



Research paper

Anthelmintic effect and tissue alterations induced *in vitro* by hydrolysable tannins on the adult stage of the gastrointestinal nematode *Haemonchus contortus*



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ABSTRACT

Haemonchus contortus constitutes a severe problem for ruminant's production, it is the most frequent nematode parasite both in temperate and tropical regions, induces economical losses, and shows increasing resistance to currently available anthelmintics. Tannins are secondary metabolites that naturally fulfill defense functions in plants, representing a non-conventional, natural alternative in the treatment of gastrointestinal parasites in sheep. The objective of this work was to explore the *in vitro* anthelmintic activity of hydrolysable tannins on adult stage of *Haemonchus contortus*. Adults nematodes were obtained directly from the abomasum of ovines, and a dose response curve was performed with tannins extract at 0, 2, 4, 8, 25 and 50 mg/mL, and a time response curve at 0.5, 1, 2 and 24 h at 37 °C. Hydrolysable tannins decreased motility and induced mortality of *H. contortus*. We observed cuticle disruption around the mouth and reproductive organs, as well as evisceration. After 24 h of exposition, 8 mg/mL induced 83% of mortality and with 25 mg/kg 100% of mortality was achieved. The LD₅₀ was 3.54 mg/mL, while LD₉₀ was 10 mg/mL. We propose hydrolysable tannins as an alternative to contribute in the nematode control in ruminants.

1. Introduction

Gastrointestinal nematodes represent a major health problem worldwide in grazing sheep, goat and cattle production systems and control of these parasites has been complicated by the emergence of resistant nematodes to the commercially available anthelmintics. The profitability of livestock activities can be diminished significantly by the effects of parasites; nematode parasites have such an impact on herd health that they can potentially make production less profitable or even unprofitable (Charlier et al., 2014).

Haemonchus contortus (*H. contortus*) is the nematode parasite most frequently found both, in temperate and tropical regions, influencing

both animal health and production, and consequently, inducing important economic losses (Waller and Chandrawathani, 2005; Domke et al., 2013; de Cezaro et al., 2016). This parasite is located in the abomasum, and its hematophagous activity causes hemorrhages on the mucosa of the abomasum, gastritis, anemia and associated complications, leading to death in severely affected animals. The resistance of *H. contortus* to anthelmintics becomes a worldwide growing phenomena (Emery et al., 2016); thus, finding and developing alternative substances that could contribute to this nematode control becomes important.

Tannins are secondary metabolites that naturally fulfill defense functions in plants, and conventionally they have been grouped into

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hydrolysable tannins (HT) and condensed tannins (CT), or proanthocyanidins (Hoste et al., 2012). Although tannin-rich forages are known to increase protein uptake and to reduce gastrointestinal nematode infections in grazing ruminants, most published research involves forages with condensed tannins (CT), while published literature lacks information on the anthelmintic capacity, nutritional benefits, and antioxidant capacity of alternative forages containing hydrolysable tannins (HT) (Katiki et al., 2013). For hydrolysable tannins, among the activities determined on a molecular basis are the chemical, biological and pharmacological actions such as superoxide anion scavenging, apoptosis, antitumor, anti-EVB, anti-MRSA and anti-plasmin inhibitory activities, etc., in addition to their fundamental activities, i.e., binding to proteins, large molecular compounds and metallic ions, and antioxidant activities (Okuda and Ito, 2011). Tannins have been used as an additive that have the function of modulating the fermentation in the rumen, mainly improving the rumen by-pass of essential amino acids, which also results in a better productive performance of the animals (Mezzomo et al., 2011; Rivera-Méndez et al., 2016). Furthermore, the use of tannin rich plants represents a non-conventional, natural alternative in the treatment of gastrointestinal parasites in sheep, and tannin rich plants have been the most largely studied group of bioactive plants in veterinary medicine (Hoste et al., 2012, 2016). They have anthelmintic effect dependent on the genus of the plant, the parasite species, and the parasite stage (Paolini et al., 2003a,b, 2005), and, although the effect of tannins in the control of intestinal nematodes has been evaluated *in vitro* and *in vivo* in small ruminants, only CT have been explored (Hoste et al., 2006; Athanasiadou et al., 2007). HT have broad biological and pharmacological activity (Okuda and Ito, 2005) and as biological agents are even more potent than CT thus, the perception can be that at the doses referred for the use of CT, they could be toxic (Haslam, 1974 cited by Martínez-Ortíz-de-Montellano et al., 2010). However, using low doses in amount not enough to induce toxicity in animals, HT have shown ability to improve the absorption of nutrients and the productive performance of the animals (Frutos et al., 2004) and it has been suggested that they have an *in vivo* anti-parasitic effect (Corona-Palazuelos et al., 2016). Regarding to the potency of different groups of tannins, it has been also reported that at the doses of 20 and 25 mg/mL, HT-rich, or both CT- and HT-rich, extracts were significantly more lethal to adult *C. elegans* than extracts containing only CT. HT and CT are high in antioxidant capacity, with ORAC values ranging from 1800 mol to 4651 mol of trolox equivalents/g (Katiki et al., 2013).

