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Effects of Sulla Condensed Tannins on the Degradation of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase (Rubisco) And On The Viability Of Three Sheep Gastrointestinal Nematodes *In vitro*

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Abstract: Condensed tannins (CT) extracted from sulla (*Hedysarum coronarium*) were incubated with total soluble leaf protein extracted from white clover (*Trifolium repens*) and rumen fluid in order to determine the effects on the degradation of the large subunit (LSU) and small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). In the absence of CT, incubation with rumen fluid resulted in a rapid degradation of the LSU and SSU, although degradation of the LSU tended to be faster than that of the SSU and the degradation rate was significantly ($P<0.001$) higher than that in the incubations without rumen fluid. Addition of 150, 300 and 600 μg per ml of CT from sulla to the incubations decreased ($P<0.05$-0.001) the rate of degradation of both LSU and SSU of Rubisco. At all concentrations of CT, addition of polyethylene glycol (PEG) eliminated the inhibition attributable to CT. The effect of CT extracted from sulla on the viability of the infective third-stage (L3) larvae of three gastrointestinal nematodes (*Haemonchus contortus, Ostertagia circumcincta and Trichostrongylus colubriformis*) was tested by a larval migration inhibition (LMI) assay *in vitro*. The LMI assay measures the ability of the test material to immobilise the larvae and inhibit their passage through 20 μm nylon mesh sieves. Extracts of CT from sulla were added to rumen and abomasal fluids (collected from sheep fed lucerne chaff) to provide concentrations similar to these observed in the abomasal digesta of sheep when CT-containing forages were fed (50-1000 μg ml^{-1}). Incubation of L3 larvae in these fluids containing sulla CT reduced the viability, relative to the larvae in the control incubations (no CT added). This study showed that the larvae of *T. colubriformis* were more resistant ($P<0.001$) to the inhibitory effect of CT from sulla than were the larvae of the other nematodes. When the larvae of these nematodes were incubated in rumen fluid containing 1000 μg CT ml^{-1}, fewer larvae of *T. colubriformis* (37%) failed ($P<0.001$) to pass through the sieves than those of *O. circumcincta* (59%) and *H. contortus* (72%). Addition of 2 μg PEG μg^{-1} CT to the incubations eliminated the inhibitory effect of CT on larval viability through inactivation of CT, particularly in the rumen fluid.

Key words: *Trichostrongylus colubriformis, Haemonchus contortus, Ostertagia circumcincta*, nematode larvae, paralysis, sulla, protein degradation, condensed tannins

Introduction

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

Proprietary anthelmintic drenches, the current 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 and Leathwick *et al.*, 2001). Anthelmintic resistance, increasing concern about anthelmintic residues in animal products and findings that regular drenching can not remove the effect of the parasite completely (Coop *et al.*, 1982) indicate the need to explore alternative methods of gastrointestinal nematode parasite control.

Forages such as sulla (*Hedysarum cornarium*), which contain CT have had dramatic effects 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 whether this effect is due to CT forming insoluble protein complexes in the rumen which result in an increased supply of protein to the small intestine facilitating a better immune response against the infection, or direct specific antiparasitic properties of CT.

The purpose of this research was to investigate the direct effect of CT extracted from sulla on three sheep

gastrointestinal nematodes, *Haemonchus contortus, Ostertagia circumcincta* and *Trichostronglus colubriformis* using an *in vitro* assay and to investigate the impact of sulla CT on the degradation of the total soluble leaf protein (Rubisco) by mixed rumen microorganisms *in vitro*.

Materials and Methods

Experimental Design: In this study, four series of *in vitro* experiments were conducted. The first series of experiments tested the ability of Sephadex LH-20 treated extracts from sulla to reduce the rate of proteolysis of Rubisco protein in the rumen compared to control incubations which did not contain CT. All incubations were undertaken with and without the addition of polyethylene glycol (PEG; MW 3,500) to inactivate the CT (Aerts *et al.*, 1999).

Another three series of *in vitro* experiments were undertaken to determine the effect of CT extracted from sulla on the motility of the third stage (L3) exsheathed larvae of the sheep nematodes *H. contortus, O. circumcincta* and *T. colubriformis* using an LMI assay. Instead of phosphate buffered saline (PBS) which is usually used as a medium in this assay (Molan *et al.*, 2000a,b), rumen and abomasal fluids were used to provide an environment close to the *in vivo* environment.

