effect of CT on adult *H. contortus*. In vivo trials indicated reduction in faecal egg counts in lambs fed diets containing CT. Feed intake and nutrient digestibility of CT-fed sheep was lower and nitrogen balance was higher as compared to control. Maximum weight gain was observed in animals fed diets containing 3% CT. The direct anthelmintic effect of CT, therefore, was evidenced by inhibited egg hatching; whereas, faecal egg counts reduction in sheep was through improved nutrient utilization.

Keywords: Condensed tannins; Anthelmintic; Sheep; Nutrition; *Haemonchus contortus*; Digestibility

1. Introduction

Helminthiosis is one of the major constraints in small ruminant production. An increase in the supply of digestible protein (DP) has been reported to improve the resilience and resistance of sheep to gastrointestinal nematodes (GINs) (Van Houtert and Sykes, 1996; Donaldson et al., 1997).

Microbial actions alter both the quantity and quality of protein supply to small intestine due to combined effects of degradation and synthesis in the rumen (Church and Santas, 1981). Protection of dietary protein from rumen degradation can increase protein availability/absorption in the small intestine (Waghorn and Shelton, 1995; Haslam, 1993; Coop and Kyriazakis, 1999). Condensed tannins (CT) also called as proanthocyanidin (PA) are expected to bind strongly to protein and protect them from degradation by rumen microbes. Proanthocyanidin containing forages have been reported to minimize the detrimental effects including diarrhoea due to heavy load of internal parasites (Niezen et al., 1995; Robertson et al., 1995). It seems that consumption of plants containing CT may affect GIN numbers and animal performance in a

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number of ways that involve direct effect on the parasite (Athanasiadou et al., 2000) and indirect effect through improved protein supply (Niezen et al., 1993, 1995). Forages containing CT have, therefore, been suggested as an alternate to parasite management (Niezen et al., 1995; Barry et al., 2001; Min et al., 2002a,b). The objective of the present study was to evaluate the role of CT in exerting their anthelmintic effects against *Haemonchus contortus* through improvement in nutrient utilization in sheep. In addition, however, direct in vitro anthelmintic effect of CT was also investigated.

2. Materials and methods

2.1. In vitro anthelmintic activity of CT

The in vitro anthelmintic activity of CT extracts prepared from commercially available tannin {Kenya source, used in textile industry and containing 1.19% g CT/kg DM as determined by Butanol–HCl reagent method (Porter et al., 1986)} was investigated by egg hatch test (Coles and Simpkins, 1977) and paralysis/motility of adult *H. contortus* (Sharma et al., 1971).

2.1.1. Collection of H. contortus worms and eggs

Adult *H. contortus* were collected from the abomasa of sheep, slaughtered at Faisalabad abattoir (Maqsood et al., 1996). The worms were picked manually using artery forceps and placed in a bottle containing PBS (pH 7.2). Female worms were separated from males by grossly witnessing the blood filled intestine spirally coiled around white ovary giving an appearance of barber's pole worm (Soulsby, 1982). The adult worms were used for egg collection and/or paralysis/mortality test.

Female worms were separated from males and washed thrice in lukewarm PBS (pH 7.2) and transferred to 0.9 percent normal saline solution and incubated at 37 °C for 24 h. The worms were removed from normal saline solution after 24 h and eggs laid by them were collected by standard faecal procedures by sedimentation using slow centrifugation (Soulsby, 1982). The eggs were then washed thrice in distilled water and adjusted to a known density in water using the McMaster technique (Whitlock, 1960).

2.1.2. Egg hatch test

Stock solution (8 μg/ml) was made by dissolving 8 mg of tannin in 5 ml of Dimethyl sulfoxide (DMSO) and making to 1 l by adding 0.1% NaCl. Likewise, stock solution (8 μg/ml) was made by dissolving 8 mg of oxfendazole (Glaxo-Welcome) in 5 ml of DMSO and making to 1 l by adding 0.1% NaCl. Ten serial dilutions of tannin and oxfendazole (0.00625–3.2 μg/ml) were made in wells of titration plates. Two milliliter of fresh eggs (n = 300), within 3 h post-collection, were taken in each well of a 24 well titration plate (Flow Laboratories). A 10 μl of tannin from each dilution was added to the experimental wells and 10 μl from each dilution of oxfendazole was added in other wells which served as positive control while the negative control well received only 10 μl of the diluent (0.1% NaCl). Plate was incubated at 27 °C for 48 h. After incubation, two drops of Lugol's iodine were added and at least 100 of the remaining eggs (dead and embryonated) and hatched larvae were counted. Each experiment was conducted in triplicate.

