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The effects of condensed tannins extracted from different plant species on egg hatching and larval development of *Teladorsagia circumcincta* (Nematoda: Trichostrongylidae)

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Abstract: The effects of condensed tannins (CTs) extracted from five species of plants on egg hatching and larval development of *Teladorsagia circumcincta* (Stadelmann, 1894) (syn. *Ostertagia circumcincta*) were evaluated using *in vitro* bioassays. The extracts of CTs were obtained from *Lotus pedunculatus* (LP), *Lotus corniculatus* (LC), *Dorycnium pentaphyllum* (DP), *Dorycnium rectum* (DR) and *Rumex obtusifolius* (RO). The results of egg hatching assay showed that about 53%, 68%, 51%, 60% and 46% of the eggs hatched when *in vitro* incubations contained 900 μg/ml of CTs from LP, LC, DP, DR and RO, respectively ($P< 0.001$ relative to control incubation), while in control incubations (no CT added) 87% of the eggs hatched. In the larval development assay, development was allowed to proceed for 7 days, by which time 89% of the hatched larvae in control wells (no CTs) had reached the infective third stage (L3). In incubations containing 200 μg CT from LP, LC, DP, DR and RO/ml, about 8%, 15%, 14%, 8% and 4% of the eggs attained full development to L3 larvae, respectively ($P< 0.001$ relative to control incubation). Only 1% of the eggs were able to develop to L3 larvae in incubations containing 400 μg CT extracted from LC/ml, whilst in the incubations containing the same concentration of other CTs the eggs were not able to develop to L3 larvae. It seems that CTs are not only slowing down the larval development but also kill the undeveloped larvae. At 400 μg/ml, for example, CT from LP, LC, DP, DR and RO killed 67%, 48%, 68%, 93% and 91% of first-stage (L1) and second-stage (L2) larvae, respectively. This study shows that CTs are able to disrupt the life cycle of nematodes.

Keywords: *Teladorsagia circumcincta*, egg hatching, larval development, condensed tannins

It is well established that gastrointestinal nematode parasites decrease livestock production. At present, most nematode parasite control is protective and based on the regular use of anthelmintics (Vlassoff and McKenna 1994). Although the regular use of anthelmintics is effective in removing the existing worm burdens (Parkins and Holmes 1989) and protects against serious disease and mortality, it does not necessarily prevent the exposure of animals to high levels of pasture contamination and hence production losses can still occur if there is re-infection between treatments with anthelmintics (Brunsdon 1980). Thus the development of anthelmintic resistance and the increasing concern about anthelmintic residue (Sangster 1999, Leathwick et al. 2001) highlight the need to find alternative non-chemical parasite control strategies for controlling internal parasites. One approach may be to include plants that contain condensed tannins (CTs) into the grazing rotation (Robertson et al. 1995) or use the CT extracts as a drench.

CTs are polyphenolic compounds found in the leaves, stems, buds, flowers and bark of many species in the plant kingdom (Terrill et al. 1992). It is well known that in ruminants CTs can protect the dietary protein from microbial degradation in the rumen and consequently increase the proportion of dietary protein reaching the intestine (Waghorn et al. 1994) and high protein intakes have been associated with increased immunocompetence in young sheep (Coop and Holmes 1996). In addition, forages containing CTs are able to reduce the negative effects of intestinal nematodes upon reproductive performance in different ruminant species (Niezen et al. 1995, Robertson et al. 1995, Marley et al. 2003, Min and Hart 2003).

Studies in New Zealand (Niezen et al. 1995, 1998, Waghorn et al. 1994), United Kingdom (Athanasiadou et al. 2000a, b, Marley et al. 2003), France (Paolini et al. 2003, Bahuaud et al. 2006) and United States (Min and Hart 2003) have shown that some plant species that contain CTs may reduce the degree of parasite infections and improve sheep and goat performance. Although the underlying mechanisms for such beneficial effects have not been determined, both direct (Athanasiadou et al. 2000a, b) and indirect (Aerts et al. 1999, Waghorn and

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Molan 2001) effects of CTs on parasites in the gut were suggested to be behind these effects.

