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Effect of condensed tannins from *Pinus radiata* bark on *Trichostrongylus colubriformis* larvae and adult worms in sheep

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ABSTRACT

A trial was undertaken to investigate the effect of condensed tannins (CT) extracted from pine (*Pinus radiata*) bark on gastrointestinal (GI) nematodes. Gastrointestinal parasitism is a significant cost to the sheep industry affecting health and productivity. Previous research has shown CT from some forage species can inhibit parasite egg hatching, larval development and reduce worm numbers in the GI tract. However, the forages containing CT tend to be less productive than ryegrass pasture, so this research investigated pine bark extract (PBE) which contains CT and is abundant in New Zealand, on *Trichostrongylus colubriformis* nematodes. In the *in vitro* incubations, 150 µg CT/ml prevented larval development and halved egg hatching. Effects of CT *in vivo* (35g/day) were evaluated using 12 wether sheep, fitted with abomasal cannulae and infected with *T. colubriformis* larvae. The sheep were either drenched at six hourly intervals or the PBE was infused into the abomasum over four days. Neither treatment affected faecal egg count (1,700/g faeces), egg hatching or development of eggs to L3 larvae. Lambs were slaughtered after four days of treatment. Nematode numbers were similar for all treatments (average 21,100/animal). Despite inhibitory effects on hatching and development *in vitro*, PBE had no anthelmintic benefits for the sheep and may reduce intakes.

Keywords: anthelmintic; pine bark; tannin; sheep; *Trichostrongylus colubriformis*.

INTRODUCTION

Condensed tannins (CT) are phenolic compounds which are present in the foliage and seed coats of a variety of forage plants, and also the bark of pine trees (*Pinus radiata*). These compounds may provide benefits for ruminants when diets contain sufficient protein, provided the concentration and astringency are not excessive (Waghorn, 2008). Estimated production of pine bark is about 1 million tonnes/annum from New Zealand forests (Bogan, 2007). The bark has high concentrations of phenolic compounds, including CT, which could provide a low cost source of CT for mitigating effects of gastrointestinal parasites, with additional benefits for production.

Field trials have shown that lambs with substantial parasite burdens are able to maintain high levels of productivity when fed forages containing CT (Birdsfoot trefoil (*Lotus corniculatus*); Lotus major (*L. pedunculatus*); Sulla (*Hedysarum coronarium*)), relative to pasture or lucerne (Robertson *et al.*, 1995) and lotus major reduced faecal egg counts (FEC), whilst sulla lowered worm burdens (Niezen *et al.*, 1998). The reasons for good growth rates of parasitised lambs are not clear, but CT increase plant protein flow to the intestine, and CT in *L. corniculatus* increase amino acid absorption (Waghorn, 2008). Protein supplementation can improve the ability of young sheep to tolerate worm burdens (Sykes & Greer,

2003) and may improve host immune responses (Niezen *et al.*, 2002a).

When forages containing CT are fed to sheep, faecal egg hatching and larval development can be reduced (Niezen *et al.*, 2002b), but the extent of inhibition is lower than the 60% to 80% reduction in larval development of *Trichostrongylus colubriformis* under *in vitro* conditions (Molan *et al.*, 1999; 2002; 2003). The reasons for lesser effects *in vivo* are perplexing, because the carbon component of CT passes through the intestinal tract of sheep (Terrill *et al.*, 1994) and digestion of other dietary components effectively increases the CT concentration in faeces. However, digestion may affect the structure of CT, and binding with proteins at near-neutral pH in the rumen (Jones & Mangan, 1977) reduces its capacity to affect nematodes in the intestine, therefore pine bark extract (PBE) was given to sheep via oral and abomasal routes in this trial. Oral drenching will allow the CT to bind with protein in the rumen, whereas an abomasal infusion will reduce opportunities for binding and maximise opportunities for CT to associate with the *T. colubriformis* in the small intestine.