2.1.3. Paralysis/mortality of adult H. contortus

Ten worms were exposed in triplicate to the tannin extracts (25, 50 and 100 mg/ml) in PBS. The positive and negative controls were levamisole (0.55 mg/ml) and PBS, respectively. The mortality of the worms or inhibition of their motility at different hours post-exposure was used as the criterion for anthelmintic activity. The dead worms were easily recognized by their straight flat appearance with no movements at the head and tail regions of the body. The motility was observed on 0, 2, 4, 6 and 8 h post-exposure. Finally, the treated worms were kept for 30 min in the lukewarm fresh PBS to observe revival of motility.

2.2. In vivo anthelmintic activity of CT

Eighteen lambs having almost uniform characteristics (nature and intensity of helminth infection, age, weight, etc.) were purchased from Livestock Experiment Station, Rakh Ghulaman (Punjab, Pakistan) and used to evaluate the anthelmintic activity of tannins at different levels. The nature and intensity of helminth infection was determined by faecal egg counts (Thienpont et al., 1979) and coproculture (MAFF, 1986) before the start of experiment. The animals were divided into three equal groups, i.e., low and high tannin groups were fed diets containing 2 and 3% CT, respectively; whereas, animals belonging to control group were fed diet without CT.

The chemical composition of sheep diet is given in Table 1. All animals were prearranged on restricted feeding and the diets were formulated isoenergetic (1.5 Mcal/kg) and iso-nitrogenous (2.68%). During experimental period, the animals were kept in separate pens. The experiment lasted for 140 days. The first 20 days were given for adaptation to the sheep diet without

Table 1
Ingredients and chemical composition of experimental diets (DM basis)

	Diets[a]		
	Control (without CT)	LT (2% CT)	HT (3% CT)
Ingredients			
Maize	28	28	28
Rice polishing	16	16	16
Wheat bran	25.5	23.3	22.3
Maize gl. M. 30%	9	9	9
Maize oil cake	5	5	5
Canola meal	7	7	7
Cane molasses	7	7	7
Mineral mix	2	2	2
Urea	0.5	0.65	0.7
Tannin	–	2.0	3.0
Chemical composition (%)			
DM	71.9	71.9	71.9
OM	75.9	74.4	73.6
N	2.63	2.63	2.63
NDF	33.3	32.7	32.4

[a] Iso-energetic (1.5 Mcal/kg) and iso-nitrogenous (2.68%); CT, condensed tannin; control diet contained 28% fodder and 72% concentrate; LT, contained 28% fodder and 70% concentrate with added 2% tannin; HT, contained 28% fodder and 69% concentrate with added 3% tannin.

supplementation of CT. The diets were prepared twice in a week and stored at room temperature. Faecal egg counts from all the experimental sheep were done on days 0, 15, 30, 45, 60, 75, 90 and 120 of the experiment (Thienpont et al., 1979).

2.3. Nutrient intake, digestibility and weight gain

Complete collections of the faeces were made to determine the nutrient digestibility according to the procedure described by Nisa et al. (2004). Faeces were collected daily for 4 days towards the end of experiment, dried at 55 °C, bulked, mixed and sampled at the end of experiment. Daily collections of urine were acidified with 50% H_2SO_4. Feed, orts and faecal samples were analyzed for DM, N and OM (AOAC, 1990) and NDF (Van Soest et al., 1991). The animals were weighed at the start of experiment and thereafter weekly to assess the growth performance of sheep.

2.4. Statistical analyses

The data obtained from in vivo results were analyzed through general linear model using repeated measures analysis of variance option and obtained the least square means of interaction and then these means were plotted (StatSoft, 1999). The effect of treatments on nutrient intake, digestibility and nitrogenous balance was compared by the least significant difference test (Steel et al., 1997).

3. Results

3.1. In vitro anthelmintic activity of CT

Tannin extracts and oxfendazole inhibited egg hatching at different concentrations and a dose-dependent inhibitory response was observed (Fig. 1.). The maximum eggs hatched in 0.1% NaCl (86.7%). There was no effect of tannin extracts on the adult *H. contortus* in comparison to control (data not shown). However, 9 of the 10 worms exposed to levamisole were found dead at 8 h post-exposure; whereas, nine of the 10 worms survived and remained active at 8 h post-exposure in PBS.