As part of a programme in the discovery of potential new anthelmintics from plant sources, extracts from several forage legumes and trees were tested against helminths of sheep and deer (Molan et al. 2000a, b, 2002). These studies have shown that CTs extracted from *Lotus pedunculatus* auct. (Fabales: Fabaceae), *Lotus corniculatus* L. (Fabales: Fabaceae), *Dorycnium pentaphyllum* (L.) Ser. (Fabales: Fabaceae), *Dorycnium rectum* Scop. (Fabales: Fabaceae) and *Rumex obtusifolius* L. (Caryophyllales: Polygonaceae) can inhibit egg hatching and larval development of *Trichostrongylus colubriformis* (Giles, 1892) under laboratory conditions.

The objective of the present study was to investigate the inhibitory effects of CTs extracted from five plants, *L. pedunculatus* (LP), *L. corniculatus* (LC), *D. pentaphyllum* (DP), *D. rectum* (DR), and *R. obtusifolius* (RO), on egg hatching and the larval development of the sheep nematode, *Teladorsagia circumcincta* (Stadelmann, 1894) (syn. *Ostertagia circumcincta*) under laboratory conditions.

MATERIALS AND METHODS

Experimental design. Three sets of experiments were conducted. In the first experiment, the egg hatch assay was used to evaluate effects of CT extracts (at 0, 50, 100, 200, 400, 600 and 900 μg/ml) from each of five plant species (LP, LC, DP, DR and RO) on hatching of *Teladorsagia circumcincta* eggs *in vitro*.

In the second experiment, a larval development assay was used to evaluate the effects of CTs extracted from the same forages on the development of eggs to L3 larvae. Eggs were incubated with a range of CT concentrations (0, 25, 50, 100, 200, 300, 400 and 500 μg/ml) from each of these five plant species in culture medium for 7 days at 24°C.

The third set of experiments was conducted in order to evaluate the effects of CTs on the viability of L1 and L2 larvae that could not develop into L3 larvae after 7 days of incubation. The proportion of dead larvae was calculated in incubations involving each CT.

Preparation of condensed tannins (CTs). 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) 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 CT extracts were freeze-dried and stored at –20 °C.

Preparation of eggs. The eggs were collected from the fresh faeces of lambs experimentally infected with *Teladorsagia circumcincta*. Eggs were recovered from about 50 g of faeces by mixing 200 ml of water, after which the suspension was poured through a 1mm-mesh screen assisted by a gentle jet of water to give about 2 l of eluate. The eluate was progressively washed through sieves with 100 μm and then 20 μm size which retained the eggs. The eggs were washed off the 20-μm sieves and centrifuged at 3700 rpm for 7 min to precipitate the eggs. The supernatant was discarded and the eggs were further cleaned from organic debris by centrifugation in magnesium sulphate (20%) for 7 min at 3700 rpm. The supernatant was then filtered through 60-μm sieve and the eggs were collected and washed on a 20-μm sieve after which they were transferred to graduated tubes and allowed to settle. The supernatant was removed to reduce the volume to 15 ml and the number of eggs estimated in ten 10 μl samples enabling the final volume of the suspension to be adjusted to approximately 1500 eggs/ml for storage at 4 °C.

Egg hatch assay. A stock solution of CT was prepared by dissolving CTs in distilled water, from which working solutions were prepared by further dilution with distilled water. Twenty microlitres of the working solutions were pipetted into each well of 48-well tissue culture plates (Costar, Cambridge, MA) together with a known number of eggs (about 100 eggs) and made up to 2 ml with distilled water to give final concentrations ranging from 50 μg to 900 μg CT/ml.

Assays were conducted in triplicate. Eggs in distilled water alone were used as controls. The eggs were incubated at 24 °C for 26 hours, and then a drop of aqueous iodine was added to each well to kill the eggs and hatched larvae. The numbers of eggs and hatched larvae were counted and then the percentage of eggs hatched (number of L1 larvae/number of eggs in culture × 100; Hubert and Kerboeuf 1984) was calculated.

