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# The effect of condensed tannins from seven herbages on *Trichostrongylus colubriformis* larval migration *in vitro*

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**Abstract.** The effects of condensed tannins (CT) extracted from seven forages on the motility of the economically important nematode, *Trichostrongylus colubriformis* (Giles, 1892), were evaluated by using a larval migration inhibition (LMI) assay. The assay involved incubation of third stage (L3) exsheathed *T. colubriformis* larvae with CT extracted from *Lotus pedunculatus*, *Lotus corniculatus*, sulla (*Hedysarum coronarium*), sainfoin (*Onobrychis viciifolia*), *Dorycnium rectum*, *Dorycnium pentaphyllum* and dock (*Rumex obtusifolius*) and measurement of larval migration through nylon mesh with a 20μm pore size. At 100 μg $ml^{-1}$, CT from *L. pedunculatus, L. corniculatus*, sulla, sainfoin, *D. rectum, D. pentaphyllum* and dock inhibited 20%, 10%, 15%, 25%, 28%, 32% and 27% of the larvae, respectively from passing through the sieves compared to controls (no CT added). At 1000 μg CT $ml^{-1}$, CT purified from *D. pentaphyllum* had the highest inhibitory activity (63%) against 1-month old larvae followed by sainfoin (59%), *L. pedunculatus* (57%), *D. rectum* (53%), dock (50%), sulla (40%) and *L. corniculatus* (37%). Seven-month old larvae were more sensitive to the action of CT than 1-month old larvae ($P < 0.001$). Addition of 2 μg polyethylene glycol ([PEG] per μg CT; to remove the effect of CT) eliminated 81-93% of the CT activity ($P < 0.001$) compared to incubations without PEG. The impact of CT on larval migration suggests a possible role for these plants in ruminant diets as a means to reduce dependence upon proprietary anthelmintics.

Subclinical helminth infections have resulted in up to 40% depression in live weight gain and a 6-30% reduction in food intake by lambs (Poppi et al. 1990). Wool production has been reduced by 40% and milk production by 15% in parasitised sheep (Steel and Symons 1982, Sykes 1982). Parasite control in sheep over the past 30 years has been achieved almost exclusively by the use of proprietary anthelmintics (Vlassoff and McKenna 1994) and resistance of nematodes to these drenches has been reported world-wide, whilst parasite control can no longer be achieved in some regions (Waller 1994). Alternative strategies for parasite control are urgently needed and one approach may be to include plants which contain condensed tannins (CT) into the grazing rotation (Robertson et al. 1995). CT can increase the proportion of dietary protein reaching the intestine (Waghorn et al. 1987, 1994) and high protein intakes have been associated with increased immuno-competence in young sheep (Coop and Holmes 1996). Some studies (Niezen et al. 1995, Robertson et al. 1995) have shown that CT-containing forages such as sulla have had significant effects on intestinal nematodes in sheep but they could not determine whether the effect was indirect, i.e. by increasing the amount of protein reaching the small intestine which may increase the animal resistance to the parasites or to their direct effect on the nematodes in the small intestine.

The objective of the present study was to determine whether CT exerted a direct effect on the larvae of the economically important sheep nematode, *Trichostrongylus colubriformis* (Giles, 1892) by using the larval migration inhibition (LMI) assay.

## MATERIALS AND METHODS

**Experimental design.** *In vitro* experiments were undertaken to determine the effect of CT extracted from *Lotus pedunculatus*, *Lotus corniculatus*, sulla (*Hedysarum coronarium*), sainfoin (*Onobrychis viciifolia*), *Dorycnium rectum*, *Dorycnium pentaphyllum* and dock (*Rumex obtusifolius*) on the *in vitro* motility of the third stage (L3) exsheathed larvae of the sheep nematode *T. colubriformis*.

Condensed tannins are routinely purified using affinity chromatography with Jackson et al. (1996) reporting that the Sephadex LH-20 extract of *L. corniculatus* contained predominantly CT.

