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Effect of purified condensed tannins from pine bark on larval motility, egg hatching and larval development of *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Nematoda: Trichostrongylidae)

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Abstract: The effects of condensed tannins (CTs) extracted from pine bark on egg hatching, larval development and the viability of infective L3 larvae of *Trichostrongylus colubriformis* (Giles, 1892) and *Teladorsagia circumcincta* (Stadelmann, 1894) (syn. *Ostertagia circumcincta*) were evaluated using *in vitro* bioassays. Significant inhibitory effects of CTs were obtained on the viability of the infective larvae, egg hatching and larval development of both nematodes. In all bioassays, the larval stages of *Te. circumcincta* were significantly ($P < 0.05$) more susceptible to the inhibitory effects of CT than those of *Tr. colubriformis*. At 1 000 µg/ml, CTs from pine bark inhibited 48% and 69% of the infective larvae of *Tr. colubriformis* and *Te. circumcincta*, respectively, from passing through the sieve relative to the control incubations (no CT added; $P < 0.0001$). At the same concentration, CTs were able to inhibit 36% and 47% of the eggs of the two parasites, respectively, from hatching relative to the control incubations without CTs. Moreover, at 150 µg/ml, the CTs were able to inhibit 88% and 95% ($P < 0.0001$ relative to control incubation) of L1 larvae of the two nematodes, respectively, from attaining the full development to L3 larvae in comparison with the control incubations without CTs. At 200 µg/ml, CTs were able to inhibit completely the larval development in both nematodes. Addition of 2 µg polyethylene glycol (PEG; tannin inhibitor) per µg CT eliminated up to 87% of the CT activity ($P < 0.0001$) compared to incubations without PEG. In conclusion, this study shows that CTs are able to disrupt the life cycle of nematodes and their effects varied according to the parasite species and stage.

Keywords: condensed tannins, nematodes, anthelmintic activity, *in vitro*, bioassays

Gastrointestinal nematodes cause significant production losses in grazing ruminants and their control is achieved mainly by anthelmintics (Vlassof and McKenna 1994). The increased incidence of anthelmintic resistance in farmed sheep, goats and cattle in New Zealand and worldwide, rising consumer concerns about chemical use on farms combined with the finding that regular drenching cannot remove the effects of the parasite entirely (Jackson and Coop 2000, Leathwick et al. 2001), places limitations on the continued use of anthelmintics. Therefore, alternative parasite control strategies are needed.

Some studies have shown that condensed tannins (CTs)-containing forages have had significant effects on intestinal nematodes in sheep (Niezen et al. 1995), but they could not determine whether the effect was indirect or direct. Our previous research (Molan et al. 2000a,b) showed for the first time that CTs extracted from a wide range of forages have direct inhibitory effects against the larvae of sheep and deer gastrointestinal nematodes and lungworms *in vitro* and have the ability to slowdown the hatching process as well as to prevent the hatched larvae from attaining full development to infective larvae (Molan et al. 2002, Schreurs et al. 2002, Molan and Farag 2010). This may lead to disruption of the parasite life cycle and reduce or prevent pasture contamination with infective larvae.

Radiata pine, *Pinus radiata* Don (Coniferopsida: Pinaceae), is native to the coast of southern California, USA and Mexico (Millar 1999). Pine bark extract is rich in phenolic compounds such as catechin, epicatechin, quercetin, dihydroquercetin, taxifolin, phenolic acids and procyanidins. It has been used in traditional medicine in Europe and North America for long time for many diseases and illnesses (Packer et al. 1999). Kim et al. (2005) investigated the inhibitory effect of pine bark extract on carbohydrate-hydrolysing enzymes and the hypoglycaemic effect in diabetic mice. The authors found that pine bark extract showed inhibitory effect against salivary α-amylase and yeast α-glucosidase. Recently, Molan et al. (2009) investigated the effect of purified CTs extracted from pine bark on the sporulation of oocysts of three eimerian species and reported that water-soluble CT extracts have anticoccidial activity as evidenced by their ability to decrease significantly the sporulation of the oocysts of *Eimeria tenella* (Railliet et Lucet, 1891), *E. maxima* (Tyzzer, 1929) and *E. acervulina* (Tyzzer, 1929), under laboratory conditions.