3.2. In vivo anthelmintic activity of CT

The nematode eggs recovered from the experimental sheep were identified on egg morphology as *Trichuris ovis* and by subsequent larval culture as *H. contortus*, *Trichostronglyus* spp., *Oesophagostomum columbianum*. The overall effect of different treatments on eggs per gram of faeces of individual lamb's revealed time- as well as dose-dependent response (Fig. 2). The data revealed a gradual reduction in faecal egg counts, which differed significantly on days 60–120 from the day 0 values in sheep fed on diets supplemented with CT both at 3 and 2% levels (Fig. 3). There was no difference in FEC of sheep fed a diet without CT.

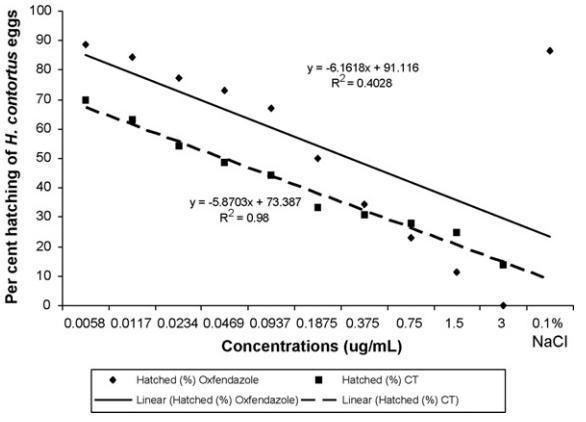

Fig. 1. Regression analysis of percentage of eggs hatched in various concentrations of oxfendazole and condensed tannins in vitro.

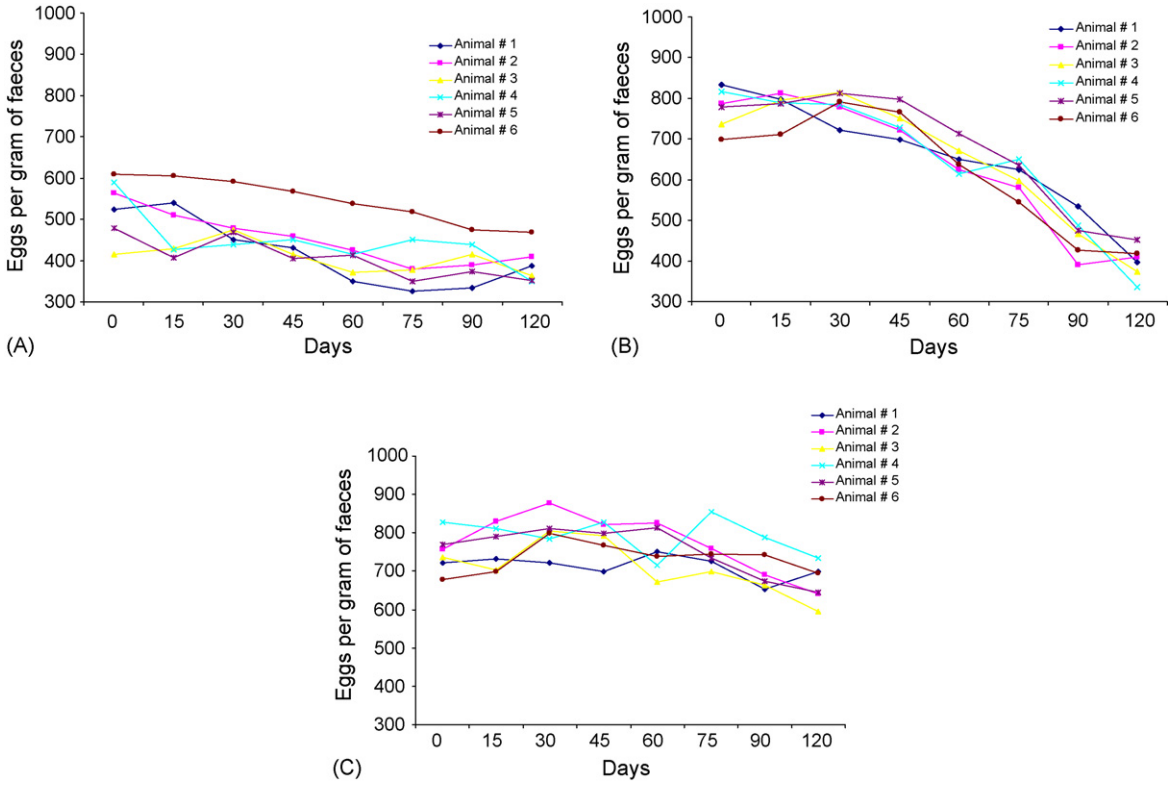

Fig. 2. Time-dependent response of tannin on faecal egg output of individual experimental animals (A) tannin 2%, (B) tannin 3% and (C) control.