**Preparation of CT extracts.** The CT extracts were prepared using the method of Jackson et al. (1996). The frozen whole plants 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 and approximately 25 g of the material was redissolved in 150 ml of 1:1 methanol/water (v/v). This material was placed on a column containing 200 ml of Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and washed with 2000 ml of 1:1 methanol/water before eluting the CT with 200 ml of acetone: water

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(70:30 v/v). The forages were harvested on twelve separate occasions over a four-month period and Sephadex LH-20 extracts prepared. The CT extracts for each plant were freeze dried, bulked and stored at -20 °C.

**Assay procedure.** The larval migration inhibition (LMI) bioassay procedure developed by Wagland et al. (1992) and modified by Rabel et al. (1994) was used to determine the inhibiting effect of purified CT against *T. colubriformis*. The method involved preparation of test solutions with CT and of L3 larvae which were combined and incubated in the wells of tissue culture plates (Costar, Cambridge, MA). The freeze dried CT extracts were dissolved in phosphate buffered saline (PBS; 0.1 M phosphate, 0.05 M NaCl; pH 7.2) and serially diluted with PBS immediately prior to incubation. Concentrations of CT in the incubations were 50, 100, 200, 300, 400, 600, 800 and 1000 μg $ml^{-1}$. The majority of the incubations used 1-month old *T. colubriformis* larvae but 7-month old larvae (collected from the same infected sheep and held at 4°C) were also used to evaluate the importance of larval age in this type of assay. The larvae were exsheathed in sodium hypochlorite solution (0.025% available chlorine; Rabel et al. 1994), washed five times with PBS and concentrated to 1,500 larvae $ml^{-1}$ PBS. One hundred microlitres of larvae solution (~150; third stage of larvae, L3) were added to wells containing both negative controls (no CT) and a range of CT concentrations from each of seven plant species.

In order to demonstrate that CT were responsible for the anthelmintic activity a series of incubations were undertaken with and without polyethylene glycol (PEG; 2 μg $μg^{-1}$ CT). The addition of PEG prevents CT from binding to protein (Jones and Mangan 1977) enabling the effect of CT to be deduced by comparing incubations with added PEG (CT inactive) to incubations without PEG (CT active).

All 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 20 μm mesh at one end) and left overnight (16-18 hours) at room temperature to enable the active larvae to migrate through the sieves for counting. The 20 μm mesh size was selected in order to ensure that active migration of the larvae through the sieve was determined. The cross-diameter of L3 larvae is 25 μm (Rabel et al. 1994) which is slightly larger than the mesh and would thus prevent the larvae "falling" through the sieve. Four replicate samples were run for each concen-tration of each CT as well as negative controls.

**Calculation of data and statistical analyses.** The number of larvae which had migrated through the sieves were counted using 40× magnification and the % LMI was determined according to Rabel et al. (1994) using the following equation;

$$\% \text{ LMI} = \frac{A - B}{A} \times 100$$

Where A = number of larvae migrating through sieves in negative control wells (containing no CT), and B = number of larvae migrating through sieves in treatment wells (containing CT).

The significance of differences among treatment means in each experiment was assessed using GLM (general linear models) procedures (SAS, version 6).

