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Effects of an extract from sulla (*Hedysarum coronarium*) containing condensed tannins on the migration of three sheep gastrointestinal nematodes *in vitro*

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ABSTRACT

The effect an extract of sulla (*Hedysarum coronarium*) containing condensed tannins (CT) had on the viability of the infective third-stage larvae (L3) of three gastrointestinal nematodes (*Haemonchus contortus, Ostertagia circumcincta* and *Trichostrongylus colubriformis*) was tested by the larval migration inhibition (LMI) assay *in vitro*. The LMI assay measures the ability of test material to immobilize larvae and prevent their migration through 20 mm nylon mesh sieves. The extract from sulla was added to rumen (RF) and abomasal fluid (AF), collected from sheep fed lucerne (*Medicago sativa*) chaff, to provide concentrations of CT similar to those observed in the rumen and abomasal digesta of sheep fed CT-containing forages (50-1000 mg/ml). Incubation of L3 larvae in rumen and abomasal fluid containing the sulla extract reduced the migration of larvae compared to those in control incubations (no extract added). This study showed that the larvae of *T. colubriformis* were more resistant ($P<0.001$) to the inhibitory effects of the sulla extract than were the larvae of the other nematodes. When the larvae of these nematodes were incubated in rumen and abomasal fluid containing 1000 mg CT/ml, the LMI values for *O. circumcincta* (RF 59%; AF 79%) and *H. contortus* (RF 72%; AF 81%) were significantly ($P<0.001$) greater than for *T. colubriformis* (RF 37%; AF 26%). Addition of 2 mg polyethylene glycol (PEG)/ mg CT to the incubations partially reduced the effects of the sulla extract on larval migration, particularly in the rumen fluid, suggesting that CT was responsible for most but not all of the inhibitory effect of this extract.

Keywords: sulla; condensed tannins; sheep; gastrointestinal nematodes.

INTRODUCTION

Gastrointestinal helminth parasites cause significant production losses in grazing ruminants throughout the world, particularly in young and periparturent sheep, goats and cattle (Sykes, 1994). Gastrointestinal nematodes cause extensive protein losses in sheep (Kimambo *et al*., 1988), redirect protein synthesis away from skeletal muscles and into repair of gut tissues (MacRae, 1993) and consequently, depress both liveweight gain and wool production (Steel and Symons, 1982, Poppi *et al*., 1990; Niezen *et al*., 1995).

Proprietary anthelmintic drenches, the predominant means of internal parasite control, cost New Zealand farmers millions of dollars every year. Resistance to anthelmintic drenches amongst the major nematode parasites of sheep and goats has now reached alarming proportions throughout the world and threatens the future viability of continued small ruminant production in many countries (Waller, 1999). Anthelmintic resistance, the increasing concern about anthelmintic residues in animal products (Sykes, 1982) and the finding that regular drenching can not completely remove the detrimental effects of parasites (Coop *et al*., 1982) demonstrate the urgent need to explore alternative methods of gastrointestinal nematode parasite control.

Forages that contain condensed tannins (CT) like sulla (*Hedysarum coronarium*) have had a dramatic effect on intestinal parasite numbers. In parasitised lambs grazing sulla, total worm burdens were 58% lower than in similar lambs grazing lucerne (Niezen *et al*., 1995). However, it is not clear what it is in sulla that is responsible for these effects. It could be due to the ability of CT to increase the supply of dietary protein to the small intestine (Waghorn *et al*., 1987, 1994) by reducing microbial degradation of plant protein in the rumen (McNabb *et al*., 1996; Aerts *et al*., 1999). The CT in sulla could also have direct effects on mature parasites in the gastrointestinal tract that reduces their viability or some as yet, unidentified property in sulla maybe responsible.

The objective of this research was to investigate the direct effects of an extract from sulla that contained CT on three sheep gastrointestinal nematodes, *Haemonchus contortus, Ostertagia circumcincta* and *Trichostrongylus colubriformis* using an *in vitro* larval migration assay that measures the viability of the parasite.

