IN VITRO EFFECT OF CONDENSED TANNIN EXTRACT FROM ACACIA (*Acacia mearnsii*) ON GASTROINTESTINAL NEMATODES OF SHEEP

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ABSTRACT:- MINHO, A.P.; BUENO, I.C.S.; GENNARI, S.M.; JACKSON, F.; ABDALLA, A.L. ***In vitro* effect of condensed tannin extract from acacia (*Acacia mearnsii*) on gastrointestinal nematodes of sheep.** [Efeito *in vitro* do extrato de tanino condensado de acácia (*Acácia mearnsii*) em nematóides gastrintestinais de ovinos]. *Revista Brasileira de Parasitologia Veterinária*, v. 17, supl. 1, p.144-148, 2008. Área de Sanidade Animal, Instituto Agronômico do Paraná, Rod. Celso Garcia Cid, km 375, Cx. Postal 481, Londrina, PR 86001-970, Brazil. E-mail: apminho@iapar.br

The aim of this study was to determine the inhibitory effects of condensed tannin extract from acacia on the feeding of first-stage larvae (L1) of *Haemonchus contortus, Trichostrongylus vitrinus* and *Teladorsagia circumcincta.* The experiment was developed such that the inhibition of feeding for each of the nematode species could be evaluated. L1 recovered from fecal samples from a donor with monospecific infection was incubated in several dilutions of acacia extract (AE). The LD_{50} was determined for the three species of nematodes. Polyethylene glycol (PEG) was added to all dilutions of AE to inactivate the condensed tannins (CT) from acacia and to confirm their effects on L1. The impact of CT on larval feeding inhibition was detected for all the species of nematodes (*H. contortus, T. colubriformis* and *T. circumcincta*). There were differences between the aqueouswater control and CT treated groups ($P < 0.01$). The LD_{50} values were 0.043, 0.038 and 0.050 (SE = 0.0024), for *H. contortus, T. vitrinus* and *T. circumcincta*, respectively. A difference was detected between the AE and AE + PEG treatments ($P < 0.01$). Analysis of these results suggested that the direct effect of CT on L1 of the nematodes studied could be used as an alternative means for controlling nematodes in sheep.

KEY WORDS: Condensed tannin extract; Anthelmintic effect; Nematodes; *In vitro*.

RESUMO

O objetivo desse trabalho foi determinar os efeitos inibitórios dos taninos condensados (CT), provenientes do extrato de acácia, na alimentação de larvas do primeiro estádio (L1) de *Haemonchus contortus, Trichostrongylus vitrinus* e *Teladorsagia circumcincta.* Para isso foi utilizado o método *in vitro* de inibição da ingestão de bactérias por L1 dos respectivos nematóides. Larvas recuperadas de fezes de ovinos doadores com infecção monoespecífica foram incubadas em diluições seriadas do extrato de acácia (AE). A DL50 foi determinada para as três espécies de nematóides. Polietilenoglicol (PEG) foi adicionado a todas as diluições de AE para inativar os taninos condensados (CT) da acácia e para confirmar seus efeitos sobre as L1 O impacto dos CT na inibição da ingestão das larvas (LFI) foi detectado sobre todas as espécies de nematóides utilizados (H. contortus, T. colubriformis e T. circumcincta). Houve diferença entre o controle aquoso e os grupos tratados com CT (P < 0,01). Os valores de DL50 foram de 0,043; 0,038 e 0,050 (EP = 0,0024) para H. contortus, T. vitrinus e T. circumcincta respectivamente. Foi detectada diferença entre os tratamentos AE e AE + PEG (P < 0,01). A análise dos resultados sugere que o efeito direto dos CT sobre as L1 dos nematóides estudados pode ser promissor no controle alternativo de nematóides em ovinos.

PALAVRAS-CHAVE: Extrado de tanino condensado, efeito anti-helmíntico, nematóides; *in vitro*.