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 (70:30 v/v). The Sephadex LH-20 extracts were freeze-dried and stored at -20°C until required.

Extraction of Total Soluble Protein: Total soluble leaf protein was extracted from white clover (*Trifolium repens*) by grinding one part leaves with 3 parts freshly prepared artificial saliva (pH 6.8; McDougall 1948) that was saturated with CO_2 gas. The resulting slurry was strained through two layers of cheesecloth and then centrifuged at 5800 g for 15 min at room temperature to remove all particulate plant material. The amount of protein in the extract was determined by the Coomassie Blue dye binding method (Bradford 1976). Bovine serum albumin (Sigma) was used as a standard.

The Preparation of Rumen Fluid: A Border Leicester-Merino castrated male sheep fitted with a rumen cannula (55 mm id) and fed fresh perennial ryegrass/white clover pasture *ad lib*, was used to supply rumen fluid for the incubations. After 1-2 h fasting, rumen fluid was collected and strained through 4 layers of cheesecloth into a prewarmed Dewar flask that had been flushed with CO_2 and immediately used for the incubations.

***In vitro* Incubation of Rubisco with Rumen Fluid and Sulla CT:** In 5 ml tubes fitted with one-way valves, 1.07 ml of the white clover protein extract (containing 2 mg protein), 0.53 ml of CO_2-saturated artificial saliva (pH 6.8-6.9) and 0.4 ml of rumen fluid were added. Before the addition of the rumen fluid 0, 150, 300 or 600 μg of condensed tannins extracted from sulla per ml were added. All experiments were done in the presence or absence of 2 μg PEG/ μg CT. The tubes were flushed with CO_2 and incubated at 39°C in a water bath.

Aliquots (20 μl) were removed from each test tube after 0, 1, 2, 4, 6 and 8 hours of incubation and added to Eppendorf tubes containing 20 μl of protein loading buffer (Aerts *et al.*, 1999) and ß-mercaptoethanol (4:1) and stored at -20°C for gel electrophoresis. After each sampling, the *in vitro* incubation tubes were flushed with CO_2 gas.

Sodium Dodecyl Sulphate -polyacrylemide Gel Electrophoresis (SDS-PAGE) and Densitometry: The samples were denatured by heating at 95°C for 5 minutes, centrifuged and aliquots of the samples representing 8 μg of protein at zero time were fractioned by SDS-PAGE according to the method described by Jackson *et al.* (1996). After electrophoresis, the large subunit (LSU) and small subunit (SSU) of Rubisco were detected by staining gels with Coomassie Brilliant Blue for 20 minutes. The LSU and SSU were quantified by densitometry. The amount of protein present in the *in vitro* rumen prior to incubation was set to zero and the percentage of protein degraded during incubation was determined for each time point.

Calculation of Data and Statistical Analyses: The percentage of each protein degraded after 1, 2, 4, 6 and 8 h

incubation was calculated by the following equation:

degradation = (A - B)/A X 100

Where A = the intensity of the LSU or SSU of Rubisco on SDS-PAGE gels prior to incubation (0 h)and B = the intensity of the LSU or SSU of Rubisco on SDS-PAGE gels at subsequent time periods.
The significance of differences among treatment means in each experiment was assessed using GLM (general linear models) procedures (SAS, version 6).

Parasite *in vitro* Assays
Source of Rumen and Abomasal Fluids: For the parasite *in vitro* assays, 4 sheep fitted with a rumen and abomasal cannulae were housed in metabolism crates indoors and fed Lucerne (*Medicago sativa*) chaff and pellets and used as donors of rumen and abomasal fluid. The rumen and abomasal fluid was collected from all sheep, pooled together, strained through two layers of cheesecloth and centrifuged twice at 15 000 rpm (to get rid of particulate materials which would block the sieves) and used immediately in the assay.