MATERIALS AND METHODS

Experimental Design

Three *in vitro* experiments were undertaken to determine the effect of an extract from sulla that contained CT on the motility of the third stage (L3) exsheathed larvae of the sheep nematodes *H. contortus, O. circumcincta* and *T. colubriformis* using the larval migration inhibition (LMI) assay. The 20 mm mesh size was selected in order to ensure that active migration by the larvae was required for passage through the sieve. The cross-sectional diameter of the L3 larvae of these nematodes (23-25 mm; as measured by Sigmascan; Scientific Measurement system, Version 1.10, USA) is slightly larger than the mesh size and this prevents the larvae from simply "falling" through the sieve. In order to provide an environment more closely approximating the *in vivo* situation the phosphate-buffered-saline (PBS) that is normally used as a buffer in this assay, was replaced with rumen (RF) and abomasal (AF) fluid. In the first experiment, the activity of the sulla extract L3 larvae of *H. contortus* was evaluated by incubating the larvae in rumen and abomasal fluid in the presence or absence of 2 mg polyethylene glycol (PEG) per mg 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 inactivate) to incubations without PEG (CT active). The second and the third experiments were identical to the first experiment except that L3 larva of *O. circumcincta* and *T. colubriformis* were used, respectively.

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Collection of Rumen and Abomasal Fluid

Four sheep with rumen and abomasal cannulae were housed indoors in metabolism crates for a total of 8 weeks (fed chaffed lucerne; *Medicago sativa*) and used as a source of rumen and abomasal fluid. The rumen and abomasal fluid was collected from the four sheep, pooled together, strained through two layers of cheesecloth and centrifuged twice at 15,000 rpm (to remove particulate material that would otherwise block the sieves) and used immediately in the assay. The pH of rumen and abomasal digesta was also determined.

Preparation of the Extract of Sulla

The extract was 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 (70:30 v/v). Condensed tannins are routinely purified using affinity chromatography with Sephadex LH-20 as a matrix. However, Jackson *et al.* (1996) reported that the Sephadex LH-20 extract of *Lotus corniculatus*, although containing predominantly CT, also contained other non-CT phenolic compounds. Therefore, the term "extract of sulla" has been used to reflect such possibilities rather than the more specific term "CT", to describe the preparation that was used in this study.

Larval Migration Inhibition Assay Procedure

The larval migration inhibition (LMI) bioassay (Rabel *et al.*, 1994) was used to determine the inhibitory effect of a sulla extract containing CT against *H. contortus*, *O. circumcincta* and *T. colubriformis*. The method involves incubation of sulla extract with L3 larvae in the wells of 48-well tissue culture plates (Costar, Cambridge, MA). The larvae of each nematode were exsheathed in sodium hypochlorite solution (0.025% available chlorine), washed five times with tap water and suspended in PBS at a concentration of 1,500 larvae ml^{-1} prior to use in the LMI assay (Molan, *et al*., 2000). One hundred microlitres of this larvae solution (~ 150 L3 larvae) was added to each well. The wells contained 400 ml of either rumen or abomasal fluid to which sulla extract had been added to give a range of CT concentrations (0, 50, 100, 200, 400, 800 and 1000 mg/ml). The plates were incubated for 2 h at 37 ^{o}C after which the contents of each well were transferred to sieves (7 mm ID with 20 mm mesh at one end) and left for 16-18 hours at room temperature to enable the active larvae to migrate through the sieves. After incubation the material that had passed through the sieves was collected and the larvae contained in that material counted using a microscope. Four replicate samples were run for each concentration of CT as well as negative controls containing rumen or abomasal fluids and larvae only. The larvae were obtained from the Parasitology Laboratory, Institute of Veterinary, Animal and Biomedical Sciences, Massey University and were used within 2 months of collection.

Calculation of Data and Statistical Analysis

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

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

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 general linear model procedures (SAS, version 6).

RESULTS

The mean pH of the rumen and abomasal fluid used in the LMI bioassay was 7.2 and 3.1, respectively.