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INTRODUCTION

Recent surveys have identified anthelmintic effects from many bioactive substances (GITHIORI et al., 2006), particularly from condensed tannin (CT) sources (NIEZEN et al., 2002; HORDEGEN et al., 2003; ATHANASAIDOU et al., 2005; MARLEY et al., 2003). Some *in vitro* methods can be used to investigate the efficacy of potential anthelmintic substances towards nematode parasites: assays on larval development (LD), larval migration inhibition (LMI), larval feeding inhibition (LFI) and egg hatching (EH) (COLES et al., 1988; AMARANTE et al., 1997). Athanasiadou et al. (2001a) reported that LMI and LFI are the best assays for testing the anthelmintic effect of bioactive substances *in vitro*.

The role of tanniniferous forage or tannins extracts in parasite control has been studied around the world. Tannins are part of a group of polyphenol substances that contain factors affecting food taste and protein availability. They are classified according to their chemical structure, as phlorotannins, hydrolysable tannins or condensed tannins (BARRY; MCNABB, 1999). Quebracho extract (QE) has been found to be effective against *Haemonchus contortus* and *Trichostrongylus colubriformis* infections in goats (PAOLINI et al., 2003a,b, 2005). Athanasiadou et al. (2000) and Butter et al. (2000) suggested that QE from the bark of *Schinopsis* spp. had direct anthelmintic action on *T. colubriformis* in sheep.

Moreover, Minho et al. (2008) demonstrated that acacia extract (AE) from the bark of *Acacia molissima* had a direct anthelmintic effect in sheep that were naturally infected with *H. contortus*. AE is a fine powder containing approximately 15% CT that is water-soluble. In that experiment, sheep were drenched with it at a concentration of 1.6 g/kg LW, diluted in warm water. The animals received approximately 3.6 and 5.0 g of CT per day for the first and second treatment, respectively. Acacia has been chosen as a major source of CT for many *in vivo* and *in vitro* assays because the *Schinopsis brasilienses* tree (the source of QE) is an endangered species in Brazil.

The use of AE for *in vivo* and i*n vitro* studies can eliminate a considerable problem: the variation in CT levels in tanniniferous plants. Athanasiadou and Kyriazakis (2004) reported that the concentration of these bioactive metabolites could vary between different regions, seasons of the year and even within the same grazing period. Moreover, CT analysis is complicated by the diversity of structures found within this group of compounds (polymers of flavonol units).

Polyethylene glycol (PEG) is used worldwide in studies on tannins because of its tannin binding capability (MAKKAR, 2003). Through this, it inactivates CT and its effects on *in vitro* assays. Therefore, PEG tests can estimate the direct effect from CT and rule out the possibility of deleterious effect caused by other toxic compounds in AE.

The main objective of this study was to determine the potential inhibitory effect of CT derived from AE, on first-stage larvae (L1) of *H. contortus*, *Teladorsagia circumcincta* and *T. vitrinus*.

MATERIALS AND METHODS

Condensed tannin source

The CT extract source used was acacia extract[1] (A*cacia mearnsii*; synonym: *A. molissima*). This extract contains 15% CT when analyzed by means of the HCl-butanol method (PORTER et al., 1986).

First-stage larvae (L1)

Eggs were obtained from feces collected from donor lambs carrying monospecific infection of Moredun[2] ovine anthelmintic-susceptible isolates of *H. contortus, T. circumcincta* or *T. vitrinus*. The eggs were incubated at 27ºC for 24h to recover the L1.

Larval feeding inhibition (LFI)

Stock solution (10 mg/ml) was produced by dissolving the AE in distilled water. Seven serial dilutions of tannin extract (1.25 mg/ml to 0.02 mg/ml) were made, deriving from this. The L1 were incubated in duplicate for 2 h, at room temperature (approximately 22°C), in several dilutions of AE plus a distilled water control. After this incubation, the L1 were exposed to fluorescein isothiocyanate (FITC)-labeled *Escherichia coli*[3].

