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Effects of condensed tannins and sesquiterpene lactones extracted from chicory on the viability of deer lungworm larvae

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ABSTRACT

The objective of this study was to determine the effects of condensed tannins (CT) and a crude extract containing sesquiterpene lactones (CSL) extracted from chicory (*Cichorium intybus*) on the viability of the first (L1) and third stage (L3) larvae of deer lungworm, *Dictyocaulus viviparus in vitro* using the larval migration inhibition (LMI) assay. The CT and CSL immobilised larvae and inhibited their migration through 25 mm nylon mesh sieves. Incubation of L1 larvae in rumen fluid (collected from deer fed pasture) containing 100, 400, and 1000 mg CT/ml, inhibited 12%, 28% and 41% of the larvae from passing through the sieves, respectively while the incubation of L3 larvae with rumen fluid containing the same concentrations inhibited 26%, 37% and 67% of L3 larvae from passing through the sieves, respectively. CT were more effective ($P < 0.001$) at reducing the viability of lungworm L1 and L3 larvae when added to the rumen fluid (pH 6.6) than when added to the abomasal fluid (pH 3.0). L3 larvae were more sensitive to the action of CT than L1 larvae. Addition of 2 mg polyethylene glycol/mg CT eliminated most of the inhibitory effect of CT against L1 and L3 larvae especially during incubation in rumen fluid. The CSL extract showed similar inhibitory activity in both fluids, indicating that this extract was not affected by the pH of the fluid, and they were more effective against L3 than L1 larvae.

Keywords: Condensed tannins; crude sesquiterpene lactones; chicory; lungworm; deer.

INTRODUCTION

Deer are susceptible to a variety of diseases and parasites and it has long been held that wapiti-type deer are more susceptible to parasitism than red deer in New Zealand (Watson, 1986; Bringans, 1986). Lungworm (*Dictyocaulus viviparus*) infection is the parasitic disease of farmed deer which poses the greatest risk if not controlled (Wilson and Collier, 1981; Mackintosh *et al.*, 1984; Audige *et al.*, 1998). The life cycle of this nematode is direct. The adult worms in the lungs lay eggs, which hatch in the lungs into L1 larvae. The larvae and eggs are coughed up, swallowed, passed in the faeces and continue to develop on the pasture to L3 larvae. The infective L3 larvae are then consumed with herbages, penetrate intestinal mucosa and migrate via lymphatic and blood circulation to the lungs (Haigh and Hudson, 1993).

Anthelmintic drenching is the most common method of controlling deer internal parasites. Although anthelmintic resistance has not been reported in farmed deer, it is desirable to minimise anthelmintic usage, to lower cost and to reduce the risk of development of anthelmintic resistance and carcass chemical residues. Moreover, Waldrup *et al.* (1994) reported that, although the ivermectin treatment of deer infected with lungworm did substantially reduce the infections, it did not eliminate them.

It has been found that withholding anthelmintic treatment resulted in clinical parasitism in deer grazing perennial ryegrass/white clover pasture during autumn, associated with reduction in liveweight gain (LWG), voluntary feed intake (VFI), carcass weight and a 22% reduction in number of deer reaching 92 kg live weight (50 kg CW) by one year of age, but there was no effect of withholding anthelmintic treatment on autumn LWG, VFI or carcass weight of deer grazing chicory (Hoskin *et al.* 1999). Similarly, Scales *et al.* (1995) found that parasitised lambs grazing chicory in autumn were unaffected by gastrointestinal nematodes, whilst parasitised lambs grazing grass pastures exhibited lower carcass weights than anthelmintic-treated lambs. It is not clear whether the effect of feeding chicory on internal parasites of deer and lambs is direct or indirect. The purpose of this study was to determine if the condensed tannins (CT) and crude sesquiterpene lactones (CSL) extracted from chicory have direct effects on the viability of first-stage (L1) and third-stage (L3) larvae of deer lungworm (*D. viviparus*) *in vitro*.