The anti-parasitic effect of HT has been poorly studied, despite the economic potential it could have, since, probably being more potent than condensed tannins, it is postulated that low doses would be required, which would also induce favorable gastrointestinal effects for animals. In this study, we focused on the *in vitro* evaluation of the direct anti-helminthic activity of HT on adult stage of *H. contortus*.

2. Material and methods

2.1. Ethic statement

This work does not involve the direct use of animals by any of the authors.

2.2. Parasites

The sheep used in this study were humanely slaughtered in the municipal slaughterhouse of Costa Rica, Culiacán, Mexico, to obtain meat. The authors did not participate in the sacrifice, we only intervened in the collection of the parasites. Immediately after sacrifice, the abomasum of sheep was longitudinally sectioned, its content was collected and sieved, retaining the adult stage of *Haemonchus contortus*. Also, the parasites were collected from the wall of the abomasal mucosa. The parasites were placed in saline solution and kept at 4 °C during transportation to the laboratory and during storage. All the tests

were performed within 15 days after the recovery of the parasites.

2.3. Hydrolysable tannins extract (HTE)

The hydrolysable tannins from chestnut tree (*Castanea sativa*) were obtained from NutriP® (SilvaTeam; San Michele Mondovi, Italy). Stock solution was prepared at 100 g/L in tap water and filtered with Whatman® 1.

The HTE doses were calculated from results of the second *in vivo* experiment described by Corona-Palazuelos et al. (2016), where they used bull-calves which mean bodyweight (BW) along the experiment was 231.6kg, fed a diet containing 1.358 Mcal NE_m/kg DM. From these data, its daily Dry Matter Intake (DMI) was calculate in 5.59kg according with a prediction formula, presupposed by the NASEM (2016): NE_m Intake = SBW^{0.75} * (0.2435 * NE_m - 0.0466 * NE_m² - 0.1128); where NE_m Intake is the total daily NE_m intake Mcal, SBW^{0.75} is SBW = BW*0.96, and NE_m is the NE_m content of the diet in Mcal/kg DM.

2.4. Bioassay

Solutions containing ascendant concentration of HTE (00, 2, 4, 8, 25 and 50 mg/L) were placed in Petri dishes (50 mm); 15–20 adults of *H. contortus* were placed in each one and incubated at 37 °C, 5% CO₂ in a CO₂, during 0.5, 1, 2 or 24 h. All the procedure was performed by triplicate, and repeated on three different days. Parasites were observed using a stereoscopic microscope and counted as alive when movement was evident after light stimulation, or death when mobility was not perceptible even after exposition to the brightly light of the microscope for 5 s (Martínez-Ortíz-de-Montellano et al., 2013).