Parasite 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 inhibitory effect of purified CT against *O. circumcincta, H. contortus*and *T. colubriformis* in experimental series 2,3 and 4, respectively. The method involved preparation of test solutions with CT and of L3 larvae which were combined and incubated in the wells of 48-well tissue culture plates (Costar, Cambridge, MA). The larvae were exsheathed in sodium hypochlorite solution (0.025% available chlorine; Molan and others 2000b), washed five times with tap water and concentrated to 1,500 larvae ml^{-1} water. One hundred microliters of the larvae solution (~ 150 L3 larvae) were added to wells containing 400 microliters of either rumen or abomasal fluid that contained a range of CT concentrations (0, 50, 100, 200, 400, 800 and 1000 $\mu g\ ml^{-1}$). The plates were then incubated at 37 °C for 2 hours. The plates were incubated 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. All incubations were done with and without the addition of 2 μg of polyethylene glycol (PEG) per μg of CT.
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 slightly larger than the mesh and would thus prevent the larvae "falling" through the sieve. Four replicate samples were run for each concentration of 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 x magnification and the % of the larvae that had not migrated through the sieves (%LMI) was determined according to the following equation:

% LMI = (A - B)/A X 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

It can be seen from Fig. 1 that in the incubations which did not involve rumen fluid, the LSU and SSU (Fig. 1A) of Rubisco in total soluble protein extracted from white clover was degraded more slowly ($P < 0.001$) than their counterparts in the incubations containing rumen fluid (Fig. 1B). Addition of PEG had no effect on the rate of degradation of both LSU and SSU in either incubation (Fig.1).

Addition of CT extracted from sulla reduced the rate of degradation of both LSU and SSU of Rubisco (Fig. 2A-C). Addition of 150, 300 or 600 μg sulla CT $m1^{-1}$ reduced the rate of degradation of both LSU and SSU ($P < 0.05$-0.001) of Rubisco compared to control incubations (without CT). It is worth mentioning that no significant difference was observed in the degradation rate of both LSU and SSU of Rubisco by mixed rumen microorganisms at the highest concentration of sulla CT, when compared to incubations involving no rumen fluid.
At all concentrations of sulla CT, addition of PEG increased the rate of degradation of both LSU and SSU of Rubisco

(Figs. 2A, B and C) to levels similar to control incubations that contained rumen fluid only (without CT). It can be seen from Fig. 3 that the ability of CT to protect Rubisco from microbial degradation increases with increasing concentration of CT.

Incubation of exsheathed *O. circumcincta, H. contortus* or *T. colubriformis* L3 larvae with rumen fluid collected from sheep fed lucerne containing a range of concentrations (100, 200, 400, 800, 1000 μg ml^{-1}) of CT extracted from sulla resulted in a significant ($P<0.001$) reduction in the viability of these larvae as judged by their inability to pass through the sieves in the LMI assay, compared to control incubations containing larvae and rumen fluid only (no CT added; Figs.4-6). The larvae of *T. colubriformis* seem to be more resistant to the action of CT than the larvae of *O. circumcincta* and *H. contortus*. At 400 μg ml^{-1}, for example, sulla CT inhibited 22% of *T. colubriformis*