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The CT extracts (especially when obtained from inexpensive sources such as pine bark) may provide another route for reducing parasitism compared to feeding CT-containing forages, which require specialist establishment and management and have a seasonal production (Molan et al. 2009, Min et al. 2012). By applying the phenolic and tannin components in waste pine bark to the animal health sector, the research will in the first instance create the conditions for a new bark processing industry, which will add value to, and also minimise the polluting aspects of, waste products.

The CTs from pine bark has been targeted in this work because the bark is cheap and abundant. The use of waste pine bark in animal health sector represents an ideal combination of using a problem waste from the forestry industry to enhance the production of the farming sector in a significant way. Tannins may affect ecosystem processes by a number of mechanisms including complexation with proteins or metals, inactivation of enzymes, inhibition of microorganisms and plants (Krause et al. 2003). Accordingly, removal of CTs from pine bark would be very desirable for the ecosystem.

In order to investigate whether the CTs from pine bark have direct effects on the nematodes of sheep, a series of *in vitro* experiments have been conducted to test the effect of CTs on the viability of the eggs and larvae of *Trichostrongylus colubriformis* (Giles, 1892) and *Teladorsagia circumcincta* (Stadelmann, 1894) using *in vitro* bioassays (larval migration inhibition – LMI, egg hatching – EH, and larval development – LD).

MATERIALS AND METHODS

Preparation of purified condensed tannins

Pinus radiata bark was obtained from 7–10 year old pine trees grown in Manawatu area, New Zealand. The bark was fed through a wood chipper two times and then ground in a Wiley™ mill through a 2 mm mesh. The ground bark was further reduced in size in a Cyclotech (Foss Tecator™, Sydney, Australia) rotary grinder to yield a fine powder. The finely ground bark was mixed with 70% acetone (1 : 4) containing 1 g/l ascorbic acid in a Warring Blender. Acetone extracts were reduced to the aqueous phase by evaporation under reduced pressure at 50 °C. The resulting aqueous phase was mixed with methylene chloride in a separating flask and the upper layer containing the CT was collected. Traces of methylene chloride were removed from the remaining aqueous fraction by rotary evaporation. The resulting fraction was freeze-dried, redissolved in 1 : 1 methanol/H_2O (v/v) and then purified by using a column containing Sephadex LH-20 (Pharmacia, Uppsala, Sweden). The Sephadex LH-20 extracts were freeze-dried and stored at -20 °C until required. In all bioassays, the incubations were done with and without the addition of 2 µg polyethylene glycol (PEG; tannin inhibitor) per µg of CTs to see if PEG has the ability to inactivate the action of CTs.

Larval migration inhibition (LMI) bioassay

The larval migration inhibition (LMI) bioassay procedure was used to determine the inhibitory effect of purified CTs against *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* (see Molan et al. 2000a). Briefly, the larvae were exsheathed in sodium hypochlorite solution (0.025% available chlorine; Rabel et al. 1994), washed five times with tap water and concentrated to 1 500 larvae/ml water. The bioassay involved preparation of test solutions with different concentrations of purified CTs (0, 100, 200, 400, 800, 1 000, 1 500 and 2 000 µg/ml) and of L3 larvae which were combined and incubated in the wells of 48-well tissue culture plates (Costar, Cambridge, MA, USA). The plates were incubated for 2 h at 37 °C after which solutions were transferred to sieves (7 mm ID with 20 m mesh at one end) and left overnight (16–18 h) at room temperature to enable the active larvae to migrate through the sieves for counting. The 20 m mesh size was selected to ensure that active migration of the larvae through the sieve was determined. The cross-diameter of L3 larvae (25 m; Rabel et al. 1994) 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.

Egg hatch bioassay

The eggs were collected from the fresh faeces of lambs experimentally infected with *Te. circumcincta* and *Tr. colubriformis* as described previously (Molan and Farag 2010). This bioassay has been conducted as described previously (Molan et al. 2002). Briefly, a stock solution of CT was prepared by dissolving CT in distilled water. Working solutions of CT were prepared by further dilutions with distilled water. Twenty ml of the working solutions were pipetted into each well of 48 well tissue culture plates (Costar) together with about 100 eggs and made up to 2 ml with distilled water to give CTs final concentrations ranging from 100 µg to 2 000 µg/ml. Assays were conducted in quadruplicate. Eggs in distilled water alone were used as controls. The eggs were incubated at 24 °C for 26 h. At the end of incubation a drop of Lugol's iodine was added (to stop further hatching) and the numbers of unhatched eggs and L1 larvae were counted to determine the proportion of eggs hatched (number of L1 larvae/number of eggs in culture × 100).