2.5. Scanning electronic microscopy (SEM)

Parasites exposed to different concentrations of HTE during distinct times, were fixed using 4% formalin during 24 h, washed twice during 10 min with distilled water, and twice with saline solution. Progressive dehydration with graded ethanol solutions for 1 h was performed, with three subsequent washes (1 h each) using 100% ethanol. A dried critical point process was performed utilizing extradry – CO₂. Dehydrated parasites were assembled on carbon adhesive aluminum slides, and covered with a 20 mA gold layer during 2 min. Immediately, samples were observed with a S450 scanning electronic microscopy (Hitachi) at 10–15 kw (Perez-Ponce de León et al., 2016)).

2.6. Statistical analyses

Data of alive or death nematodes were used to calculate mortality as percentage; each Petri dish constituted the experimental unit. Mortality data was analyzed by ANOVA for a completely randomized experimental design with a 6 × 4 factorial arrangement of treatments (six TE levels and four incubation times). An alpha level P ≤ 0.01 fitted to accept statistical difference and when it happened, the separation of means was performed using the Tukey test. All calculation was accomplished with the version 9 of the Statistix software (Tallahassee, 2007). The mean lethal doses (LD₅₀) and LD₉₀ were obtained through interpolation with the logarithmic values of the mortality curve at 24 h.

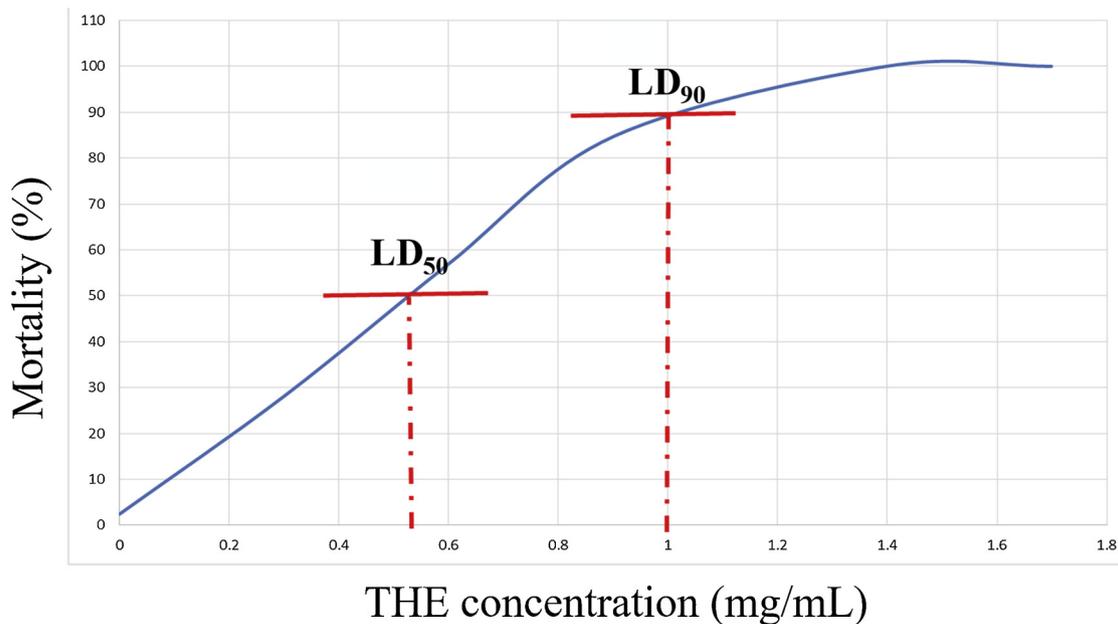
3. Results

3.1. Mortality is induced at low dose of HTE and early exposure time

Tannins had an effect dependent on the concentration of the extract and its time of exposure. At dose of 8 mg/mL, we observed a diminution in the mobility of the parasite as early as 2 h of exposition (not shown). In the control group, mortality of 2.38% was observed only at 24 h (Table 1); while with HTE, damage was observed at a dose of 4 mg/ml and 24 h of exposure, with a mortality of 54.8%, yielding a mortality of

Table 1Influence of time and dose of *in vitro* treatment with hydrolysable tannins extract on the percentage of mortality of *H. contortus* (mean \pm SE).