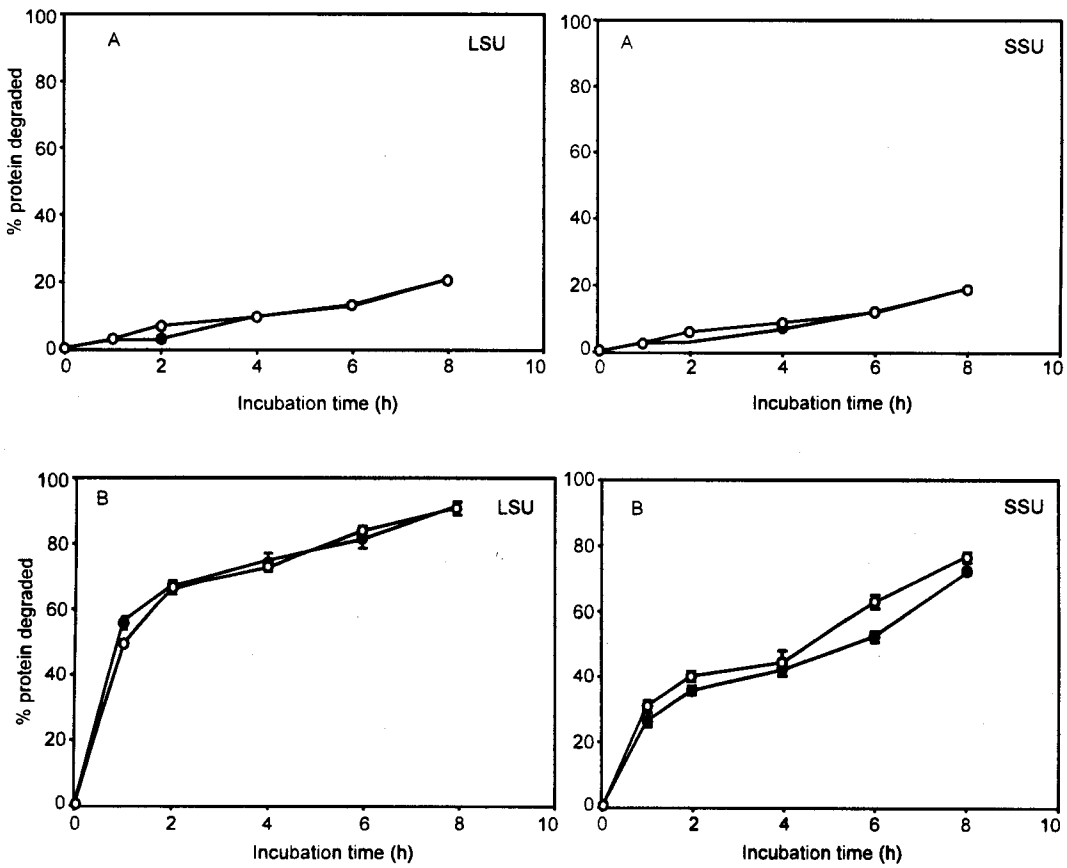

Fig. 1: The degration of the large subunit (LSU) and small subunit (SSU) of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) during *in vitro* incubation of total soluble leaf protein extracted from white clover (*Trifolium repens*) without rumen fluid (A) and with rumen fluid (B). Incubation were performed without (*) and with () the addition of polyethylene glycol (PEG; 2μg μg^{-1} CT; molecular weight 3350). Samples were removed prior to and after 1, 2, 4, 6 and 8 h of incubation. Total protein in the sample was fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)and the LSU and SSU protein was quantified by imaging densitometry. The amount of LSU and SSU protein prior to incubation was set to zero and the percentage of the degradation during incubation was determined. Means of quadruplicate incubations are shown.