Larval development bioassay

The assays were carried out in 96-well micro-titre plates (Molan et al. 2004). The assay involved mixing 40 ml of growth medium with 60 ml of larval suspension (containing approximately 100 L1 larvae) and a series of CT concentrations. Duplicate assays were run for each concentration of CTs (0, 25, 50, 100, 150 and 200 µg/ml) and four 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 (Taylor 1990). At the end of incubation period, a drop of Lugol's iodine was added to each well to stop further development and the numbers of L1, L2, and L3 larvae were counted and the mean larval development was calculated (number of L3 larvae/number of L1 larvae in the medium × 100).

Growth medium

The medium used to culture used by Hubert and Kerboeuf (1984) and modified by Molan et al. (2002) was used to culture the parasite from the L1 to the third infective larval stage. The culture consisted of a suspension of *Escherichia coli* 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, Sydney, Australia) in 100 ml of distilled

water, and sterilising it by autoclaving. The nutritive medium was prepared by dissolving 1 g of yeast extract (Y-1000; Sigma) in 90 ml of 0.85% saline solution plus 10 ml of Earle's balanced salt solution (E75 10; 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).

Calculation of data and statistical analyses

The per cent inhibition of egg hatching (EH assay), per cent inhibition of larval development (LD assay) and per cent inhibition of migration (LMI assay) were calculated by using the following equation: % inhibition = (A - B)/A × 100, where A is the number of eggs hatched (EH assay), the number of hatched larvae that managed to develop into L3 larvae (LD assay), or the number of L3 larvae migrated through the sieves in the LMI assay in control incubations, and B is the number of eggs hatched, number of L3 larvae in LD assay, or the number of L3 migrated in incubations containing different concentrations of pine bark.

To provide another parameter of comparison between the two nematodes, we calculated the concentration at which 50% (IC_{50}) of the infective larvae failed to pass through the sieves of the LMI assay, the concentration at which 50% of eggs failed to hatch and the concentration at which 50% of the hatched larvae failed to achieve full development to L3 larvae. The IC_{50} was calculated by using probit analysis and Genstat programme.

Data are presented as the mean ± standard error of the mean (S.E.M.). A *t*-test or one-way ANOVA, followed by Tukey's test, were used to detect significance among groups and different concentrations. $P < 0.05$ was considered to be statistically significant.

RESULTS

Concentration of condensed tannins in the bark

The powdered extract of pine bark used in this study contained 35% CTs, of which 31% was free, 3.5% was protein-bound and 0.5% was fibre-bound (data not included).

Effect of CT on the motility of the infective L3 larvae

The present study shows for the first time that water-soluble extracts from pine bark containing 35% condensed tannins (Molan et al. 2009) have antiparasitic activity as evidenced by the results from the larval migration inhibition (LMI) assay (Fig. 1). The CT extracts showed inhibitory effect against the larvae of both sheep nematodes as evidenced by their ability to inhibit the passage of L3 larvae of these parasites through 20 m nylon mesh sieves relative to the CT-free control incubations. About 86% of the larvae in the negative controls (no added CTs) passed through the sieves. At 1 000 g/ml, CTs prevented 48% and 69% of *Trichostronylus colubriformis* and *Teladorsagia circumcincta* L3 larvae, respectively, from passing through the sieves in comparison with the control incubations with no CTs. Statistical analysis shows that the larvae of *Te. circumcincta* were more ($P < 0.05$) susceptible to the effect of CTs than the larvae of *Tr. colubriformis*.

Addition of 2 µg polyethylene glycol (PEG; tannin inhibitor) per µg CT eliminated 65–76% and 73–82% of the CT inhibitory activity ($P < 0.0001$) against the larvae of *Te. cirumcincta* and *Tr. colubriformis*, respectively, in comparison with incubations without PEG (Fig. 1).