After another incubation lasting 18 h (at 22 °C), the quantity of viable L1 was counted using an epifluorescent microscope. Presence of fluorescence in the gut of the L1 was used as the criterion for larva viability.

To confirm the relationship between CT activity and L1 viability, each assay was repeated in the presence of PEG. The aim in using PEG was to inactivate the effect of CT due to the AE. By dissolving 1 g of PEG in 25 ml of distilled water, a stock solution was produced. Ten microliters of PEG solution were added to each Eppendorf tube before L1 incubation.

Experimental design and statistical analyses

A complete 3x8x2 factorial design (three nematode species, seven AE dilutions and two treatments – with and without PEG) was used to develop the assays. The percentage of viable L1 was determined for each AE dilution. The respective 50% lethal dose (LD_{50}) for each nematode species was calculated by logarithmic transformation of the dilutions. A 50% reduction in the number of viable larvae, compared with the distilled water control, was considered to represent the LD_{50}**.** Variance analysis between the mean L1 viability findings at different AE dilutions (with and without PEG) was performed using the Tukey test ($P < 0.01$).

RESULTS

Acacia extract inhibited larval viability (Table 1), and a dose-dependent inhibitory response was observed. Significant

[5] Commercially available condensed tannin extract from acacia (Natur N®, Seta SA, Brazil).

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[7] *E. coli* 026: B6 LPS (FITC-LPS, Sigma).

Table 1. Mean percentages (from duplicates) of first-stage larvae of *Haemonchus contortus*, *Trichostrongylus vitrinus* and *Teladorsagia circumcincta* that, in presence of acacia extract (AE), fed on FITC-labeled bacteria. Results are shown with and without the presence of polyethylene glycol (PEG).

Concentrations	*H. contortus*		*T. vitrinus*		*T. cirxumcincta*	
mg/ml	AE	AE+PEG	AE	AE+PEG	AE	AE+PEG
Controll**	73[a] (71;75)*	75[a] (71;79)*	75[a] (75;75)*	75[a] (73;77)*	74[a] (73;75)*	75[a] (72;78)*
1.25	0[d]	0[d]	0[c]	0[e]	0[d]	6[d]
0.625	0[d]	3[d]	0[c]	0[e]	0[d]	20[d]
0.31	0[d]	6[d]	0[c]	3[e]	0[d]	38[c]
0.155	0[d]	8[d]	0[c]	20[d]	2[d]	44[b]
0.08	11[c]	38[c]	4[c]	51	10[c]	71[a]
0.04	42[b]	60[b]	26[b]	65[b]	40[b]	71[a]
0.02	71[a]	71[a]	65[a]	74[a]	71[a]	72[a]

* Values (minimum; maximum).
**Control: Distilled water + FITC-labeled bacteria.
Different letters in the same column indicate significant difference ($P < 0.01$).
FITC: fluorescein isothiocyanate.

differences were observed between the control (distilled water) and the AE treatments ($P < 0.01$), with LD_{50} values of 0.043, 0.038 and 0.050 mg/ml (SE: 0.0024), for *H. contortus*, *T. vitrinus* and *T. circumcincta*, respectively. There was also a significant difference between the AE with PEG and AE without PEG treatments ($P < 0.01$), such that the LD_{50} in the presence of PEG was modified to 0.096, 0.100 and 0.250 mg/ml, respectively for *H. contortus*, *T. vitrinus* and *T. circumcincta*.

The LFI results were consistent. The distilled water and distilled water + PEG controls did not produce sufficient larval viability to produce at least 70% of the L1 that were capable of feeding on bacteria. The results were shown to be repeatable in all replicates.