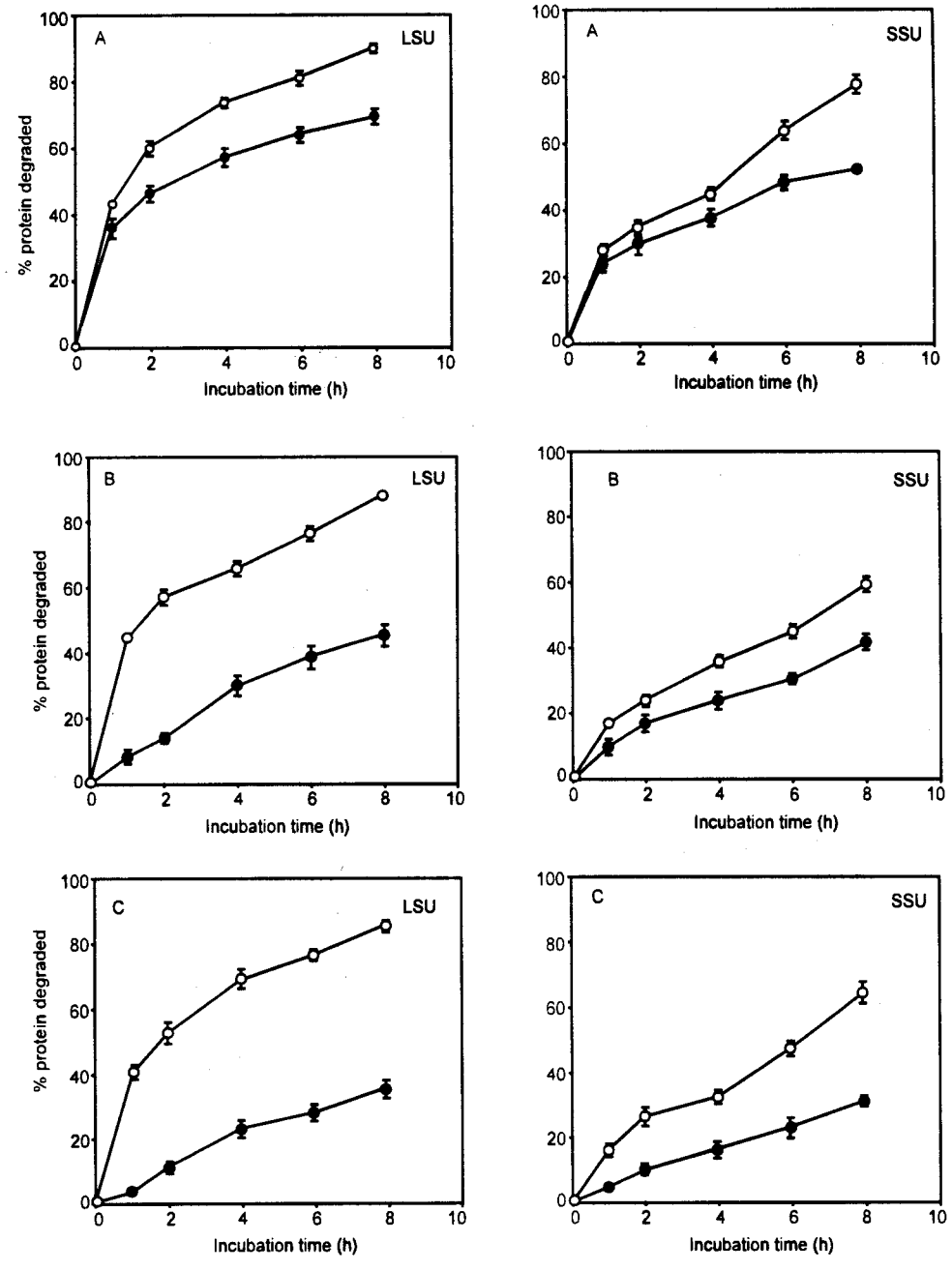

Fig. 2: The degradation of the large subunit (LSU) and small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) during *in vitro* incubation of total soluble leaf protein extracted from white clover (*Trifolium repens*) with rumen fluid and 150 μg (A), 300 ig (B) and 600 μg (C) of CT purified from sulla (*Hedysarum coronarium*)/ml. Incubations were performed without (CT active; ★) and with (CT inactive; _) the addition of polyethelene glycol (PEG; 2 μg μg^{-1} CT; molecular weight 3350). Samples were removed prior to and after 1, 2, 4, 6 and 8 h of incubation. Total protein in the samples was fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)and the LSU and SSU protein was quantified by imaging densitometry. The amount of LSU and SSU protein prior to incubation was set to zero and the percentage of the degradation during incubation was determined. Means of quadruplicate incubations and standard errors are shown

larvae from passing through the sieves compared to 41% and 65% for the larvae of *O. circumincinta* and *H. contortus*, respectively. At 1000 μg ml^{-1}, the CT inhibited 37%, 59% and 72% of the larvae of *T. colubriformis*, *O. circumincinta* and *H. contortus*, respectively from passing through the sieves.

Incubation of the larvae of these three nematodes in abomasal fluid (collected from sheep fed lucerne) containing the same concentrations of CT as in the first series of experiments also reduced the viability of the larvae ($P<0.001$) compared to the larvae in the control incubations (Figs. 4-6). Again, the larvae of *T. colubriformis* were more resistant to the inhibitory effect of sulla CT than the larvae of *O. circumincinta* and *H. contortus*. At 1000 μg ml^{-1}, 25% *T. colubriformis* larvae were inhibited from passing through the sieves compared to 79% and 81% of *O. circumincinta* and *H. contortus* larvae, respectively.

Addition of 2 μg polyethylene glycol (PEG) per μg sulla CT, reduced ($P<0.001$) the effects of sulla CT in incubations involving rumen fluid. In incubations that involved abomasal fluid, PEG was not effective in eliminating the inhibitory activity of CT except in incubations involving *O. circumcinta* where the difference was significant ($P<0.01$) between CT-active (no PEG) and CT-inactive (with PEG) treatments.