Experiment 1. The effect of the sulla extract on the migration of the L3 larvae of *Haemonchus contortus*

In incubations involving rumen fluid only, 16% of the *H. contortus* larvae were unable to pass through the sieves. Incubation of exsheathed *H. contortus* L3 larvae in rumen fluid collected from sheep fed lucerne and containing a range of concentrations (50, 100, 200, 400, 800 and 1000 mg/ml) of CT resulted in a significant ($P<0.001$) reduction in the migration of these larvae. Addition of 100, 200, 400 and 1000 mg CT/ml inhibited larval migration by 37%, 51%, 65% and 72%, respectively (Fig.1A). Addition of 2 mg PEG/mg CT resulted in significantly ($P<0.05$) less inhibition of larval migration (12%, 17%, 12% and 34% at 100, 200, 400 and 1000 mg CT/ml, respectively). However, this also suggests that PEG was unable to completely remove the effect of the sulla extract at higher CT concentrations.

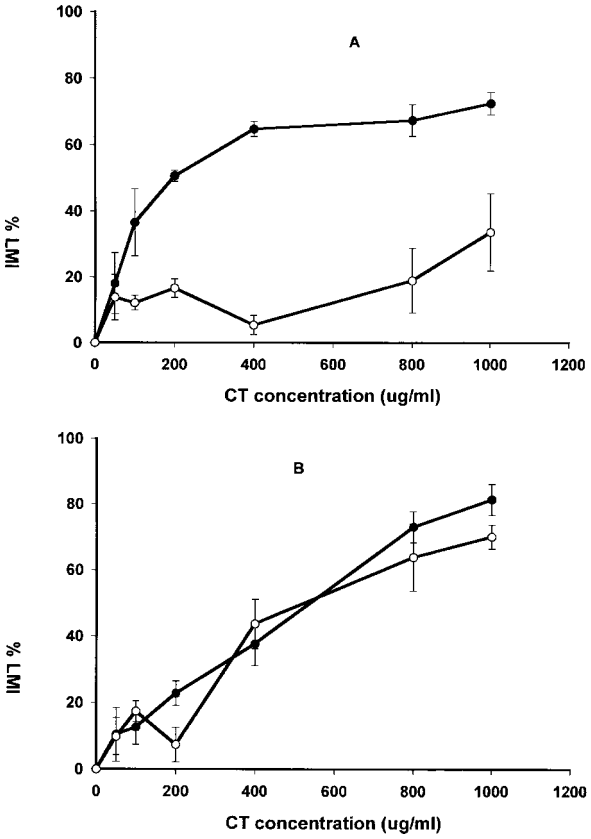
In incubations involving abomasal fluid only, 21% of the *H. contortus* larvae were unable to pass through the sieves. Incubation of L3 larvae of *H. contortus* in abomasal fluid containing these same concentrations of CT also significantly ($P<0.001$) reduced the number of larvae able to pass through the sieve compared to control incubations. Addition of PEG did not completely remove the effects of CT against the larvae incubated in abomasal fluid (Fig. 1B). This suggests that CT was not solely responsible for the effects of the sulla extract in abomasal fluid or that the interaction between CT and larvae in abomasal fluid was pH independent.

Experiment 2. The effect of the sulla extract on the migration of the L3 larvae of *Ostertagia circumcincta*

In incubations involving rumen fluid only, 18% of the *O. circumcincta* L3 larvae were unable to pass through the sieves. Incubation of exsheathed *O. circumcincta* larvae in rumen fluid containing the same range of concentrations of CT as used in Experiment 1 (50, 100, 200, 400, 800 and 1000 mg/ml) resulted in a significant ($P<0.001$) reduction in the migration of these larvae. Addition of 100 mg CT/ml resulted in a 33% inhibition of larval migration. Addition