3.3. Nutrient intake, digestibility and weight gain

The data (Table 2) revealed significantly lower nutrient intake by sheep fed diets containing CT than that without CT. Likewise; the values of digestibility parameters were also lower in sheep fed diets containing CT than that of control diet. The nitrogen balance was higher (Table 3) in sheep fed diets containing CT than those in control group (without CT). During 120 days of experiment, weight gain was significantly higher (8.2 kg; $p < 0.05$) in sheep fed diets with 3% CT than those on 2% CT (6.8 kg) and tannin-free diet (4.8 kg).

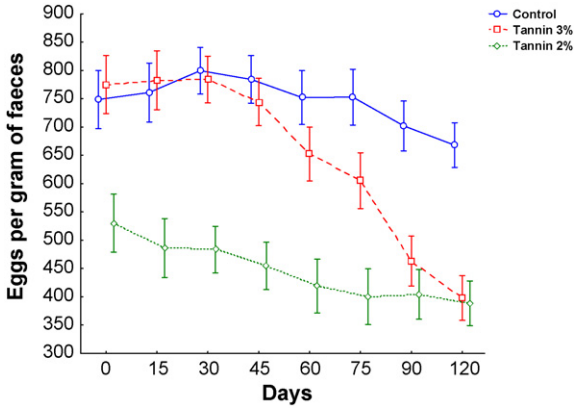

Fig. 3. Effect of different levels of condensed tannin compared with control on eggs per gram of faeces of mixed gastrointestinal nematode infection in sheep at different time intervals.

4. Discussion

About 40 years ago, Taylor and Murant (1966) reported the use of CT to reduce the soil nematode populations. Hence, it was surmised that the CT may be able to affect nematodes in the gastrointestinal tract of sheep. Therefore, the use of CT for anthelmintic purposes in animals has been a focused area of research particularly during the last 10–15 years. There are numerous reports indicating direct or indirect anthelmintic effects of CT (Barry et al., 1986; Dobson et al., 1990; Coop and Holmes, 1996; Van Houtert and Sykes, 1996; Donaldson et al., 1997; Aerts et al., 1999; Kahn and Diaz-Hernandez, 2000; Athanasiadou et al., 2001; Niezen et al., 2002; Iqbal et al., 2002).

In the present study, an attempt has been made to ascertain the mechanism of anthelmintic effects exerted by CT. The inhibitory effects on egg hatching of

Table 2
Nutrient intake and digestibility in sheep fed varying levels of condensed tannins

Parameters	Nutrient intake (kg/day)				Digestibility (%)			
	Control	LT	HT	S.E.	Control	LT	HT	S.E.
Dry matter	1.34[a]	1.20[b]	1.20[b]	0.058	71.97[a]	71.24[a]	71.08[a]	1.591
Organic matter	1.02[a]	0.89[b]	0.91[b]	0.050	73.58[a]	72.60[a]	72.50[a]	1.519
Nitrogen	0.04[a]	0.03[a]	0.03[a]	0.003	75.53[a]	73.20[ab]	71.60[b]	1.499
Neutral detergent fiber	0.45[a]	0.39[b]	0.39[b]	0.020	55.29[a]	50.99[ab]	44.01[b]	3.799

Control, diet contained 28% fodder and 72% concentrates; LT, diet contained 28% fodder and 70% concentrates with added 2% tannin; HT, diet contained 28% fodder and 69% concentrates with added 3% tannin.
a–b, values marked with the similar alphabets in a row do not differ significantly at $P \geq 0.05$.

Trichostrongylus colubriformis have been reported previously (Niezen et al., 2002) after feeding tanniferous diets to lambs. Earlier, it has been shown that CT extracted from herbage can inhibit the migration of *T. colubriformis* L3 and the L1 and L3 larvae of *D. viviparous* in in vitro assays (Molan et al., 2000) indicating that CT can be detrimental to both egg hatching and larval development. These results suggest that CT can have a marked effect on the subsequent development of nematode larvae. As CT are not absorbed in the digestive tract (Terrill et al., 1994) they become concentrated in the faeces, thus affecting the hatchability of nematode eggs resulting in lower pasture contamination.