Culture medium. The medium used to culture the parasite from the egg to the third infective larval stage was similar to that used by Hubert and Kerboeuf (1984) except that agar was omitted. This modification was necessary because CT can bind to any source of protein or carbohydrate so by omitting agar the eggs and larvae will be the main targets for CTs. The culture comprised an *Escherichia coli* suspension plus nutritive medium and Amphotericin B. The *E. coli* suspension was prepared by dissolving 15 mg lyophilised *E. coli* cells [strain W (ATCC) 9637, Sigma] in 100 ml of distilled water. The suspension was sterilised by autoclaving. The nutritive medium prepared by dissolving one gram of yeast extract (Y-1000, Sigma) in 90 ml of 0.85% saline solution plus 10 ml of Earle's balanced salt solution (E7510, Sigma). The growth medium was prepared by mixing 3 ml of *E. coli* suspension, 3 ml of the nutritive medium and 180 μl of Amphotericin B (to inhibit the growth of fungi).

Larval development assay. The assays were carried out in 96-well microtitre plates as described by Molan et al. (2002). Briefly, the assay involved mixing 40 μl of growth medium with 60 μl of egg suspension (containing approximately 100 eggs) and a series of CT concentrations. Triplicate development assays were run for each concentration of CT (25, 50, 100, 150, 200, 300, 400 and 500 μg/ml) and three control wells containing eggs, growth medium but no CTs were included in each experiment. After the addition of CTs, the plates were incubated at 24 °C for 7 days in a large covered glass petri dish sealed with paraffin film to maintain a high relative humidity and prevent the plates from drying out. At the end of incubation period the plates were kept on ice to stop further development and the numbers of unhatched eggs, first-stage (L1), second-stage (L2) and third-stage (L3) larvae (live and dead) were counted in each well and the mean larval development was calculated as the number of L3/number of eggs in culture × 100. All observations were carried out using a binocular microscope at ×40 magnification.

The mean number of hatched larvae that subsequently died was also calculated (number of dead larvae/number of total number of larvae × 100) for each concentration and control incubations. The movement and posture of the larvae were used as the main criteria for the viability of larvae. If the larva does not move within 30 seconds, it is considered dead. In addition, and from our experience, the dead larvae assume straight postures and become transparent.

Calculation of data and statistical analysis. The per cents of egg hatching and the mean larval development were calculated by expressing numbers hatched or developed as a percentage of starting numbers. Results were expressed as the mean ± S.E.M. A *t*-test or one-way ANOVA, followed by Tukey's test, was used to detect statistical significance of differences between groups. $P<0.05$ was considered to be statistically significant.

RESULTS

The results of the egg hatching assay are shown in Fig. 1. In control wells (no CTs were added), 87% of the eggs hatched. For all CTs, the number of eggs hatched decreased significantly ($P<0.05$–0.001) with increasing CT concentrations ($R^2 = 0.9304$, 0.9049, 0.9042, 0.7856 and 0.8165 for LP, LC, DP, DR and RO, respectively). At 200 µg/ml of CTs from LP, LC, DP, DR and RO, the per cents of egg hatching were 72%, 78%, 73%, 70% and 66%, respectively while at 400 µg/ml they were 66%, 73%, 69%, 63% and 63%, respectively ($P < 0.001$ for both concentrations and all the plants in comparison to the control incubation). At 900 µg/ml, the hatching rate was significantly ($P<0.001$) lower than that at 400 µg/ml of CTs extracted from all forages and lower than that in the control incubation ($P<0.001$).

In the larval development assay (Fig. 2), 89% of the eggs in control wells (no CTs were added) were able to attain full development to the third-stage larvae (L3). Exposure of eggs to 25 µg/ml or higher of CTs from all the tested plants resulted in a significant reduction in the larval development of *T. circumcincta*. At 200 µg/ml of CTs extracted from LP, LC, DP, DR and RO the per cents of eggs attaining full development to L3 larvae were 8%, 15%, 14%, 8% and 4%, respectively ($P<0.001$ for all CTs when compared with the control incubation). In the wells containing 300 µg/ml of CT from LC, DP and DR, the proportion of larval development was 5%, 4% and 3%, respectively while at this concentration, CTs from LP and RO inhibited the larval development totally. At 400 µg/ml, CTs from all the sources completely inhibited the development of eggs to L3 larvae.

The per cent of larvae that hatched and subsequently died is shown in Fig. 3. The proportion of dead larvae was calculated in order to get some information on the mode of CT action and whether they kill the larvae or just slow down the larval development.