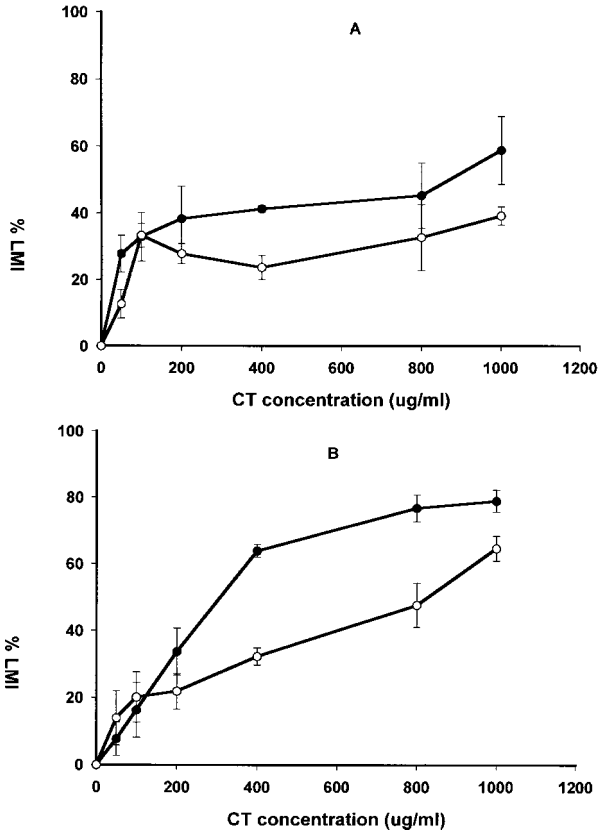
of 400 and 1000 mg CT/ml resulted in significantly (P<0.01) fewer (41 and 59% inhibition, respectively) larvae being able to pass through the sieves (Fig 2A). Addition of PEG significantly (P<0.05) reduced the effect of CT only at a concentration of 1000 mg/ml (Figure 2A). Addition of 2 mg PEG/mg CT partially removed the effects of the CT on larval migration at 400 and 1000 mg CT/ml only. Addition of PEG to incubations had no effect (P>0.05) on larval migration at all other concentrations of CT.

FIGURE 1: The effect of the condensed tannin (mg CT ml^{-1}) contained in an extract from sulla (*Hedysarum coronarium*) on the larval migration inhibition (LMI) of infective third-stage (L3) larvae of *Haemonchus contortus* during incubation in either rumen **(A)** or abomasal **(B)** fluid. The incubations were done with (-¢-) and without (-l-) the addition of polyethylene glycol (PEG; molecular weight (MW) 3500; 2 mg/mg CT). All incubations were done in quadruplicate and are presented as a mean with standard error of the mean.



In incubations involving abomasal fluid only, 19% of the *O. circumcincta* L3 larvae were unable to pass through the sieves. Incubation of larvae in abomasal fluid containing the same range of concentrations of CT resulted in a significant (P<0.001) reduction in the migration of these larvae. Addition of 100 mg CT/ml resulted in a 16% inhibition of larval migration when incubated in abomasal fluid. Addition of 400 and 1000 mg CT/ml resulted in significantly (P<0.001) fewer (64 and 79% inhibition, respectively) larvae being able to pass through the sieves (Fig 2B). Addition of PEG partially reduced (P<0.05) the effect of the sulla extract in abomasal fluid at higher CT concentrations only.

FIGURE 2: The effect of the condensed tannin (mg CT ml^{-1}) contained in an extract from sulla (*Hedysarum coronarium*) on the larval migration inhibition (LMI) of infective third-stage (L3) larvae of *Ostertagia circumcincta* during incubation in either rumen **(A)** or abomasal **(B)** fluid. The incubations were done with (-¢-) and without (-l-) the addition of polyethylene glycol (PEG; molecular weight (MW) 3500; 2 mg/mg CT). All incubations were done in quadruplicate and are presented as a mean with standard error of the mean.