This trial evaluated PBE derived from bark which had been chopped and ground, on *T. colubriformis* eggs and larvae *in vitro*, and effects on egg hatching and development when administered to lambs previously infected with *T. colubriformis* as well as FEC and intestinal worm

numbers at slaughter. Administration of PBE was either by mouth as a drench, or infusion to the abomasum.

MATERIALS AND METHODS

Bark obtained from seven to 10 year old pine trees was chopped then ground, and the fine powder mixed with boiling water (1:10 ratio) for 20 minutes. After particulate matter was removed, the solution was freeze dried. About 2 kg of PBE powder was produced from 15 kg bark. The powder contained 35% CT, of which 90% was unbound and 10% bound, as indicated by sequential butanol/HCl extraction (Terrill *et al.*, 1992). The freeze dried extract was added to water to achieve a 10% (w:w) solution, and the supernatant either drenched or infused into lambs as well as being used for *in vitro* assays.

Egg hatching and larval development assays

Egg hatch and larval development assays were made using faecal samples taken from each lamb on Day 63 (prior to PBE administration), and on Days 3 and 4 of the treatment period. *T. colubriformis* eggs were collected from faeces by soaking in cold tap water followed by a sequential filtration of the slurry to retain eggs on a sieve with 20 µm pore size. The suspension was washed with a 20% MgSO₄ solution and centrifuged as described by Molan *et al.* (2002) to obtain eggs for analyses.

The egg hatch assay (Molan *et al.*, 2002) involved incubation of about 100 eggs in 200 µL distilled water with 0, 160, 320, 640, 1,280, 1,920, 2,560 and 3,200 µg PBE/mL. These concentrations were equivalent to 0, 50, 100, 200, 400, 600, 800 and 1,000 µg CT/mL. After incubating for 24 hours at 24°C, the numbers of unhatched eggs and first stage (L1) larvae were counted from triplicate samples at each concentration.

The larval development (Molan *et al.* 2002) involved about 100 eggs incubated in triplicate in 200 µL of medium in 96-well microtitre plates. Incubation was for eight days at 25°C with sufficient PBE to give CT concentrations of 0, 10, 25, 50, 75, 100 and 150 µg/mL of medium. The number of L1, L2 and L3 larvae and eggs were counted after eight days to determine the effect of PBE on larval development.

In vivo assays

The animal experiment was approved by the Palmerston North Campus Animal Ethics Committee. Twelve Dorset/Romney wether lambs aged about 10 months and weighing between 35 and 45 kg were used. They were removed from pasture, fed lucerne pellets (800 g/d) and lucerne chaff (200 g/d) (940 g dry matter (DM)/d) twice daily (09:00 and 17:00 h) for the 70 day experimental period. Refusals were collected for DM determination over

the final 30 days, so that feed intakes could be determined. Dietary crude protein (CP) concentration was 225 g/kg DM. All lambs were given Cydectin (0.2 mg active ingredient/kg live weight; Boehringer-Ingelheim, Ingelheim am Rhein, Germany) on Days 2 and 16 to remove intestinal worms. Abomasal cannulae were installed under general anaesthesia on Days 21 to 30. All lambs were given 6000 *T. colubriformis* L3 (infective larvae) each day on Days 35 to 40 of the trial by stomach tube. Faecal samples were taken for FEC 22 days after initial larval dose on Day 56, and on Day 58 and daily from Day 60 to the end of the trial.

Lambs were allocated to treatments as follows: The four Control sheep did not receive PBE and were drenched 4 x daily with water; the four sheep in the "oral" group were given 250 mL of solution containing 25 g PBE at six hourly intervals using a drench gun, whilst the "abomasal" group received the same daily dose of PBE (100 g in 1000 mL) as a continuous infusion of about 45 mL/hour. PBE was administered to eight of the 12 sheep on Days 67, 68, 69 and 70, after which they were slaughtered.

Rumen liquor was obtained by stomach tube on Day 65 (before PBE treatment) and on Days 2 and 3 of PBE administration to measure concentrations of ammonia and volatile fatty acids (VFA). Blood samples (jugular) were taken on Days 3 and 4 of PBE administration to determine concentrations of lactate, glucose and urea.