## RESULTS

The results of the LMI assay using seven different types of CT are illustrated in Fig. 1. All CT extracts from the forages showed inhibitory effect against 1-month old larvae of the sheep parasite *T. colubriformis* as evidenced by their ability to inhibit the passage of L3 larvae of this parasite through 20 μm nylon mesh sieves relative to the CT-free controls. About 90% of the larvae in the negative controls (no added CT) passed through the sieves. At 1000 μg CT $ml^{-1}$ all seven CT caused a significant reduction in larval migration through the sieves ($P < 0.001$) with highest levels of LMI activity from *D. pentaphyllum*, sainfoin, *L. pedunculatus*, *D. rectum* and dock being 63%, 59%, 57%, 52% and 50%, respectively. The CT from *L. corniculatus* had the lowest activity (37%) and those for sulla was 40% respectively. When 400 μg CT $ml^{-1}$ was incubated with 1-month old larvae (Fig. 1), that from *D. pentaphyllum* maintained the highest LMI activity (40%) followed by *D. rectum* (36%), sainfoin (38%), *L. pedunculatus* (34%), dock (35%), sulla (27%) and *L. corniculatus* (21%). At 200 μg CT $ml^{-1}$ the highest LMI activity was achieved by *D. rectum* (38%) followed by dock (32%), sainfoin (32%), *D. pentaphyllum* (31%), *L. pedun-culatus* (21%), sulla (18%) and *L. corniculatus* (15%).

In general CT extracted from *D. pentaphyllum* was more inhibitory to larval migration than CT from *L. pedunculatus*, *L. corniculatus*, sulla, dock ($P < 0.001$) and sainfoin ($P < 0.05$). The CT from *L. pedunculatus* was significantly ($P < 0.001$) more effective than that from *L. corniculatus* and sulla but less inhibitory than that extracted from sainfoin. Sulla CT was significantly ($P < 0.001$) more effective than those purified from *L. corniculatus*.

To compare the effect of CT on 1-month and 7-month old larvae, the LMI values were determined with 400, 800 and 1000 μg CT $ml^{-1}$ (Fig. 2). All sources of CT showed that 7-month old larvae were more sensitive to CT than the 1-month old larvae ($P < 0.001$). The mean LMI activity of these three concentrations of CT extracted from *L. pedunculatus* against 7-month and 1-month old larvae were 81% and 45% respectively; 66% and 29% for *L. corniculatus*; 72% and 33% for sulla; 80% and 47% for sainfoin; 75% and 45% for *D. rectum*; 82% and 54% for *D. pentaphyllum* and 77% and 39% for CT purified from dock.

Addition of PEG (800-2000 μg $ml^{-1}$) had no significant effect on the migration of larvae through the sieves in the absence of CT, whereas the addition of 2 μg PEG $μg^{-1}$ CT to the incubations containing CT purified from *L. pedunculatus*, *L. corniculatus*, sulla and sainfoin (at concentrations of 400, 800 and 1000 μg $ml^{-1}$) eliminated 81-93% of their inhibitory effect against 1-month old larvae ($P < 0.001$; Fig. 3).

## DISCUSSION

The objective of this study was to determine if CT extracted from a range of forages exhibited inhibitory effects on the principal nematode of the small intestine of sheep. Inhibitory effects were measured using a larval migration inhibition assay. This assay has been successfully used to test the inhibitory effects of proprietary anthelmintic drenches (Wagland et al. 1992, Rabel et al. 1994, Lorimer et al. 1996).

The range of total CT concentration in the abomasal and duodenal digesta of sheep fed CT-containing diets was about 1100-2800 µg $ml^{-1}$ whilst the range of extractable CT was about 350-900 µg $ml^{-1}$ (Terrill et al. 1994). The effective concentrations of CT used for the LMI assay in the present study were within the range of free CT in digesta measured by Terrill et al. (1994), and the results of the present study therefore have physiological significance.

The results presented here show for the first time that condensed tannins extracted from diverse plant sources have the ability to inhibit the migration of infective L3 larvae of *T. colubriformis in vitro*. Taylor and Murant (1966) had previously reported nematicidal activity in aqueous extracts from raspberry canes and roots, which contained CT, but did not attribute these findings to CT *per se*. More recently Lorimer et al. (1996) screened plant extracts for anthelmintic activity, and attributed inhibitory activity in foliage from the tree, *Phyllocladus aspeniifolius* var. *alpinus* against *T. colubriformis* to plant polyphenolics, although CT may have been present.