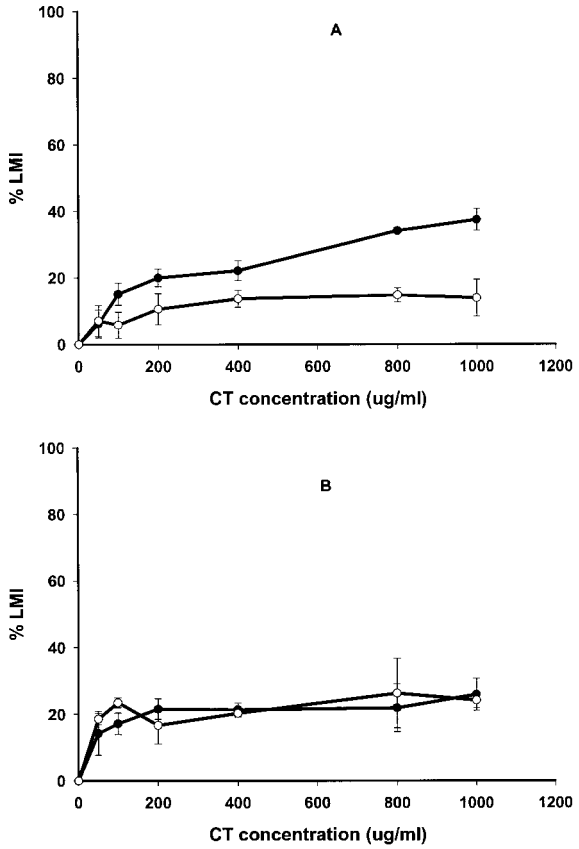


Experiment 3. The effect of the sulla extract on the migration of the L3 larvae of *Trichostrongylus colubriformis*

In incubations involving rumen fluid only, 9% of the *T. colubriformis* L3 larvae were unable to pass through the sieves. Incubation of exsheathed *T. colubriformis* larvae in rumen fluid containing the same range of concentrations of CT (50, 100, 200, 400, 800 and 1000 mg/ml) resulted in a significant (P<0.001) reduction in the migration (6-37%, respectively) of these larvae compared with control incubations (no CT added; Fig. 3A). Addition of PEG to the rumen fluid incubations partially reduced the effects of the sulla extract at 800 mg CT/ml (P<0.05) and 1000 mg CT/ml (P<0.01) only.

In incubations involving abomasal fluid only, 18% of the *T. colubriformis* L3 larvae were unable to pass through the sieves. Incubation of exsheathed *T. colubriformis* larvae in abomasal fluid containing the same range of concentrations of CT resulted in a reduction in the migration (14-26%, respectively) of these larvae compared with control incubations (no CT added; Fig. 3B). Addition of PEG had no effect on larval migration when *T. colubriformis* larvae were incubated with sulla extract in abomasal fluid.

FIGURE 3: The effect of the condensed tannin (mg CT ml^{-1}) contained in an extract from sulla (*Hedysarum coronarium*) on the larval migration inhibition (LMI) of infective third-stage (L3) larvae of *Trichostrongylus colubriformis* during incubation in either rumen **(A)** or abomasal **(B)** fluid. The incubations were done with (-¢-) and without (-l-) the addition of polyethylene glycol (PEG; molecular weight (MW) 3500; 2 mg/mg CT). All incubations were done in quadruplicate and are presented as a mean with standard error of the mean.



DISCUSSION

The principle objective of this research was to investigate the direct effects of an extract from sulla that contained CT on the viability of three sheep gastrointestinal nematodes, *Haemonchus contortus, Ostertagia circumcincta* and *Trichostronglus colubriformis* using an *in vitro* larval migration inhibition bioassay. The sulla extract significantly reduced the migration of infective (L3) larvae of *H. contortus, O. circumcinta* and *T. colubriformis* relative to control incubations (no CT added) in rumen and abomasal fluid. However, the CT present in the sulla extract was only partially responsible for the inhibitory effects of the extract observed in this study. Larimer *et al*. (1996) screened plant extracts for anthelmintic activity and attributed LMI activity in foliage from the tree *Phynocladus aspeniifolius* against *T. colubriformis* to plant polyphenolics. It is likely that the major plant polyphenolic present in this extract would have been CT. More recently Molan *et al.* (2000) used the LMI bioassay to test the biological activity of extracts containing CT from seven forages (including sulla) against *T. colubriformis* and found that the CT present in the extracts prevented 29-66% of the larvae from migrating through the nylon mesh sieves. This research supports the results presented here.