At 200 µg/ml CT from LP, LC, DP, DR and RO killed 40%, 18%, 33%, 60% and 50% of the L1 and L2 larvae, respectively after 7 days of incubation while at 400 µg/ml they killed 87%, 48%, 68%, 93% and 91% of L1 and L2 larvae, respectively. At 500 µg/ml, CT extracted from LP, DP, DR and RO killed all the larvae while those extracted from LC killed 92% of the larvae, respectively (Fig. 3).

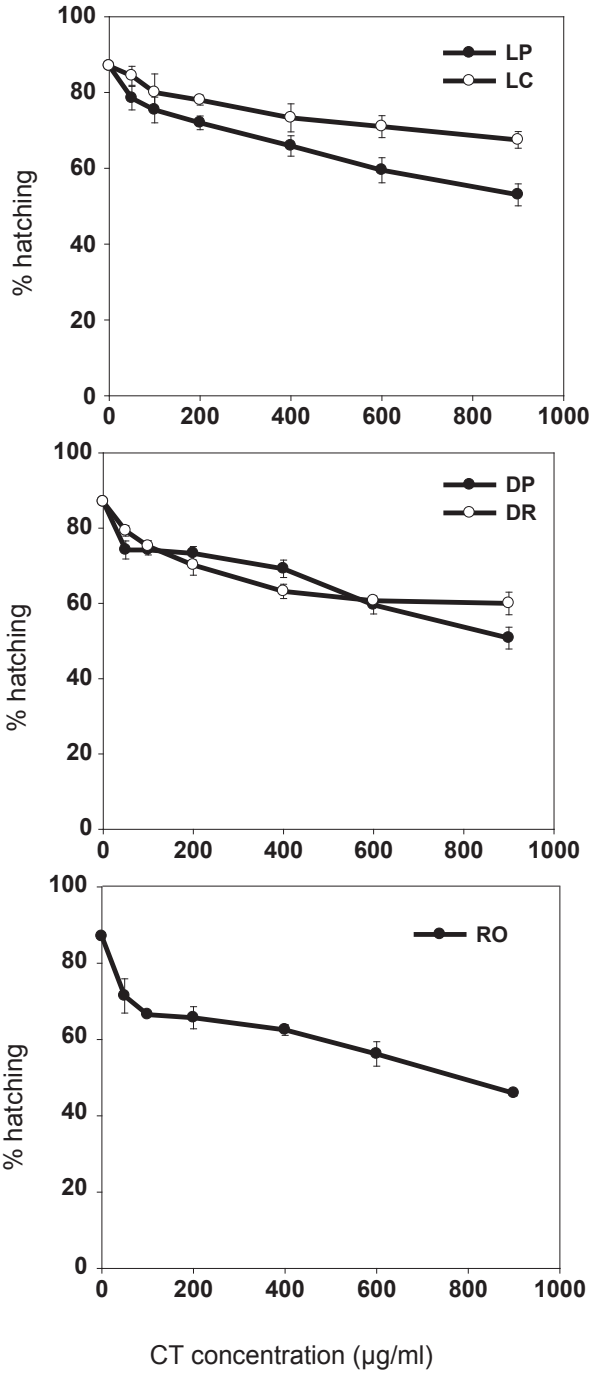

Fig. 1. The effect of condensed tannins (CTs) extracted from *Lotus pedunculatus* (LP), *Lotus corniculatus* (LC), *Dorycnium rectum* (DR), *Dorycnium pentaphyllum* (DP) and *Rumex obtusifolius* (RO) on the proportion of *Teladorsagia circumcincta* eggs hatching *in vitro*. Each point represents the mean of triplicates with the standard error of the mean (S.E.M.).