MATERIALS AND METHODS

Experimental design

Four experiments were undertaken to determine the effect of condensed tannins (CT) and crude sesquiterpene lactones (CSL) extracted from chicory on the *in vitro* motility of the first- (L1) and third-stage (L3) ensheathed larvae of the deer lungworm Dictyocaulus viviparus using the larval migration inhibition (LMI) assay. In the first experiment, the activity of CT against L1 larvae was evaluated in both rumen and abomasal fluids in the presence or absence of 2 mg polyethelene glycol (PEG) per mg CT. The second experiment was very similar to the first experiment except that L3 larvae were used instead of L1 larvae. The third experiment evaluated the effects of CSL against L1 larvae while the fourth experiment evaluated the effects of CSL against L3 larvae.

Preparation of CT and SL extracts

The CT extracts were prepared using the method of Jackson *et al.* (1996). About three kilograms of frozen chicory leaves were extracted with acetone:water (70:30 v/v) containing ascorbic acid (1 g l^{-1}) and washed five times with methylene chloride to remove chlorophyll and lipids. The aqueous defatted crude extracts were freeze dried,

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purified by column chromatography with Sephadex LH-20 (Pharmacia, Uppsala, Sweden), freeze dried and stored at -20°C.

Chicory roots were cleaned and cut into small pieces that were then stored at -15°C in the dark until they were required. A crude CSL extract was then prepared from the roots of chicory using the method of Visser and Blair (1992): CSL occur in both leaf and root tissue, but root tissue was used to eliminate problems caused by chlorophyll. About 250 g of chicory roots were crushed and added to 500 ml of RO (reverse osmosis) water and the mixture incubated in a water bath at 30°C for three hours. During this period the pH was maintained at 4.5 by the addition of H_2SO_4. After that 200 mg of cellulase and 3-ml of pectinase were added. The temperature was then increased to 40°C and the pH maintained at 4.5 ±0.1 by the addition of 50% aqueous KOH. The slurry was then centrifuged for 15 minutes at 7,500 g and the pellet discarded. The clear brown liquid was washed four times with distilled ethyl acetate in a separating funnel. After each wash the clear brown liquid was retained. After the final wash with ethyl acetate, the solution was washed with supersaturated NaCl solution. The salt and ethyl acetate layers separated and the ethyl acetate layer was retained (brown coloured layer). The solution was dehydrated by the addition of anhydrous $MgSO_4$ powder. The resulting solution was filtered to remove the $MgSO_4$ crystals and evaporated at 50°C, with the resulting brown residue being a crude CSL extract. The sesquiterpene lactone content of the crude extract was not determined.

Preparation of Lungworm larvae

Larvae were cultured from faeces collected from infected, commercially-farmed donor red deer stag calves during the autumn of 1999 when grazing perennial ryegrass/white clover pasture. This forage does not contain either CT or SL. Faeces were collected using collection bags and harnesses. Faeces were first placed in a Baermann apparatus and left overnight to allow lungworm larvae (L1) to migrate out and be tapped-off in water 12-18 hrs later. This was then sieved (250 mm) to remove any debris and added to 200 ml distilled water in a measuring cylinder and allowed to settle for 1-2 hrs. The L1 larvae were further washed and some larvae were retained for the LMI assay while the rest were placed in an incubator (25°C) and gently aerated every second day for 15-20 minutes using an air pump (Elite-801, Aquarium products, Rydalmere, NSW, Australia) for eight days or until more than 90 % of larvae had reached the infective (L3) stage. Larvae were then counted in a McMaster slide under a binocular microscope after addition of Lugols solution. The larvae were then stored in 250-ml lots in 600-ml culture bottles placed horizontally at 10°C.

The mean diameter of L1 and L3 lungworm larvae was 29 and 31 mm, respectively (Sigmascan; Scientific Measurement system, Version 1.10, USA).

Animals

Four red deer grazing perennial ryegrass/white clover pasture were used in the trial: three were fistulated in the rumen and one was fistulated in the abomasum. The rumen fluid was collected in the morning from donor deer, pooled together and centrifuged twice at 10,000 g. The abomasal fluid was collected in the morning and centrifuged twice at 10,000 g. Both rumen and abomasal fluids were used on the day of collection.