In this study, 25–100 mg/ml concentrations of CT did not exert anthelmintic effects on adult *H. contortus*. As far as ascertained, anthelmintic effect of CT on adult worms has not so far been investigated in vitro. The larval development/viability assays have, however, indicated that larval development was not affected, but the viability of infective nematode larvae was adversely affected after exposure to different concentrations (0–10%) of Quebracho tannins (Athanasiadou et al., 2001). It was speculated that the anthelmintic activity of Quebracho extract against infective larvae may be due to capacity of tannin to bind to proteins, which resulted in reduced nutrient availability, and thus larvae starvation and death. Condensed tannins may also bind to the cuticle of larvae, which is high in glycoprotein (Thompson and Geary, 1995) and cause their death. The difference in the anthelmintic effects of CT on infective larvae (Athanasiadou et al., 2001) and adult *H. contortus* (present study) could be attributed to the interspecific differences in susceptibility as demonstrated by Athanasiadou et al. (2001). It may also be attributed to the difference in stage of the parasite used. The adult worm may have become more resilient to CT compared with the larval form.

The present in vivo study have indicated anthelmintic effects against mixed gastrointestinal nematode infections in sheep fed on diets supplemented with CT. Similar findings have been reported previously after feeding the tanniferous plants to animals (Athanasiadou et al., 2000; Kahn and Diaz-Hernandez, 2000). The results of the present study indicated significant reduction in FEC on days 60 and 120 in sheep fed on diets supplemented with 3 and 2% CT, respectively. The digestibility parameters also differed significantly

Table 3
Nitrogen balance in sheep fed varying levels of tannin diets

Parameters	Diets			
	Control	LT	HT	S.D.
Nitrogen intake (kg/day)	0.04[a]	0.03[a]	0.03[a]	0.0333
Faecal nitrogen (kg/day)	0.009	0.009	0.009	0.0005
Urinary nitrogen (kg/day)	0.013[a]	0.007[b]	0.005[b]	0.0014
Total nitrogen out (urine + feces) (kg/day)	0.022[a]	0.016[b]	0.014[b]	0.0015
Nitrogen balance (g/day)	14.0[b]	17.0[ab]	18.0[a]	0.0012

Control, diet contained 28% fodder and 72% concentrates; LT 1, diet contained 28% fodder and 70% concentrates with added 2% tannin; HT 1, diet contained 28% fodder and 69% concentrates with added 3% tannin; control 2, diet contained 28% fodder and 72% concentrates; LT 2, diet contained 28% fodder and 70% concentrates with added 2% tannin; MT 2, diet contained 28% fodder and 68% concentrates with added 4% tannin; HT 2, diet Contained 28% fodder and 66% concentrates with added 6% tannin.
a–b, values marked with the similar alphabets in a row do not differ significantly at $P \geq 0.05$.

across low (2%), high (3%) and tannin-free diets. The nitrogen balance was significantly better in sheep fed on diets supplemented with CT than those of control.

Tannin feeding probably increases the supply of essential amino acid leading to an increased body growth (Aerts et al., 1999). The CT has been reported to cause a major physiological adaptation of increased growth hormone. In sheep fed CT rich forage (*L. pedunculatus*), a linear and positive correlation was found between dietary reactive CT concentration and blood plasma growth hormone level (Barry et al., 1986). Growth hormone stimulates nitrogen retention in the animal (Muir et al., 1983) that may be a contributing factor in the improved protein metabolism associated with CT (Aerts et al., 1999).

The anthelmintic effects of CT have been mainly attributed to the improved resistance and resilience to GI nematodes with an increase in the digestible protein supply (Coop and Holmes, 1996; Van Houtert and Sykes, 1996; Donaldson et al., 1997). However, many of these experiments were of a short duration, making it difficult to assess the significance of any DP-mediated effects on resistance since such effects in nematode-naïve animals generally become apparent from about 10 weeks post-infection (Dobson et al., 1990). Our results support the indirect anthelmintic effects of CT through improved resistance and resilience against GI nematodes by improved nutrient utilization. This is supported by our data on FEC (day 60 post-experiment; Table 2), improved nitrogen balance (Table 3), and adverse effect of CT on nutrient utilization and absence of anthelmintic effect in poultry (data not shown; Haseeb, 2003). Moreover, CT took about 8–9 weeks in exerting their anthelmintic effects, which would have been earlier in case of direct effects. The possibility of increased resistance through non-protein mediated effects is also remote as the experimental sheep were not naïve rather naturally parasitized with GI nematodes.

It is concluded that the direct anthelmintic effects of CT seem to be limited to the developing stages not the adult nematodes; whereas, improvement in resistance and resilience via increased DP-supply indirectly affect the nematodes. Both direct and indirect effects of CT, however, are beneficial in lowering the contamination of pastures by reducing the hatchability of nematode eggs and FEC reduction in sheep. It is suggested that controlled studies of longer duration (6–8 months) on single nematode infections with additional parameters like the worm burden, electron microscopy of nematodes and the intestinal epithelium, and albumin/globulin ratios be carried out.

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