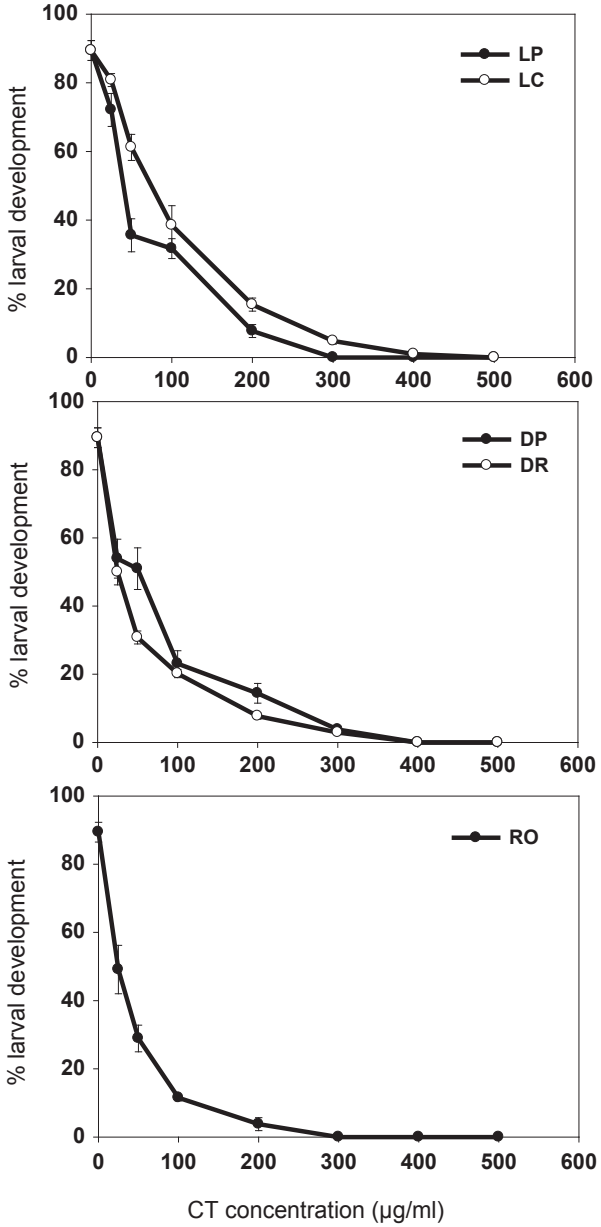

Fig. 2. The effect of condensed tannins (CTs) extracted from *Lotus pedunculatus* (LP), *Lotus corniculatus* (LC), *Dorycnium rectum* (DR), *Dorycnium pentaphyllum* (DP) and *Rumex obtusifolius* (RO) on the development of eggs of *Teladorsagia circumcincta* into infective larvae (L3) *in vitro*. Each point represents the mean of triplicate incubations with S.E.M.