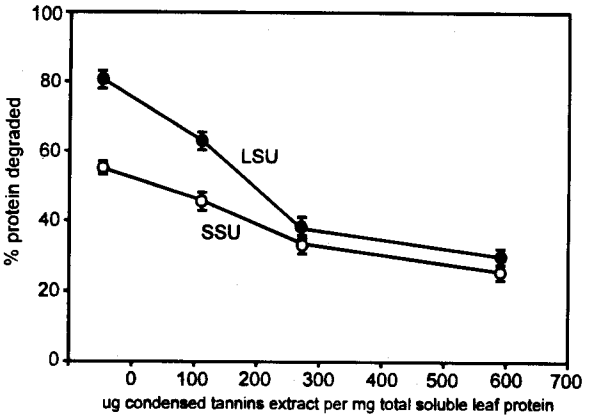

Fig. 3: The mean percentage of the large subunit (LSU;★) and the small subunit (SSU; _) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein which degraded after 4, 6 and 8 h when total soluble leaf protein from white clover (*Trifolium repens*) was incubated with rumen fluid from sheep and increasing concentrations of CT extracted from sulla (*Hedysarum coronarium*).

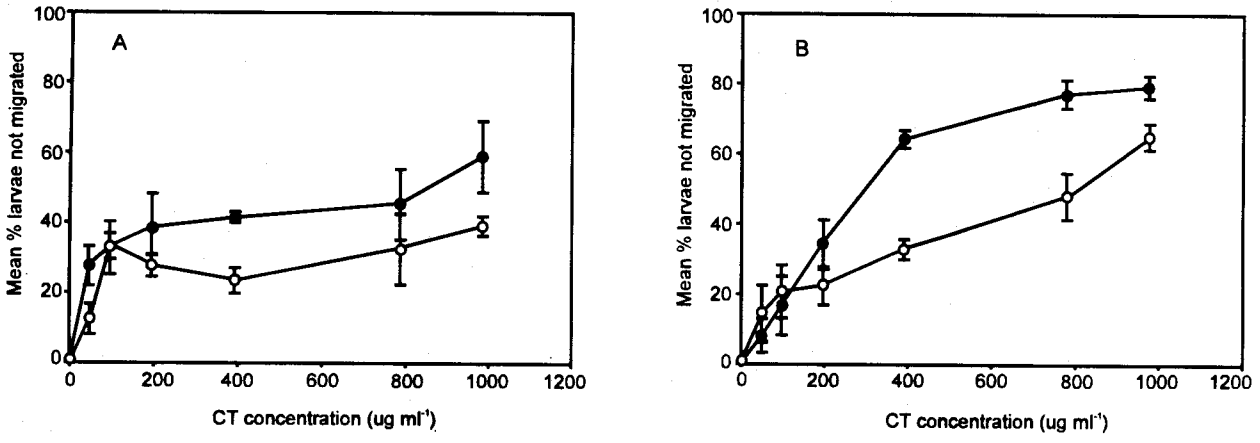

Fig. 4: The effect of condensed tannin (μg CT ml^{-1}) extracted from sulla (*Hedysarum coronarium*) on the viability of the infective third-stage (L3) larvae of *Ostertagia circumcincta in vitro*. The incubations were undertaken without (★) and with (_) the addition of polyethylene glycol. Each point represents the mean of quadruplicates with the standard error of the mean.