The present study showed that CT purified from *L. pedunculatus* were more effective than CT from *L. corniculatus* in preventing the migration of *T. colubriformis* L3 larvae *in vitro*, and this result matches those from a field trial in which parasitised sheep and

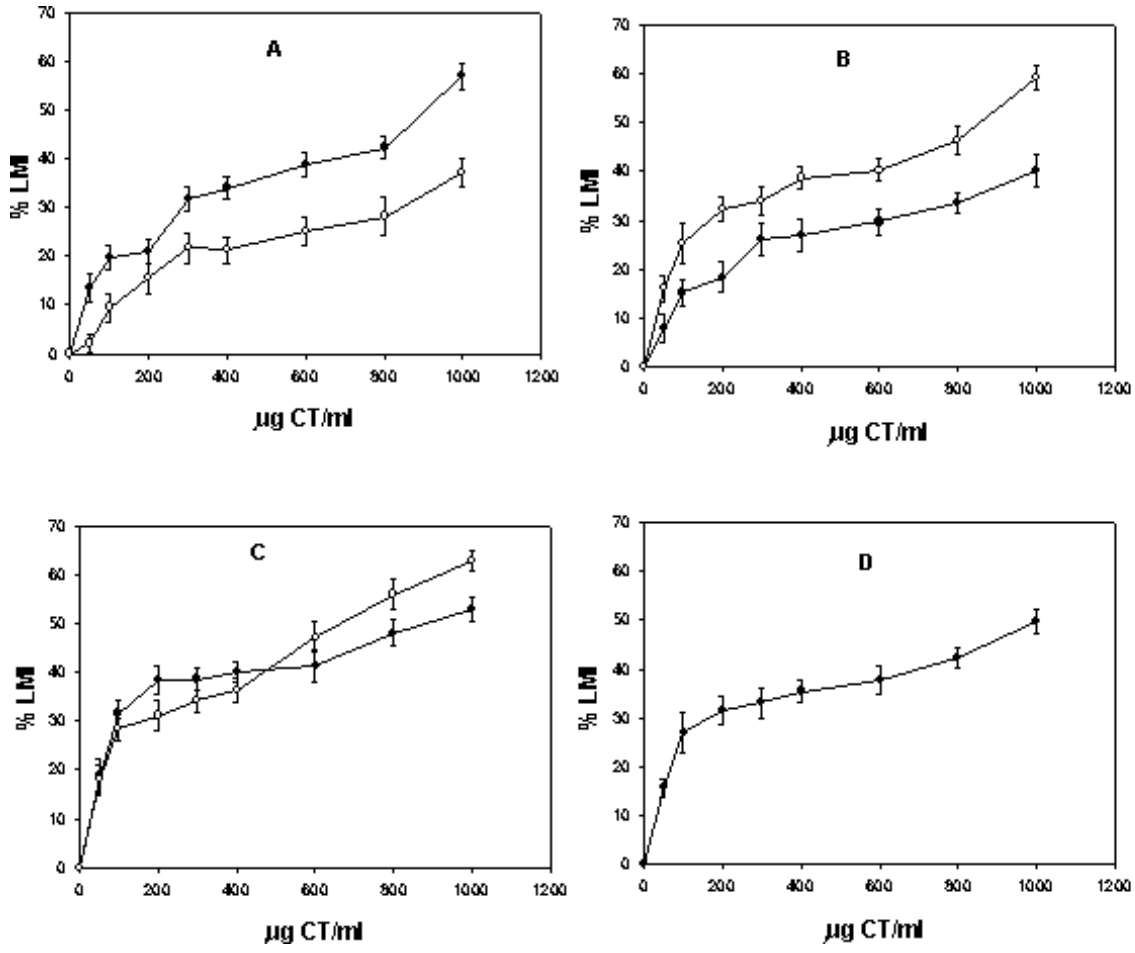


**Fig. 1**. The effect of condensed tannin (µg CT $ml^{-1}$) purified from seven herbages on larval migration inhibition (LMI) using one-month old *Trichostrongylus colubriformis* larvae *in vitro*. Each point represents the mean of quadruplicates with the standard error of the mean. **A** – *Lotus pedunculatus* (LP; ●) and *Lotus corniculatus* (LC; ○); **B** – sulla (*Hedysarum coronarium*; ●) and sainfoin (*Onobrychis viciifolia*; ○); **C** – *Dorycnium rectum* (DR; ●) and *Dorycnium pentaphyllum* (DP; ○); **D** – dock (*Rumex obtusifolius*).