***In vitro* assay**

The larval migration inhibition (LMI) bioassay developed by Wagland *et al.* (1992) and modified by Rabel *et al.* (1994) was used to determine the anthelmintic activity of purified CT and CSL extracts against deer lungworm L1 and L3 larvae. The *in vitro* method involved preparation of test solutions with CT or CSL and larvae that were combined and incubated at 37°C in the wells of tissue culture plates (Costar, Cambridge, MA). The CT extracts were dissolved in tap water and serially diluted with water immediately prior to incubation. Concentrations of CT in the incubations were 0, 100, 200, 400, 600, 800, and 1000 mg ml^{-1} (in 20-50 ml water). One hundred microlitres of larvae solution (~150 larvae) were added to wells containing both controls (no CT) and a range of CT or CL concentrations.

In order to demonstrate that CT were responsible for the inhibitory activity, a series of incubations were undertaken using CT extracted from chicory with and without the addition of polyethylene glycol, (PEG: 2 mg/mg CT), which binds specifically to and inactivates the CT (Jones and Mangan, 1977).

In order to provide an environment similar to in vivo conditions, rumen (pH 6.6) and abomasal (pH 3.0) fluids were used in the LMI assay instead of phosphate-buffered saline (PBS) that is normally used in this assay (Rabel *et al.* 1994; Molan *et al.* 2000a,b). All LMI incubations were carried out in 48-well tissue plates for 2 h at 37 °C after which solutions were transferred to sieves (7 mm ID with 25 mm mesh at one end) and left overnight (16-18 h) at room temperature to enable the active larvae to migrate through the sieves for counting. Three replicate samples were run for each concentration of CT and CSL as well as negative controls (no CT or CSL added).

Calculation of data and statistical analyses

The number of larvae which had migrated through the sieves were counted using 40 x magnification and the %LMI was determined according to Rabel *et al.* (1994) and Molan *et al.*(2000b).

The significance of differences among treatment means in each experiment was assessed using GLM (general linear models) procedures (SAS, 1985; version 5).

RESULTS

CT and CL extracted from chicory (*Cichorium intybus*) reduced the viability of the larvae of deer lungworm, D. viviparus as evidenced by their ability to immobilise them and inhibit their migration through 25 mm nylon mesh sieves. In all experiments, the activity of CT or CL increased with increasing concentrations.

Incubation of first-stage (L1) larvae in rumen fluid (collected from deer fed pasture) containing 100-1000 mg chicory CT per ml, inhibited 12%-41% of the larvae from passing through the pores of 25 mm sieves relative to control incubation containing larvae and rumen fluid only (figure

1A) whilst the incubation of L3 larvae in rumen fluid containing the same concentrations of chicory CT mentioned above resulted in the inhibition of 25-66% of larvae from passing through the sieves (Fig.1C). Addition of 2 mg PEG per mg CT to the incubations, resulted in a significant (P<0.001) reduction in the activity of CT against both L1 and L3 larvae. Incubation of L1 and L3 larvae of lungworm with abomasal fluid containing a range of chicory CT (100,200,400,800 & 1000 mg/ml) reduced significantly (P<0.001) the viability of the larvae (Figs 1B and 1D) compared to the larvae in the control incubations (no CT added). Addition of PEG reduced significantly (P<0.001) the inhibitory activity of CT against L1 larvae but not against L3 larvae incubated in abomasal fluid. Statistical analysis showed that CT were significantly more effective at reducing the viability of L1 (P<0.01) and L3 (P<0.001) larvae when added to rumen fluid than when added to abomasal fluid. At 1000 mg/ml, for example, CT in the rumen fluid inhibited 67% of L3 larvae and 41% of L1 larvae from passing through the sieves compared to 45% and 34%, respectively when they were added to the abomasal fluids. In both rumen (Figs. 1A and 1C) and abomasal (Figs. 1B and 1D) fluids, the L3 larvae were significantly (P<0.001) more sensitive to the action of chicory CT than L1 larvae.