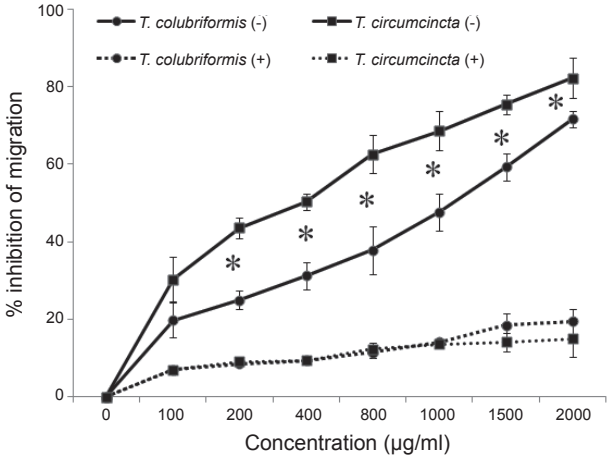

Fig. 1. The effect of condensed tannin (g CT/ml) extracted from pine bark on the motility of the infective third-stage (L3) larvae of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta in vitro*. The incubations were undertaken with (+) and without (-) addition of polyethelene glycol (PEG). Each point represents the mean ± S.E.M. of two independent experiments of duplicate incubations. Asterisks indicate statistically significant differences between the two nematodes (*P < 0.05–0.001).

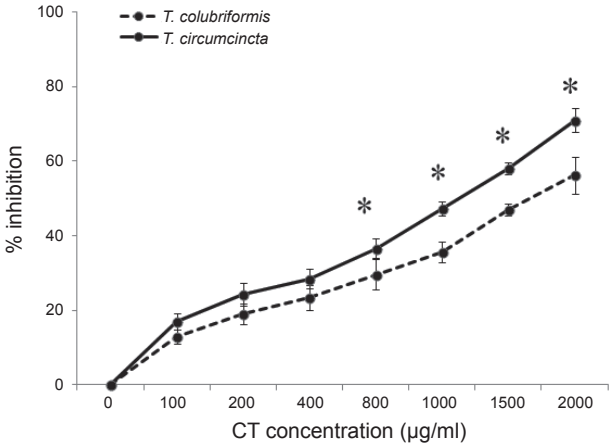

Fig. 2. The effect of condensed tannins (CT) extracted from pine bark on hatching of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* eggs *in vitro*. Each point represents the mean ± S.E.M. of two independent experiments of duplicate incubations. Asterisks indicate statistically significant differences between the two nematodes (*P < 0.05–0.001).

Effect of CT on the hatching of eggs

The results of the egg hatch assay are shown in Fig. 2. In control wells (without CTs) about 92% of the eggs hatched. It can be seen from Fig. 2 that the proportion of egg hatching decreases with increasing CT concentration in both nematodes. When the concentration was 200 µg/ml, the proportion of unhatched eggs was 19% in the incubations containing the eggs of *Tr. colubriformis* compared to 24%

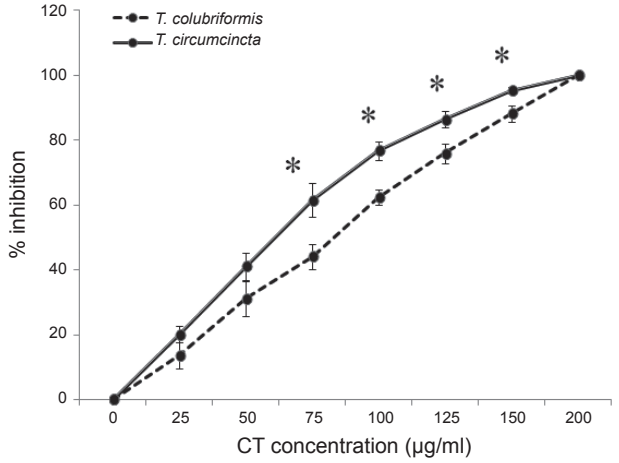

Fig. 3. The effect of pine bark condensed tannins on the development of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* first stage (L1) larvae into infective (L3) larvae. Each point represents the mean ± S.E.M. of two independent experiments of duplicate incubations. Asterisks indicate statistically significant differences between the two nematodes (*P < 0.05–0.001).

in the incubations containing the eggs of *Te. circumcincta*. At 1 000 µg/ml, bark CTs were able to prevent 36% and 47% of the eggs of *Tr. colubriformis* and *Te. circumcincta* from hatching, respectively, relative to the control incubations without CTs. Eggs of *Te. circumcincta* were more sensitive (P < 0.05) to the inhibitory effect of pine CTs than the eggs of *Tr. colubriformis*. Addition of 2 µg polyethylene glycol (PEG; tannin inhibitor) per µg CT eliminated up to 84% (data not shown) of the CT inhibitory activity (P < 0.0001) against the larvae of *Te. cirumcincta* and *Tr. colubriformis* compared to incubations without PEG.