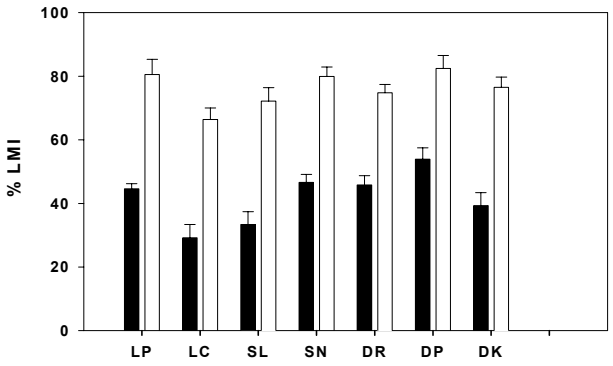


**Fig. 2**. The percentage of the larval migration inhibition (% LMI) activity of condensed tannins (CT) purified from *Lotus pedunculatus* (LP), *Lotus corniculatus* (LC), sulla (SL), sainfoin (SN), *Dorycnium rectum* (DR), *Dorycnium pentaphyllum* (DP) and dock (DK) forages against one-month (■) and seven-month (□) old *Trichostrongylus colubriformis* larvae. The LMI values were averaged from three concentrations of CT (400, 800 and 1000 µg $ml^{-1}$). Each point represents the mean of 12 observations with the standard error of the mean.

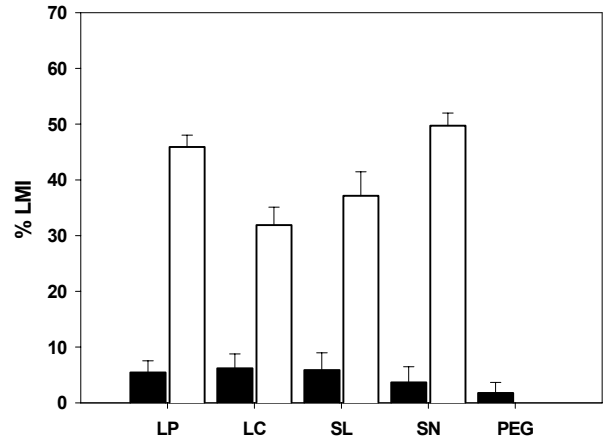


**Fig. 3**. The percentage of the larval migration inhibition (% LMI) activity of condensed tannins (CT; □ purified from *Lotus pedunculatus* (LP), *Lotus corniculatus* (LC), sulla (SL) and sainfoin (SN) forages and when CT were inactivated by the addition of polyethylene glycol (PEG; 2 µg $1\mu g^{-1}$ CT; ■) against one-month old *Trichostrongylus colubriformis* larvae. The LMI values were averaged from three concentrations of CT (400, 800 and 1000 µg $ml^{-1}$). Each point represents the mean of 12 observations with the standard error of the mean.