FIGURE 1. The effect of condensed tannins (CT; mg/ml) extracted from chicory (*Cichorium intybus*) on larval viability of ensheathed lungworm first- (L1) and third-stage (L3) larvae incubated in vitro for 2h at 37°C. All the incubations were done with (o) and without (l) the addition of 2mg polyethylene glycol /mg CT. Each point represents the mean of triplicates with the standard error of the mean.
A)   L1 larvae incubated in rumen fluid (pH 6.6).
B)   L1 larvae incubated in abomasal fluid (pH 3.0).
C)   L3 larvae incubated in rumen fluid (pH 6.6).
D)   L3 larvae incubated in abomasal fluid (pH 3.0).
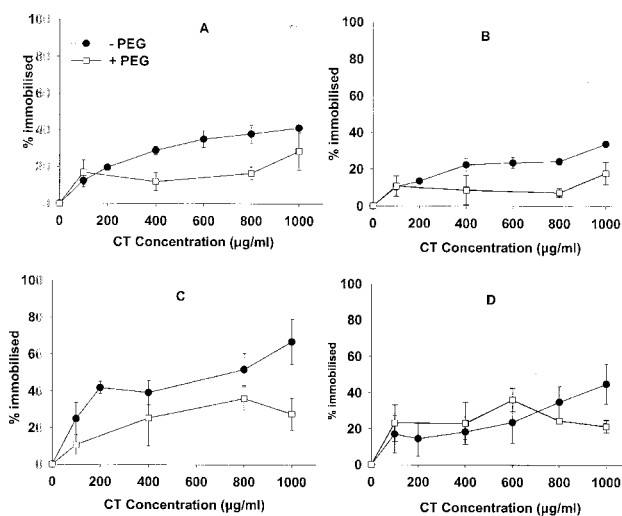
The CSL extracted from chicory inhibited 17%-32% of L1 lungworm larvae from passing through 25 mm sieves when added to the rumen fluids at concentrations of 100-1000 mg/ml (Fig. 2A). In abomasal fluids, SL inhibited 13%-28% of L1 larvae from passing through the sieves (Fig. 2B). Under the same conditions, SL reduced the viability of L3 larvae by 33%-59% when incubated in rumen fluid (Fig. 2C) and by 29-53% when incubated in abomasal fluid (Fig. 2D). As it can be seen from Figs. 2A-2D, the

CSL showed significantly (P<0.001) more inhibitory activity against L3 than L1 larvae during incubation in both rumen and abomasal fluids. Statistical analysis did not show any significant difference in the inhibitory activity of SL during incubation in rumen and abomasal fluids.

FIGURE 2. The effect of crude lactones (CL; mg/ml) extracted from chicory (*Cichorium intybus*) on larval viability of ensheathed lungworm first- (L1) and third-stage (L3) larvae incubated in vitro for 2h at 37°C. Each point represents the mean of triplicates with the standard error of the mean.
A)   L1 larvae incubated in rumen fluid (pH 6.6).
B)   L1 larvae incubated in abomasal fluid (pH 3.0).
C)   L3 larvae incubated in rumen fluid (pH 6.6).
D)   L3 larvae incubated in abomasal fluid (pH 3.0).

DISCUSSION

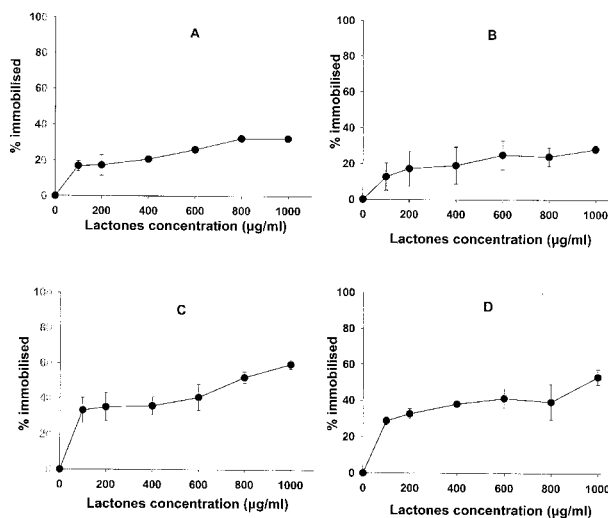The principal finding of this study was that the CT and CSL extracted from chicory have inhibitory effects on the viability of L1 and L3 larvae of deer lungworm, *D. viviparus* as evidenced by their ability to immobilise them and prevent their passage through 25 mm nylon mesh sieves, with the inhibitory effect being greater against L3 larvae. This indicates that CT and SL isolated from chicory have direct inhibitory effects on lungworm larvae and may partly explain the high growth of deer (Hoskin *et al.*, 1999) and lambs (Scales *et al.*, 1995) fed chicory in the absence of anthelmintic drenching. Recently, Molan *et al.* (2000b) found that CT extracted from Lotus pedunculatus, L. corniculatus, sulla (*Hedysarum coronarum*) and sainfoin (*Onobrychus viciifolia*) were all able to reduce the viability of deer lungworm and gastrointestinal nematode larvae.