Effect of CT on the development of first stage (L1) larvae to L3 larvae. In control incubations (without CTs), 88% of the L1 attained full development to L3 larvae. When the medium contained 100 µg/ml, CTs from pine bark prevented 62% and 77% of *Tr. colubriformis* and *Te. circumcincta* L1 larvae from attaining full development to L3 larvae, respectively in comparison with the control incubations with no CTs. At 200 µg/ml, CTs completely inhibited the development of eggs to L3 larvae in both parasites (Fig. 3). Addition of 2 µg polyethylene glycol (PEG; tannin inhibitor) per µg CT eliminated up to 87% (data not shown) of the CT inhibitory activity (P < 0.0001) against the larvae of *Te. cirumcincta* and *Tr. colubriformis* compared to incubations without PEG.

From the larval migration inhibition (LMI), egg hatching (EH), and larval development (LD) assays data, the IC_{50} values were calculated (Table 1). The results show that the eggs, L1 and L3 larvae of *Te. circumcincta* are significantly more sensitive (P < 0.0001) to the inhibitory effects of pine bark CTs than their counterparts of *Tr. colubriformis* as evidenced by the significantly lower (P < 0.0001) IC_{50} values.

Table 1. Concentrations of condensed tannins (CTs) extracted from pine bark that inhibited the passage of 50% of L3 larvae through the sieves of the larval migration inhibition (LMI) assay, inhibited the hatching of 50% of eggs, and inhibited the development of 50% of hatching larvae to infective stage larvae under *in vitro* conditions (IC_{50}). In all bioassays, the IC_{50} values for *Trichostrongylus colubriformis* were significantly lower (P < 0.05–0.0001) than those for *Teladorsagia circumcincta.*

Parasite	IC_{50} value (µg/ml)*		
	LMI	Egg hatching	Larval development
Trichostrongylus colubriformis	312	390	69
Teladorsagia circumcincta	234	320	55

* the IC_{50} was estimated by plotting X-Y and fitting the data with a straight line (line regression) after the data of the X-axis have been logarithm-transformed. IC_{50} values then estimated using the fitted line equation.

DISCUSSION

The main aim of this study was to investigate the effects of CTs extracted from pine bark on the *in vitro* viability of infective (L3) larvae of the most important gastrointestinal sheep nematodes, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta*. In addition, the effects of the same CTs on the egg hatching and larval development of the two nematodes were also evaluated under *in vitro* conditions. The CT concentrations used in this study are of a physiological importance as they are within the range of CT concentrations in the abomasal and duodenal digesta of sheep fed CT-containing forages (Terrill et al. 1994).

The results presented here show that CTs extracted from pine bark have the capacity to inhibit the migration of infective L3 larvae of both *Tr. colubriformis* and *Te. circumcincta* under *in vitro* conditions. This inhibitory activity was attributed to the CTs based on the finding that the addition of polyethylene glycol (PEG) has eliminated up to 87% of the inhibitory effect of CTs against the larvae. Molan et al. (2000a) investigated the effects of purified CTs from seven forages on the motility of *Tr. colubriformis* by using the larval migration inhibition (LMI) assay and found that the CTs were able to inhibit up to 63% of the larvae from passing through the sieves. The authors suggested that these forages may be used as a means to reduce dependence upon proprietary anthelmintics. In comparison with the CTs extracted from the forages tested by Molan et al. (2000a), it seems that CTs extracted from pine bark are more effective in inhibiting the motility of *Tr. colubriformis* and this may be attributed to the difference in the molecular weight (MW) and chemical composition. It has been reported that CTs from different plant species have different MW and chemical composition of the CTs (Foo et al. 1997, Singh et al. 1997, De Bruyne et al. 1999). Moreover, it has been shown that the inhibitory activities of CTs might be influ-

enced by the prodelphinidin : procyanidin ratios (Molan et al. 2003) and the degree of polymerisation (Novobilský et al. 2013).