At the conclusion of the trial sheep were euthanised and a 10 m section of the small intestine distal to the duodenum was excised for worm counting as described by Niezen *et al.* (1998).

Chemical and statistical analyses

The concentration of tannins in the PBE were determined by butanol/HCl assay (Terrill *et al.*, 1992), rumen ammonia by colorimetric assay and VFA by gas chromatography. Analyses of whole blood to measure glucose and lactate concentrations used a blood gas analyser (ABL System 615/610, Radiometer, Copenhagen). FEC were made using the modified McMaster technique and adult worm counts from intestinal contents (Niezen *et al.*, 1998) included identification of males, females and L4 larvae.

Treatment effects were averaged for each animal and analysed by an analysis of variance (Genstat) using mean values pre-treatment as a covariate. Data used for the covariate were either the day prior to treatment (egg hatching and larval development) or averaged for two days (faecal egg counts). Treatment effects were measured in faeces on Day 3 of PBE administration for larval development, or averaged on Days 3 and 4 (ammonia, egg hatching) or Days 2, 3 and 4 (FEC) of treatment. When there were no covariate effects, treatments were compared by an analysis of variance.

TABLE 1: Faecal egg count (FEC), egg hatching and larval development from faeces before and during the pine bark extract (PBE) treatment for Control (no PBE), Oral administration and Abomasal administration groups. None of the treatment effects were statistically significant.

Measurement	PBE treatment			Standard error of mean
	Control	Oral administration	Abomasal administration	
FEC (eggs/g faeces)				
Before PBE treatment	1,916	1,880	1,766	145
During PBE treatment	1,551	1,526	1,797	319
Egg hatching (%)				
Before PBE treatment	85.4	88.1	86.4	2.8
During PBE treatment	73.8	61.2	65.5	5.6
Larval development (%)				
Before PBE treatment	91.6	85.3	86.8	3.62
During PBE treatment	71.5	53.9	75.7	8.05

TABLE 2: Concentration of rumen ammonia before and during the pine bark extract (PBE) treatment and concentrations of rumen volatile fatty acids (VFA) and blood glucose and lactate during the PBE period of treatment for Control (no PBE), Oral administration and Abomasal administration groups. Bolding of P value indicates significance ($P < 0.05$).

Measurement	PBE treatment			P value	Standard error of mean
	Control	Oral administration	Abomasal administration		
Rumen ammonia (mmol/L)					
Before PBE treatment	13.6	14.1	12.0	0.50	0.9
During PBE treatment	9.8	9.4	11.0	0.69	1.6
Rumen VFA (mmol/L)					
Major VFA ¹	8.18	8.42	8.02		
Minor VFA ²	0.29	0.45	0.28		
Blood metabolites (mmol/L)					
Glucose	4.3	4.7	3.7	0.01	0.1
Lactate	2.5	3.8	2.3	0.24	0.6

¹Acetate, Propionate and Butyrate

²Isobutyrate, Valerate and Isovalerate

RESULTS

PBE effects on egg hatching and larval development

In vitro cultures showed PBE had a major effect on egg hatching (Figure 1) with 100 µg CT halving the number hatched after 24 hours. Although fewer than 10% of eggs hatched when CT concentrations were 400 µg/mL or higher, hatching was not completely prevented with higher concentrations. The impact of CT was greater on larval development, and 150 µg/mL prevented development.

In vivo evaluation of PBE

All sheep ate the majority of the feed offered from the commencement of the trial until about

14 days after larval administration (Day 50), when some sheep in each group showed a reduced intake for five to seven days. Over the seven days prior to the commencement of PBE administration average intakes (g DM/day ± standard deviation) of the four sheep in each treatment were: Control 939 ± 106, Oral 929 ± 172 and Abomasum 961 ± 79. After PBE administration commenced, some sheep, especially those in the Abomasal group, had lower feed intakes, so mean values over the final three days were 906 ± 120; 897 ± 185 and 630 ± 322 g DM/day for the respective groups.