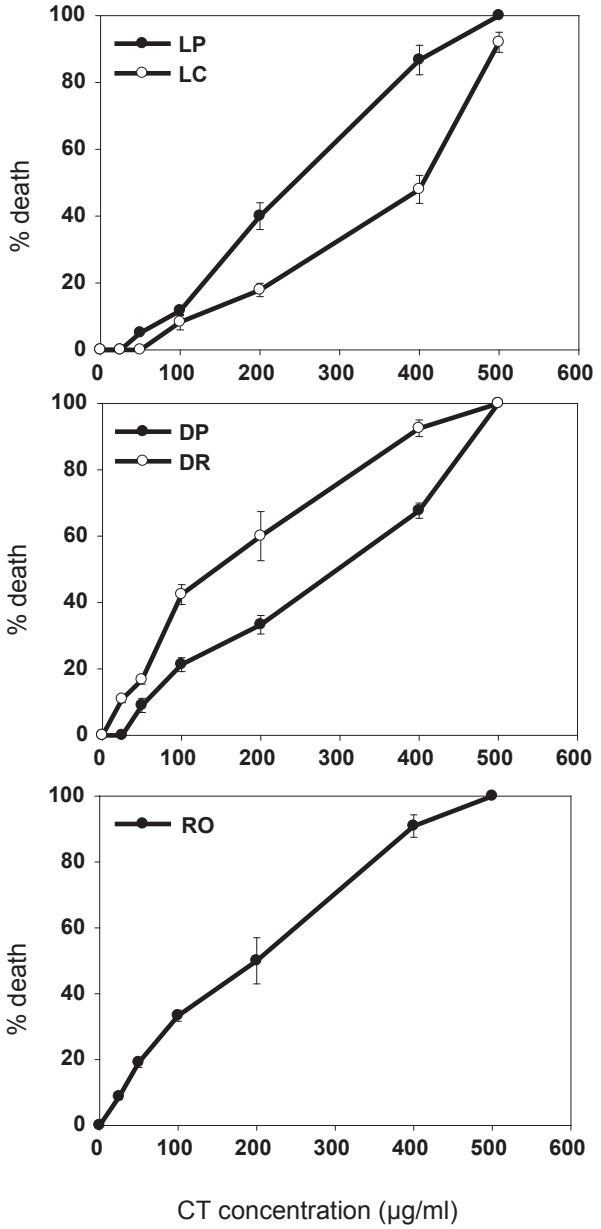

Fig. 3. The effect of condensed tannins (CTs) extracted from *Lotus pedunculatus* (LP), *Lotus corniculatus* (LC), *Dorycnium rectum* (DR), *Dorycnium pentaphyllum* (DP) and *Rumex obtusifolius* (RO) on the viability of the first-stage (L1) and the second-stage (L2) larvae of *Teladorsagia circumcincta* developed from eggs that were exposed to different concentrations of CTs for 7 days. Each point represents the mean of triplicate incubations with S.E.M.

DISCUSSION

The principal finding of this study was that condensed tannins extracted from LP, LC, DP, DR and RO had profound activity (as measured by egg hatch and larval development assays) against the eggs of *Teladorsagia circumcincta in vitro* when used in concentrations lower than those recorded in the digesta of ruminants fed CT-containing forages. Although further work is needed to determine the effects *in vivo*, the results presented here may indicate that the CTs have the ability to disrupt the life cycle of nematodes by preventing the development of eggs into the infective (L3) stage and this in turn could prevent or reduce the contamination of the farms with the viable eggs. The results of the present study may support the results of the studies that attributed the beneficial effects of

CTs to a direct toxic effect on the parasites of ruminants. Under *in vivo* conditions, Butter et al. (2000) found that feeding a model CT, quebracho tannin, reduces the faecal egg counts in lambs experimentally infected with *Trichostrongylus colubriformis*. Moreover, the administration of Quebracho Extract, a commercially available extract rich in condensed tannins, resulted in a 50% reduction in the number of eggs and adult worms recovered from the small intestine of sheep and in reduced fecundity of abomasal nematodes in goats (Athanasiadou et al. 2000a, b, Athanasiadou et al. 2001, Paolini et al. 2003).

Recently, Hoste et al. (2006) reported in their review that some of the most convincing evidence supporting a role for condensed tannins in affecting important nematode biological processes has been obtained from analytical *in vitro* assays comparing the activity of specific biochemical fractions obtained either from green tea (Molan et al. 2004) or sainfoin (Barrau et al. 2005). With both plants, the results confirmed that the antiparasitic activity, measured by the inhibition of larval migration (LMI), was associated mainly with the fractions containing condensed tannins. Moreover, when flavan-3-ols and their galloyl derivatives, which are the basic monomer units of condensed tannins, were added to a *T. colubriformis* egg culture, they significantly inhibited hatching. Similarly, the same compounds significantly affected larval development and third-stage larval migration in a dose-dependent manner (Molan et al. 2003).

Although the mechanisms by which CT inactivate the eggs are not known, CTs may be able to inactivate enzymes responsible for hatching process. The hatching of nematode eggs is initiated by environmental stimuli which lead to the release of so called 'hatching enzymes' (Sommerville and Rogers 1987), which include proteases, lipases, chitinases, beta-glycosidases and leucine aminopeptidases. Inhibition of some of these enzymes has been shown to reduce the rate of egg hatch or even stop the process completely (Rogers and Brooks 1977). Condensed tannins have been shown to inhibit endogenous enzyme activities (Oh and Hoff 1986, Horigome et al. 1988).

The CTs added to the media containing eggs and the other components of the medium (bacteria and yeast) could therefore be swallowed by the hatching larvae, and/or interact with the protein surface of eggs and L1 larvae and/or interact with the bacterial cells and yeast (Molan et al. 2002). If one or all of these hypotheses are correct, it is suggested that interaction of CTs with bacterial cells and yeast will result in formation of complexes which are either precipitated and not consumed by the larvae or swallowed by the larvae and cause damage to the mucosal lining of the gastrointestinal tract of the larvae. Other possible mechanisms are that swallowing of free or bound CTs may cause damage to the pharyngeal muscles and prevent the larvae from feeding because the larvae were allowed to develop in the presence of an abundant supply of bacteria and therefore did not have to forage for food. This may explain the inhibition of the development of L1 to L3 larvae and also the high percentage of death among the L1 larvae hatched from CT-treated eggs.