DISCUSSION

In the present study, the results show that AE had an anthelmintic effect on the nematodes studied (*H. contortus*, *T. vitrinus* and *T. circumcincta*). While first-stage larvae of nematodes need to feed on bacteria or other microorganisms in the environment in order to remain alive, the same is true for second-stage larvae (URQUART et al., 1996). Evaluation of L1 food can show whether these larvae will remain alive over the near future, thereby estimating their development as far as third-stage larvae.

From in vivo assays, Minho et al. (2008) reported that drenching animals with CT was associated with significant reductions in FEC ($P < 0.01$) and the worm burden in the abomasum ($P < 0.01$), but not in the small intestine ($P > 0.05$). From the same work, it was found that animals weighing about 30 kg normally received about 48 g of AE per day, equivalent to 7.2 g of CT. Most of the CT are released together with excrement, reaching a concentration of about 6 mg of AE/g of feces. This is more than is needed to improve the harmful effect of L1 larvae on the environment. The significant deleterious effect of AE, reported during *in vitro* assays on larval migration inhibition in relation to *T. circumcincta* (MOLAN et al., 2004) and on egg hatching inhibition in relation to *H. contortus* (IQBAL et al., 2007), may support this hypothesis.

Analysis of the results following the addition of PEG suggests that it acts to increase the viability of L1, as shown by the number of viable larvae in the AE + PEG group. Since the function of PEG is to inactivate the tannins, this shows that CT has an effect on L1 of the nematode species studied. Likewise, when PEG was added during the LFI assay, thereby inactivating the CT, the LD_{50} increased considerably. Butter et al. (2000) and Athanasiadou et al. (2000) suggested that CT from quebracho extract (from the bark of *Schinopsis* spp.) had a direct anthelmintic action on *T. colubriformis* in sheep. Cenci et al. (2005) studied sheep that were drenched with AE (3.2g CT/animal once week for 13 weeks) reported that CT had direct action on the parasites studied (*H. contortus*, *T. colubriformis* and *Cooperia* spp.). The direct action of CT in most *in vitro* studies can be defined as strong and dose-related, with efficacy levels approaching 100% (KETZIS et al., 2006).

The direct anthelmintic effect of AE on L1 of the nematodes studied reaffirms the potential use of this CT source as an alternative means for endoparasitosis control in sheep flocks. However, its practical use depends on studies on its deleterious effect on ovine. Hervás et al. (2003) analyzed the side effects of quebracho on sheep and found that doses of 1.5 g quebracho/kg LW did not cause macro or microscopic lesions in the intestines, liver or kidneys of the animals used in the experiments.

Both the direct and indirect effects of CT are beneficial in lowering the contamination of pasture, through reducing the hatchability of nematode eggs and the fecal egg count (IQBAL et al., 2006). It is possible that alternative controls such as the use of AE cannot resolve the worldwide problem regarding the development of anthelmintic-resistant worm populations. However, such controls can reduce the selection pressure on nematode populations, thereby reducing the indiscriminate use of anthelmintic drugs.

Others experiments are needed in order to determine the CT concentration in the digestive tract of sheep drenched with AE. Based on this concentration, *in vivo* assays could be developing to determine the real effect of these compounds on gastrointestinal nematodes. In particular, such tests could determine the minimum effective dose of CT compounds that can be used on sheep flocks, based on the LD_{50} described in this study.

CONCLUSIONS

It is concluded that doses of AE greater than or equal to 0.155 mg/ml presented a deleterious effect on first-stage larvae of *Haemonchus contortus*, *Trichostrongylus vitrinus* and *Teladorsagia circumcincta*, such that approximately 100% of the L1 were unviable. A dose of 0.08 mg/ml was able to

inactivate approximately 90% of L1. Therefore, this dose has potential anthelmintic use in alternative parasite controls.

It was implied that most of the direct effect of AE on L1 coming from CT was seen when the presence of PEG ensured viable larvae at concentrations of two, four or eight times higher than 0.155 mg/ml, for *T. vitrinus*, *H. contortus* and *T. circumcincta*, respectively.

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