Neither oral nor abomasal administration of PBE reduced FEC of *T. colubriformis* (Table 1), which averaged 1,625 eggs/g ($P = 0.650$). In contrast to *in vitro* measurements, PBE did not affect either egg hatching percentage ($P = 0.314$) or development of eggs to L3 larvae ($P = 0.613$). Oral administration of PBE resulted in 54% of eggs in faeces developing to L3 larvae, compared to 72% and 76% for Control and Abomasal groups respectively. Total worm numbers recovered at slaughter were

similar ($P = 0.83$) for Control (19,900), Oral (21,600) and Abomasal (21,830) groups, with male:female ratios of 1.42 to 1.47 across all treatments.

Rumen ammonia and VFA concentrations were measured to assess the impact of PBE on ruminal proteolysis and digestion. Neither ammonia nor VFA concentrations were affected by treatment (Table 2). The proportions of the principal VFA were acetate 0.67 propionate 0.21 and butyrate 0.12. There was a higher proportion of minor VFA from sheep receiving the oral PBE dose than other groups ($P < 0.01$). Oral administration of PBE did not affect blood glucose or lactate concentrations but sheep receiving abomasal PBE had a significantly ($P < 0.01$) lower glucose concentration than other groups.

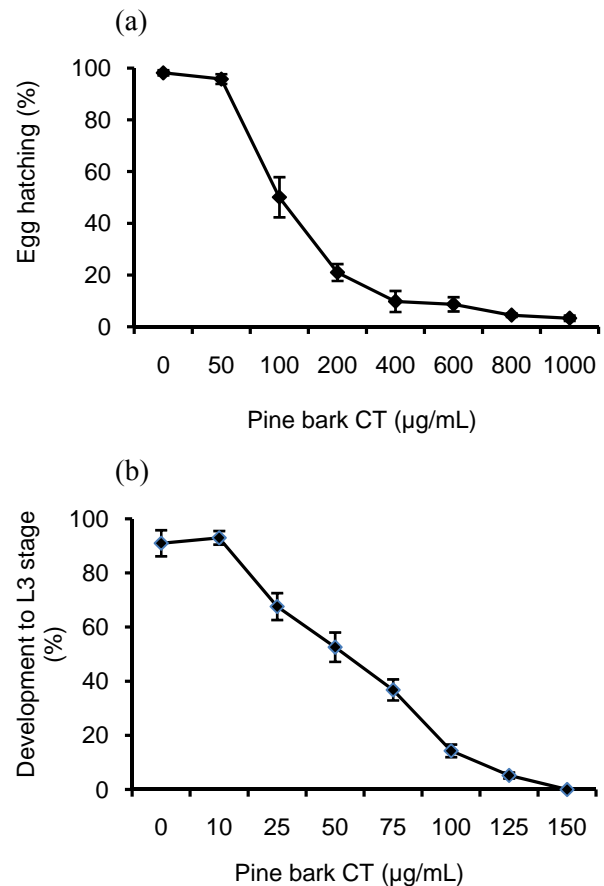
DISCUSSION

In vitro measurements suggested a potent inhibition by PBE, with a 50% reduction in egg hatching in a solution containing about 100 µg CT (in PBE)/mL, whereas purified CT extracted from seven plant species required about 1,000 µg/mL for a similar level of inhibition (Molan *et al.*, 2002). In contrast, the concentrations of CT required for a 50% inhibition of larval development to the L3 stage (50-100 µg/mL) were similar to extracts from other forage species evaluated by Molan *et al.* (2002). Reasons for differences in effectiveness of CT for inhibition of egg hatching are not clear, but may be associated with CT structure, or non-CT constituents in the PBE.