A- L3 larvae incubated in rumen fluid

B- L3 larvae incubated in abomasal fluid

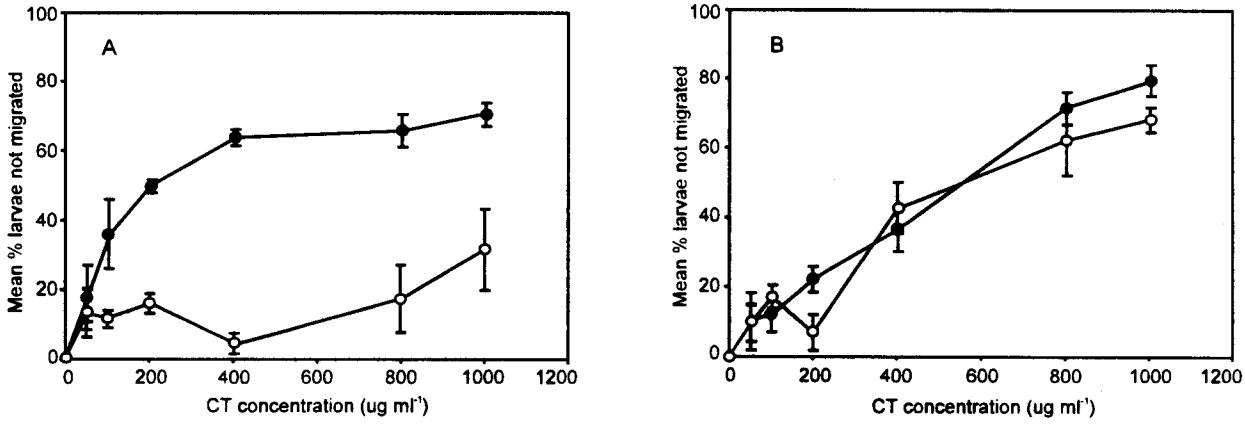

Fig. 5: The effect of condensed tannin (μg CT ml^{-1}) extracted from sulla (*Hedysarum coronarium*) on the viability of the infective third-stage (L3) larvae of *Haemonchus contortus in vitro*. The incubations were undertaken without (*) and with (_) the addition of polyethylene glycol. Each point represents the mean of quadruplicates with the standard error of the mean.
A- L3 larvae incubated in rumen fluid
B- L3 larvae incubated in abomasal fluid

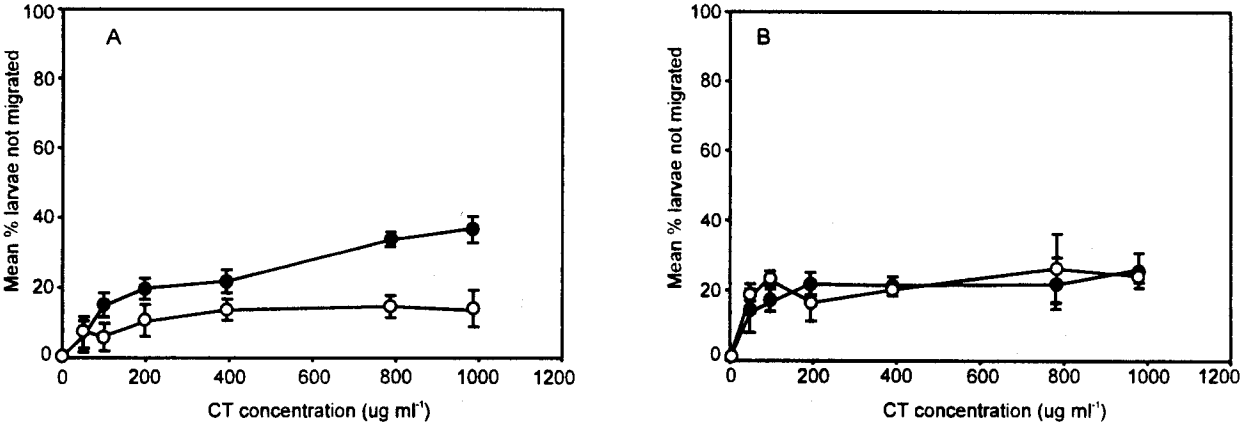

Fig 6: The effect of condensed tannin (μg CT ml^{-1}) extracted from sulla (*Hedysarum coronarium*) on the viability of the infective third-stage (L3) larvae of *Trichostrongylus colubriformis in vitro*. The incubations were undertaken without (*) and with (_) the addition of polyethylene glycol. Each point represents the mean of quadruplicates with the standard error of the mean.
A- L3 larvae incubated in rumen fluid
B- L3 larvae incubated in abomasal fluid

Discussion

The present results demonstrate that the CT from sulla are very effective at reducing the rate of degradation of LSU and SSU of Rubisco by mixed rumen micro-organisms when added to *in vitro* incubations containing rumen fluid. Although the mode of action of CT is not fully known, the reduction of proteolysis may be attributed to direct effects of CT on microbial proteolytic enzyme activity or to indirect effects on rumen metabolite concentrations, which can regulate proteolytic activity in some bacteria (Waghorn *et al.*, 1994).

At 600 μg CT mg^{-1} Rubisco protein, the CT from sulla completely protected Rubisco from degradation by mixed rumen microorganisms as there was no significant difference in the degradation rate between incubations

containing this concentration of sulla CT and incubations containing Rubisco only (no rumen fluid added). This finding is of particular importance because Rubisco is the principal dietary protein (Mangan and West 1977) and about 70% of dietary protein can be degraded to ammonia in the rumen of sheep fed fresh forages (Ulyatt *et al.*, 1975). McNabb *et al.* (1996) found that CT in *Lotus pedunculatus* reduced the degradation of Rubisco protein by rumen microorganisms and increased the proportion of this protein that was digested in the small intestine of sheep. This means that by feeding sulla, more protein will be available to ruminants grazing this fresh forage and this will increase their performance and may increase their resistance to gastrointestinal nematode parasitism. There are a number of reports that suggest that an increase in the supply of digestible protein will improve the resilience and resistance of sheep to gastrointestinal nematodes and to compensate for the endogenous losses of protein in the gut (Coop and Holmes 1996; Donaldson *et al.*, 1997).