Although the mechanisms by which CT reduce larval migration are not known, the failure of a high proportion of larvae that have been exposed to extracts containing CT to pass through the pores of sieves is indicative of paralysis. The LMI assay is dependent on active migration of larvae through pores in a sieve that have a diameter that is slightly less than the mean diameter of the L3 larvae. Inability of larvae to pass through such sieves following incubation in the sulla extract suggests that the extract interfered with neurophysiology or neuromuscular coordination in the larvae. The ability of PEG to partially reverse the effects of the sulla extract suggests that CT per se interfered with the larvae in this way. This mode of action is not inconceivable because it is similar to the mode of action of Levamisole and Ivermectin (Behm and Bryant, 1985; Wagland *et al*., 1992). Condensed tannins have been shown to have a range of activities against enzymes and bacteria that suggests they may be capable of interfering with the neurophysiology of larvae. Condensed tannins inhibited endogenous enzyme activities (Oh and Hoff, 1986; Horigome *et al*., 1988) and were potent inhibitors of rat liver cyclic AMP-dependent protein kinase (Wang *et al*., 1996). The antimicrobial activity of CT has been well documented (Scalbert 1991; Bae *et al*., 1993; Jones *et al*., 1994; Molan *et al*., 1997). McAllister *et al* (1994) reported that CT could cause cellulolytic bacteria to dissociate from substrates possibly as a consequence of CT-surface interactions. Jones *et al.* (1994) suggested that CT might penetrate the cell wall and cause a loss of intracellular constituents.

This study has shown that the larvae of *T. colubriformis* were more resistant than the larvae of *H. contortus* and *O. circumcincta* to the action of the sulla extract and to the CT in that extract. When the larvae of these nematodes were incubated in rumen fluid containing 1000 mg CT/ml, 43% of *T. colubriformis* larvae failed to pass through the sieves compared to 66% and 76% for *O. circumcincta* and *H. contortus*, respectively. Although it is difficult to compare between the CT contained in the sulla extract and the anthelmintic drugs, Jill and Lacey (1993, 1998) found similar differences in the sensitivity of these three nematodes to the action of paraherquamide and ivermectin.

Addition of 2 mg PEG/ mg CT to the incubations partially reduced the inhibitory effect of the CT on larval migration, particularly in the rumen fluid. 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). Similarly, Molan *et al*. (2000) found that addition of PEG to incubations containing *T. colubriformis* L3 larvae and CT extracted from seven different forages significantly reduced the inhibitory effect of the CT. However, PEG failed to completely eliminate the inhibitory activity of the sulla extract. This suggests that other phenolic compounds often present in Sephadex LH-20 extracts (Jackson *et al* 1996), or other inhibitory compounds already present in the digesta that do not respond to the action of PEG may have also affected the viability of the larvae.

It has been reported that the binding of CT to protein 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). The mean pH of the abomasal fluid used in this study was 3.1 and therefore, the reduction in larval

migration when larvae of all nematodes were incubated in abomasal fluid containing a range of CT concentrations is a unique and difficult observation to explain. However, it suggests that the mode of action that CT has against gastrointestinal nematodes may not be related to the well-documented pH-dependent interaction that CT has with protein.

CONCLUSIONS

This study shows that the CT-containing forage, sulla may have direct inhibitory effects against the infective L3 larvae of the three principal gastrointestinal nematodes of sheep. The results also support the conclusion that feeding sulla to sheep may be an alternative method for controlling internal parasites, reducing our dependence on anthelmintic drenching as the sole method for controlling these parasites.

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