The *in vivo* findings were in marked contrast to *in vitro* inhibition. The absence of effects of PBE on FEC over the four days prior to slaughter is comparable with findings from sheep fed *Lotus corniculatus* (5.5% CT in the DM) and *Dorycnium rectum* (17% CT in the DM), but *L. pedunculatus* (12% CT in the DM) did halve FEC (Niezen *et al.*, 2002b). Sulla (*Hedysarum coronariun*) seems always to lower FEC, and can lower worm numbers in sheep (Niezen *et al.*, 1995; 1998). *Lespedeza cuneata* (Sericea *Lespedeza*) which contained 4.6% CT in the DM reduced FEC and daily output of eggs from goats by more than 70% (Min *et al.*, 2004) and effects of CT extract from *Quebracho* (about 4.5%) reduced FEC and *T. colubriformis* worm numbers in sheep on some occasions, but not others (Athanasidou *et al.*, 2001). Buttler *et al.* (2000) suggested the concentration of dietary CP, or CT:CP ratio may affect efficacy of CT as an anthelmintic, and this could account for differences between *in vitro* and *in vivo* results from this study. However the CT:CP ratios reported with fresh forage diets were unrelated to effects on FEC (Niezen *et al.*, 1995; 1998; 2002a; Min *et al.*, 2004). In their review of anti-parasitic effects of plant secondary compounds, Athanasidou and Kyriazakis (2004) concluded *in vivo* responses often, but not always, occurred when CT were given, but results were variable, possibly due to variations in CT structure, level of intake and availability in the intestinal tract of the animal.

The efficacy of CT from several forage types for inhibition of egg hatching and larval development was investigated by Molan *et al.* (2003). They related efficacy to the type of flavan-3-ol units comprising the CT, and suggested anthelmintic effects were greatest when the prodelphinidin: cyanadin ratio was high, as in *L. pedunculatus* (4:1). The polyphenolics in pine bark comprise similar proportions of prodelphinidin and cyanadin (1:1 ratio; Czochanska *et al.*, 1979) which

FIGURE 1: *In vitro* assays of pine bark extract showing effects of condensed tannin (CT) concentrations in media on percentage of *Trichostrongylus colubriformis* eggs either hatching (a), or developing into L3 larvae (b) following incubation. Data are means of triplicate assays. Pine bark extract contained about 35% condensed tannin. Error bars represent the standard deviation about the mean from triplicate assays.



may not be as efficacious for inhibiting intestinal nematode larvae.

The availability of CT in the intestinal tract was addressed in part by the Abomasal infusion treatment. Oral drenching will allow CT to bind with protein in the rumen, forming stable complexes (Jones & Mangan, 1977) which dissociate in the abomasum, so the unbound tannin could affect *T. colubriformis*, which are located in the intestine. However, the rapid rise in intestinal pH (Terrill *et al.*, 1994) is likely to create CT-protein complexes about 1 metre distal to the pylorus so the CT will again associate with proteins, perhaps including intestinal nematodes and the mucosa. Efficacy of abomasal relative to oral administration for affecting *T. colubriformis* is not known, but results of this study did not support one route being superior to the other. Research by Niezen *et al.* (2002a) has shown CT have greater effects on the abomasal-dwelling *Teladorsagia circumcincta* (previously *Ostertagia circumcincta*), than *T. colubriformis*.

Oral administration of PBE had a minor effect on feed intakes over the four days it was given, but two sheep began to refuse feed after two days of drenching, and one sheep objected strongly to the PBE drench, so prolonged dosing might have had a detrimental effect on intakes. More important was the marked reduction in intakes of the Abomasal treatment which commenced in some sheep on the second day of infusion, and all sheep were affected by Day 4.

The reduced intakes of the Abomasal sheep were unexpected as were the significantly lower blood glucose concentrations suggesting possible damage attributable to the infusion. However, this was not evident at slaughter. The reduction in blood glucose concentrations in the Abomasal group may have been a consequence of lower intakes.

CONCLUSION

Pine bark extract that contains CT reduced the egg hatching and larval development *in vitro*, but did not reduce FEC or intestinal worm numbers when given to sheep infected with *T. colubriformis* nematodes. Daily administration of 100 g pine bark extract provided about 35 g CT/day, equivalent to about 3.5% of dry matter intake. Continuous infusion into the abomasum, as a more direct method for affecting established worms, resulted in rapid reduction in feed intake. PBE as used in this trial was deleterious to the sheep and provided no benefits.

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