It has been reported that most anthelmintic drugs can cause paralysis of the pharynx in nematodes (Grzywacz 1983, Avery and Horvitz 1990, Gill et al. 1995) and prevent pharyngeal pumping, resulting in a closed oesophagus. Although it is difficult to compare between CTs and anthelmintic drugs, CTs may mimic this action of anthelmintics. These possibilities suggest that the CTs are potentially able to affect the larvae both externally and internally. Molan et al. (2000b) checked the viability of the trapped larvae (exposed to CTs extracted from seven forages) of *T. colubriformis* that could not pass through the sieves and found that 83–93% of the larvae were alive but their movements were sluggish, suggesting partial paralysis. If CTs can cause paralysis to the body musculature (Molan et al. 2000b), then they could also paralyse pharyngeal muscles. The unique character of CTs of being poorly absorbed from the small intestine (Clausen et al. 1990, Robbins et al. 1991, Terrill et al. 1994) may make the approach of the present study more practical and consequently the eggs shed by the adult worms will be exposed to the action of CTs throughout their development. Furthermore, the concentrations of CTs used in the present study (25–900 μg/ml) were much lower than concentrations of CTs in the gut and faeces (Waghorn and Molan 2001) of sheep after consumption of fresh CT-containing forages. It is important to mention here that care must be taken when extrapolating from *in vitro* to *in vivo* conditions because some changes occur in CTs during digestion (Terrill et al. 1994) and may reduce their impact upon larval development so it is important to evaluate the effects of CTs from different plants in parasitized ruminants on egg hatching and larval development.

In comparison with the results of our previous study (Molan et al. 2002), the eggs and L3 larvae of *T. colubriformis* seem to be more susceptible to the effects of CTs than those of *T. circumcincta*. This might be the result of differences in susceptibility among parasite species or stages or to the location of the nematode in the body. Similar differences have been observed in other *in vitro* assays comparing the consequences of the same tannin-rich extracts on a range of nematode species or stages (Paolini et al. 2004). The exposure of the eggs of *T. circumcincta* to acidic environment in the abomasums and then to alkaline environment may make the eggshell more rigid and consequently more resistant to the action of CTs. Although the CTs used in the present study were extracted from the same plants, the time of harvest and the chemical composition of CTs should be taken in consideration in this regard. Under *in vivo* conditions, however, Athanasiadou et al. (2000a, b) reported that when established adult

nematodes were exposed to condensed tannins in sheep, a reduction in worm fecundity and worm numbers was observed for the intestinal nematodes, whereas no changes were recorded for the abomasal nematodes. This may be related to the fact that the abomasal species are exposed to the negative effect of tannins for a shorter time than the intestinal nematodes as evidenced by the finding of Paolini et al. (2003), who found that when goats received condensed tannins before being infected with third-stage larvae, specific differences in activity have also been described, with reductions in worm numbers of 33%, 70%, and 66% for *Haemonchus contortus*, *T. circumcincta* and *T. colubriformis*, respectively, when compared with unsupplemented controls.

The differences in the activities of the CTs extracted from the different plant species may be due to the structural variations. Forage CTs are polymers of flavan-3-ol units with a considerable range of structural variation and their biological properties depend on their structure in terms of monomer units, their degree of polymerisation and interflavanoid linkages (Foo et al. 1996, 1997). In all assays, CTs from *Lotus corniculatus* showed the lowest activity in comparison with the CTs from other plants. Aerts et al. (1999) reported that at certain concentrations, CTs extracted from *L. pedunculatus* were more effective at reducing the degradation of the plant protein (Rubisco) than CTs from *L. corniculatus* when both plants were harvested and processed at the same time. In addition, the tannins from *L. pedunculatus* contain a predominance of prodelphinidin-type subunits (Foo et al. 1997); whereas, the tannins from *L. corniculatus* have predominantly procyanidin-type subunits (Foo et al. 1996). It has been shown that the reactivity of CTs increases with increasing prodelphinidin content (Jones et al. 1976).

In summary, the data obtained from these experiments suggest that CTs from certain plants can inhibit some key biological processes in *T. circumcincta*. CTs may be useful in blocking the nematode cycle of pasture contamination and larval development but these results need to be confirmed under *in vivo* conditions using the CTs from these plants as a drench.

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