The results presented here showed that addition of CT extracted from sulla, to the abomasal and ruminal fluids containing infective (L3) larvae of *H. contortus, O. circumcinta* or *T. colubriformis* had inhibitory activity relative to the control incubations containing larvae and ruminal or abomasal fluid with no CT added. 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, although they did not determine the CT concentration. Recently, Molan *et al.* (2000b) used the LMI assay to test the biological activity of CT extracted from seven different forages against the sheep nematode, *T. colubriformis in vitro.* They found that when *in vitro* assays contained CT extracted from sulla and other forages, the CT reduced the viability of L3 larvae as evidenced by their ability to inhibit 29-66% of the larvae from passage through the nylon mesh sieves. It was also found that CT from sulla and other forages were able to inactivate the first-stage (L1) larvae of deer lungworm and L3 larvae of deer gastrointestinal larvae by inhibiting their migration through the LMI assay sieves (Molan *et al.*, 2000a). Recently Molan *et al.* (2002) found that CT extracted from sulla and other forages were able to slowdown the hatching process of *T. colubriformis* eggs and to inhibit the development of the eggs into infective larvae under *in vitro* conditions.

Although the mechanisms by which CT immobilize the larvae 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. The LMI assay is dependent on active migration of larvae through the 20 micron pores in the sieves, which are slighty less than the mean (25 micron) diameter of L3 larvae (Rabel *et al.*, 1994), suggesting an interference with neurophysiology or neuromuscular coordination of the larvae. This would mimic the action of Levamisole and Ivermectin (Behm and Bryant 1985; Wagland *et al.*, 1992). Condensed tannins have been shown to inhibit endogenous enzyme activities (Oh and Hoff 1986; Horigome *et al.*, 1988) and were a potent inhibitor 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 and Molan *et al.*, 2001). McAllister *et al.* (1994) reported that CT could cause cellulolytic bacteria to disasociate from substrates possibly as a consequence of CT-surface interactions. Jones *et al.* (1994) suggested that CT may also penetrate the cell wall and cause a loss of intracellular constituents. Sulla CT used in this study may act similarly by penetrating the wall of the larvae and immobilizing body musculature.

This study showed that the larvae of *T. colubriformis* were more resistant ($P < 0.001$) to the inhibitory effect of CT from sulla than were the larvae of *H. contortus* and *O. circumcincta* . When the larvae of these nematodes were incubated in rumen fluid containing 1000 μg CT/ml, 37% of *T. colubriformis* larvae failed to pass through the sieves compared to 59% and 72% for *O. circumcincta* and *H. contortus*, respectively. Although it is difficult to compare between CT and the anthelmintic drugs, Gill and Lacey (1993, 1998) found great differences in sensitivity to the action of paraherquamide and ivermectins between these three nematodes.

Addition of 2 μg polyethelene glycol (PEG)/ μg CT to the incubations eliminated the inhibitory effect of CT on larval viability through inactivation of CT particularly with 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.* (2000b) found that addition of PEG to the incubations containing *T. colubriformis* L3 larvae and a range of CT, extracted from seven different forages including sulla, eliminated most of the inhibitory effect of CT. This means that PEG has the ability to reverse the inhibitory effect of CT when added directly to the incubations containing the larvae and CT.

The results of this study support the hypothesis that CT may affect parasites both directly and indirectly. This study shows that sulla CT have direct inhibitory effect against the larvae of sheep gastrointestinal nematodes and suggests that feeding sulla to ruminants may be used as an alternative method for controlling internal parasites in order to reduce our dependence on anthelmintic drenching as the sole method for controlling internal parasites. This

study also shows that sulla CT have the ability to slowdown the process of proteolysis of Rubusco by mixed rumen microorganisms which suggests that they may affect the parasites indirectly by improving the performance of the host.

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