The results of the present study show that CT were more effective at reducing the viability of lungworm larvae when added to the rumen fluid than when added to the abomasal fluid. This is probably due to difference in reactivity of CT that occurs at different pH values. It has been reported that the binding of CT to proteins occurs only in the pH range 3.5-7 and that CT do not form strong complexes with proteins at pH <3.0 (Jones and Mangan, 1977). Less binding of CT to larval protein, therefore, probably occurred with abomasal fluid than when rumen fluid was used. The results of this study are of physiological importance because the range of CT concentrations used for the LMI assay in the present study was within the range of free CT (About

200-1000 mg/ml) in digesta of ruminants fed CT-containing forages (Terrill *et al.*, 1994). The reduction in LMI in the presence of PEG, especially when added to rumen fluid, confirmed that the effect as directly due to the action of CT.

It is interesting to note that CSL show similar inhibitory activity against the larvae of lungworm when added to rumen and abomasal fluids. In contrast to the CT, pH probably does not affect the reactivity of CSL. The addition of PEG did not affect the biological activity of SL (data not shown) which may indicate that CSL inhibit these larvae by a different mechanism to CT.

Although the mechanisms by which CT and CSL affect the viability of the lungworm larvae are not known, the failure of a high percentage of larvae that had been exposed to CT and CSL extracted from chicory, to pass through the pores of the sieves is indicative of inhibitory effect. The philosophy behind the LMI assay is that the larvae have to squeeze themselves in order to pass through the pores of the sieves (the pore diameter is less than the diameter of the larvae) which means that only the active larvae can pass through while the inactive ones (immobilised) can not. The failure of the larvae to pass through the sieves may suggest an interference with neurophysiology or neuromuscular co-ordination. Molan *et al.* (2000) checked the viability of the trapped larvae (exposed to CT extracted from seven forages) and found that 83-93% of the larvae was alive but their movements were sluggish suggesting partial paralysis. Condensed tannins have been shown to inhibit endogenous enzymes activities (Oh and Hoff, 1986; Horigome *et al.*, 1988) and CT isolated from 18 plant species were found to be potent inhibitors of rat liver cyclic AMP-dependent protein kinase (Wang *et al.*, 1996). The inhibitory effect of CT, therefore, probably involves enzyme inactivation.

CONCLUSION

This study shows that CT and CSL extracted from chicory have anthelmintic –like activity against the larvae of deer lungworm, *D. viviparus* and grazing this herb may be used as an alternative method for controlling lungworm in deer in order to reduce reliance on anthelmintics as the as the sole method for controlling internal parasites in ruminants.

REFERENCES

Audige, L.J.M.; Wilson, P.P.; Morris, R.S. 1998. A survey of internal parasites and parasite control n New Zealand deer farm. *New Zealand Veterinary Journal* **46:** 203-215.

Bringans, M. 1986. Post-quarantine experience with imported elk. Deer Branch, New Zealand Veterinary Association Course No. 3: 183-189.

Haigh, J.C.; Hudson, R.J. 1993. Farming Wapiti and Red Deer. Mosby-Year Book, Inc.U.S.A.

Horigome, T.; Kumar, R.O.; Kamoto, K. 1988. Effects of condensed tannins prepared from leaves of fodder plants on digestive enzymes in vitro and in the intestine of rats. *British Journal of Nutrition* **60:** 275-285.

Hoskin, S O.; Barry T.N.; Wilson P.R.; Charleston W.A.G.; and Hodgson J. 1999. Effects of reducing anthelmintic input upon growth and faecal egg and larval counts in young farmed deer grazing chicory (*Cichorium intybus*) and perennial ryegrass (*Loliun perenne*)/ white clover (*Trifolium repens*) pasture. *Journal of Agricultural Science, Cambridge* **132:** 335-345.