Although it is difficult to explain the reason, the results clearly showed that the L3 larvae of *Te. circumcincta* were more susceptible to the inhibitory action of pine bark CTs than those of *Tr. colubriformis*. Similarly, Paolini et al. (2004) tested the effect of CTs from three woody plants on parasitic nematodes *in vitro* and reported that *Te. circumcincta* and *Haemonchus contortus* (Cobb, 1898) were more susceptible to the inhibitory effects of plant extracts than *Tr. colubriformis*. Molan et al. (2004) investigated the effect of CTs extracted from sulla (*Hedysarum coronarium* Koenig) on the viability of three sheep nematodes (*Te. circumcincta*, *H. contortus* and *Tr. colubriformis*) using LMI assay and found that the larvae of *Tr. colubriformis* were more resistant to the inhibitory effect of CTs than the larvae of the other two nematodes.

It seems that the *in vitro* assays are useful in determining the effects of extracts and natural products on adult and larval stages of parasites as evidenced by the results of some studies that showed a positive relationship between *in vitro* and *in vivo* results. Molan et al. (2000a,b) and Molan and Farag (2010), showed that purified CTs extracted from various forages and medicinal plants reduced the motility of L3 larvae, minimised the rate of egg hatching, and the development of eggs and L1 larvae into infective L3 larvae under *in vitro* conditions. These results agree well with those observed in ruminants (Athanasiadou et al. 2000a,b, Paolini et al. 2003a,b).

Recently, Min et al. (2012) conducted an *in vivo* study to determine the effects of ground pine bark (PB) supplementation on animal performance, rumen fermentation, blood variables, faecal egg counts (FEC) and carcass traits in Kiko crossbred male goats and reported that supplementation of PB reduced FEC, improved performance and favourably modified rumen fermentation. Our *in vitro* results tend thus to support previous *in vivo* data obtained with pine bark by Min et al. (2012) and those by Paolini et al. (2003b) who tested the quebracho tannins on the two parasitic stages of *Te. circumcinta* and *Tr. colubriformis*.

The results clearly show that nematode larval development is more sensitive to the activity of pine bark CTs than egg hatching as evidenced by the significantly lower IC_{50} values in the larval development than that in the egg hatching. This may be related to the difference in the length of the exposure time to CTs in the two bioassays. In the egg hatching assay, eggs were exposed for only one day whereas the in the larval development assay the hatching larvae were exposed for 7 days. Moreover, the egg shell may protect the larvae inside the egg from the harmful effect of CTs in the egg hatching assay whereas the hatched larvae were in direct contact with the CTs in the larval development assay. This trend of activity supports the results of our previous *in vitro* studies on CTs extracted from different forages (Molan et al. 2002, 2003, Molan and Farag 2010).

The inhibitory effects of pine bark were attributed to the CT contents. The use of polyethylene glycol (PEG) to eliminate effects of CTs was demonstrated by Molan et al. (2004) who showed preferential binding between PEG and CTs relative to protein and CTs under *in vitro* conditions. Moreover, addition of 2 µg PEG per µg CTs to the incubations eliminated up to 93% of the inhibitory effect of CTs on larval viability through inactivation of CTs (Molan et al. 2000a, 2004). The authors concluded that the elimination of the inhibitory effect of CTs against the larvae in the LMI assay confirms that CTs is the source of the inhibitory action.

Although the mode of action by which CTs affects the eggs and larvae of the nematodes is not precisely known, the failure of the CT-exposed larvae to pass through the sieves may indicate sort of paralysis and interference with neurophysiology or neuromuscular coordination of the larvae (Molan et al. 2000a, 2004). The ability of CTs to bind to proteins and to change their physical and chemical properties (Aerts et al. 1999, Min et al. 2003, Molan et al. 2004) may be another mechanism, due to the fact that nematode cuticle is a proline- and hydroxyproline-rich structure (Thompson and Geary 1995). In support of this hypothesis, Hoste et al. (2006) reported that incubation of adult *Tr. colubriformis* with chestnut extracts has led to a disruption of the cuticle as shown by scanning electron microscopy after contact with CTs in comparison with the adult worms incubated in phosphate-buffered solution (control).

The efficacy of the low concentrations of CTs used in this study suggests that CTs may offer a potential route for minimising development of eggs into infective larvae under farming conditions. In addition, this study showed clearly that the effects of pine bark CTs varied according to the parasite species and stage.

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