lambs grazing *L. pedunculatus* were better able to withstand the infection with abomasal and intestinal nematodes than those grazing *L. corniculatus* (Robertson et al. 1995). The LMI activity of CT purified from sulla reported in the present study complements the finding of Niezen et al. (1995) who found that lambs infected with *T. colubriformis* and grazing sulla had lower faecal egg counts and lower adult worm burdens at slaughter than those grazing lucerne. Moreover, field trials with sheep fed sulla (Niezen et al. 1995) and *L. pedunculatus* (Robertson et al. 1995) have indicated that animal performance was affected only to a minor degree by a substantial parasite burden when these diets were fed, relative to sheep given proprietary anthelmintics, and to those grazing *L. corniculatus*. It was suggested that the CT in sulla and *L. pedunculatus* may have affected the larvae or adult nematodes *in situ,* although evidence for this hypothesis was not provided. Recently, Hoskin et al. (1997) have found a significantly lower proportion of *Trichostrongylus axei* worms in red deer fed sulla compared to lucerne or *L. corniculatus*.

The CT from different species of forage legumes differ in molecular (MW) weight and in chemical composition (Jones et al. 1976, Foo et al. 1996, 1997) and these structural variations may contribute to differences in LMI activity of CT in this study. The average MW of the CT from *L. pedunculatus*, for example, is 2200 and this is higher than the MW of CT from *L. corniculatus* (1900). In addition, the CT from *L. pedunculatus* contains a predominance of prodel-phinidin-type subunits (Foo et al. 1997) while the CT from *Lotus corniculatus* has predominantly procyanidin-type subunits (Foo et al. 1996). It has been reported that CT with high MW interact more strongly with enzymes and other proteins than CT with low MW (Beart et al. 1985, Horigome et al. 1988, Kawamoto et al. 1996), and the reactivity of CT increases with increasing pro-delphinidin content (Jones et al. 1976).

Although the mechanisms by which CT inhibit larval migration are not known, the failure of a high proportion of larvae which had been exposed to purified CT to pass through the pores of the sieves is indicative of paralysis because when the viability of the trapped larvae was checked we found that 87-94% (results not shown) of the larvae were alive but their movements were sluggish. The LMI assay is dependent on active migration of larvae through the 20 micron pores in the sieves, which is slightly less than the mean (25 microns) diameter of *T. colubriformis* L3 larvae (Rabel et al. 1994), so the failure of the larvae to pass through may suggest an interference with neurophysiology or neuromuscular co-ordination of the larvae.

Condensed tannins have been shown to inhibit endogenous enzyme activities (Oh and Hoff 1986, Horigome et al. 1988) and CT isolated from 18 plant species including *L. pedunculatus* and *L. corniculatus* were potent inhibitors of rat liver cyclic AMP-dependent protein kinase (Wang et al. 1996). Several studies have shown that CT have anti-microbial effects (Scalbert 1991) by altering surface morphology (Bae et al. 1993) and are inhibitory to bacterial growth and to

proteolytic activities in some but not all rumen bacterial species (Jones et al. 1994, Molan et al. 1997). Condensed tannins can cause cellulolytic bacteria to dissociate from substrates (McAllister et al. 1994) possibly as a consequence of CT-surface interactions but Jones et al. (1994) suggested that CT may also penetrate the cell wall and cause a loss of intracellular constituents. In the present study, the CT may act similarly by penetrating the wall of the larvae and affect on the muscular activity.

The greater sensitivity of 7-month old larvae to CT than 1-month old larvae has important implications for comparison of LMI activity between experiments. The 7-month old larvae were susceptible to effect of CT irrespective of source.

The use of PEG to eliminate effects of CT was demonstrated by Jones and Mangan (1977) who showed preferential binding between PEG and CT relative to protein and CT under *in vitro* conditions. PEG had been effective in removing the effects of CT in nutrition studies for determining digestibility and animal performance (Waghorn et al. 1987, 1990, 1994). It is therefore important that PEG was able to eliminate most of the CT effect in this LMI assay and this confirmed that CT was the source of the inhibition.

The inhibitory activity of seven sources of CT complements benefits arising from reduced protein degradation in the rumen (Waghorn et al. 1987, 1990, 1994, McNabb et al. 1996) which has improved the performance of grazing ruminants and may also reduce the dependence on proprietary anthelmintic drenches.

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