Jackson, F.S.; McNabb, W.C.; Barry, T.N.; Foo, Y.L.; Peters, J.S. 1996.
The condensed tannins of a range of subtropical and temperate forages and the reactivity of condensed tannin with ribulose-1,5-bisphosphate carboxylase (Rubisco) protein *Journal of the Science Food and Agriculture* **72:** 483-492.

Jones, W.T.; Mangan, J.L. 1977. Complexes of the Condensed Tannins of Sainfoin (Onobrychis viciifolia Scop.) with fraction 1 leaf Protein and with Submaxillary Mucoprotein, and their Reversal by Polyethylene Glycol and pH. *Journal of Science, Food and Agriculture* **28:** 126-136.

Mackintosh, C.G.; Manson, P.C.; Bowie, J.Y.; Beatson, N.S. 1984. Anthelmintics against lungworm (*Dictyocaulaus viviparus*) in red deer (*Cervus elaphus*). *Proceedings of a Deer Course for Veterinarians. Deer Branch of the New Zealand Veterinary Association* **1:** 69-77.

Molan, A.L.; Hoskin, S.O.; Barry, T.N.; McNabb, W.C. 2000a. The effect of condensed tannins extracted from four forages on deer lungworm and gastrointestinal nematode larval viability. *Veterinary Record* (in press).

Molan, A.L.; Waghorn, G.C.; Min, B.R.; McNabb, W.C. 2000b. The effect of condensed tannins from seven herbages on Trichostrongylus colubriformis larval migration in vitro. *Folia Parasitologica* **47:** 39-44.

Oh, H.I.; Hoff, J.E. 1986. Effect of condensed grape tannins on the in vitro activity of digestive proteases and activation of their zymogens. *Journal of Food Science* **51:** 577-580

Rabel, B.; McGregor, R.; Douch, P.G.C. 1994. Improved bioassay for estimation of inhibitory effects of ovine gastrointestinal mucus and anthelmintics on nematode larval migration. International *Journal for Parasitology* **24:** 671-676.

Scales, G.H.; Knight, T.L.; Saville, D.J. 1995. Effect of herbage species and feeding level on internal parasites and production performance of grazing lambs. *New Zealand Journal of Agricultural Research* **38:** 237-247.

Statistical Analysis System. 1985. User's Guide: Statistics, Version 5. North Carolina,

SAS Institute Inc., U.S.A.

Terrill, T. H., Waghorn, G.C., Woolley, D.J., McNabb, W.C.; Barry, T.N. 1994. Assay and digestion of 14 C-labelled condensed tannin in the gastrointestinal tract of sheep. *British Journal of Nutrition* **72:** 467-477.

Visser, F.R.; Blair, K.A. 1992. Identification of the compounds responsible for the taint in milk from cows fed on Puna Chicory. Report to the New Zealand Dairy Research Institute.

Wagland, B.M.; Jones, W.O.; Hribar, L.; Bendixen, T.; Emery, D.L.A. 1992. A new simplified assay for larval migration inhibition. *International Journal of Parasitology* **22:** 1183-1185.

Waldrup, K.; Mackintosh, C.G.; Johnstone, P.D.; Labes, R.E. 1994. The effects of parasitism on weaner deer: Parallel studies with red deer hinds and wapiti hybrid hinds. *Proceedings of Deer Course for Veterinarians. Deer Branch of the New Zealand Veterinary Association*. **11:** 193-202.

Wang, B.H.; Foo, L.Y.; Polya, G.M. 1996. Differential inhibition of eukaryote protein kinases by condensed tannins. *Phytochemistry* **43:** 359-365

Watson, T.G. 1986. Efficiency of drenching red deer and wapiti with particular reference to Elaphostrongylus cervi and Dictyocaulus viviparus. *Deer Branch, New Zealand Veterinary Association Course* **No. 3:** 170-182

Wilson, P. R.; Collier A. 1981. Lungworm in deer: A survey of veterinary practices. *Proceedings of a Deer Course for Veterinarians. Deer Branch of the New Zealand Veterinary Association* **1:** 78-93.