Time	Hydrolysable tannins dose					
	0 g/L	2 g/L	4 g/L	8 g/L	25 g/L	50 g/L
0.5 hour	0 ^{CA}	6.06 \pm 3.2 ^{CA}	0 ^{CA}	3.03 \pm 1.6 ^{CA}	3.03 \pm 2.0 ^{CA}	0 ^{CA}
1 hour	0 ^{CA}	6.06 \pm 3.2 ^{CA}	0 ^{CA}	3.03 \pm 1.6 ^{CA}	14.64 \pm 3.0 ^{CA}	8.11 \pm 8.1 ^{CA}
2 hours	0 ^{CA}	6.06 \pm 3.2 ^{CA}	0 ^{CA}	23.2 \pm 2.0 ^{CA}	28.5 \pm 3.2 ^{CA}	13.6 \pm 8.2 ^{CA}
24 hours	2.38 \pm 1.3 ^{CA}	33.3b \pm 19.2 ^{CA}	54.8 \pm 16.9 ^{abCB}	83.3 \pm 9.6 ^{abB}	100 ^{ab}	100 ^{ab}

a, b, c Distinct lowercase letters in a same row indicates statistical difference, $P < 0.01$.A, B Distinct capital letters in a same column indicates statistical difference, $P < 0.01$.**Fig. 1.** Lethal doses (LD₅₀, LD₉₀) of HTE. Interpolation of LD₅₀ (3.54 mg/mL) and LD₉₀ (10 mg) according to mortality induced by THE on *H. contortus* at 24 h after treatment.

83.3% at a dose of 8 mg/ml regarding to control ($P \leq 0.01$). A dose of 25 mg/mL was enough to induce 100% of mortality of *H. contortus* at 24 h of incubation. According to mortality at 24 h, the LD₅₀ of HTE was 3.54 mg/mL, while LD₉₀ was 10 mg of HTE/mL (Fig. 1).

3.2. Normal structure of *H. contortus*

As shown in Fig. 2, with the control treatment no changes were observed in the parasite morphology; in the anterior region, the oral cavity can be observed without alterations, the cuticular lines are continuous and a cervical papilla without damage is observed (Fig. 2A); the natural folds and the intact cuticle are observed in the middle region (Fig. 2B); in the caudal region of a female, the vulvar fold is shown without alteration (Fig. 2C), and the bursa copulatrix without alteration in a male is observed (Fig. 2D).

3.3. Damage at low dose and early exposition time

At 24 h, we observed damage mainly in the middle region of the parasite, although there is still the presence of lines along the cuticle without evident modification of the structure, incision was observed in the cuticle until its rupture with the expulsion of the internal organs (Fig. 3A). The caudal region also underwent changes in the vulvar region of the female, where the cuticle is no longer observed, exposing the muscle of the parasite and allowing the release of the uterus and eggs (Fig. 3B). At 24 h, the parasites suffered severe damage, the structure of the cuticle was lost, and the lines along the body and the circular rings

that form the cuticle are no longer observed. There was a disruption with the consequent release of internal material (Fig. 3C).

3.4. Mortality and complete destruction of the parasite structure

A massive structure destruction is shown in Fig. 4, where loss of cuticle continuity, destruction of the cuticle and release of body contents was observed (Fig. 4A); Fig. 4B shows a process similar to the deterioration of layers of the cuticle until it ruptures and separation from the body. As it could be expected, with higher HTE concentration (50 mg/mL) the damage was extended at earlier exposition times, cuticular rupture was observed from the first 4 h, and after 24 of exposure, only fragments of the parasite body were recovered, as shown in Fig. 5, damage was observed through the entire body.

4. Discussion

It is well known that several variables such as growing conditions, soil, season of harvesting, storage, processing, among other factors, can result in differences in the concentrations of secondary metabolites of plants. To minimize these effects, commercially available standardized dry extracts of tannins were used in this study.

The study of importance of tannins as an anti-parasitic on gastro intestinal parasites has focused in the activity of condensed tannins (Martínez Ortíz de Montellano et al., 2010; Assefa et al., 2017), while the effect of hydrolysable tannins is scarcely studied. Williams et al. (2014) and Engström et al. (2016) reported that tannins induce damage

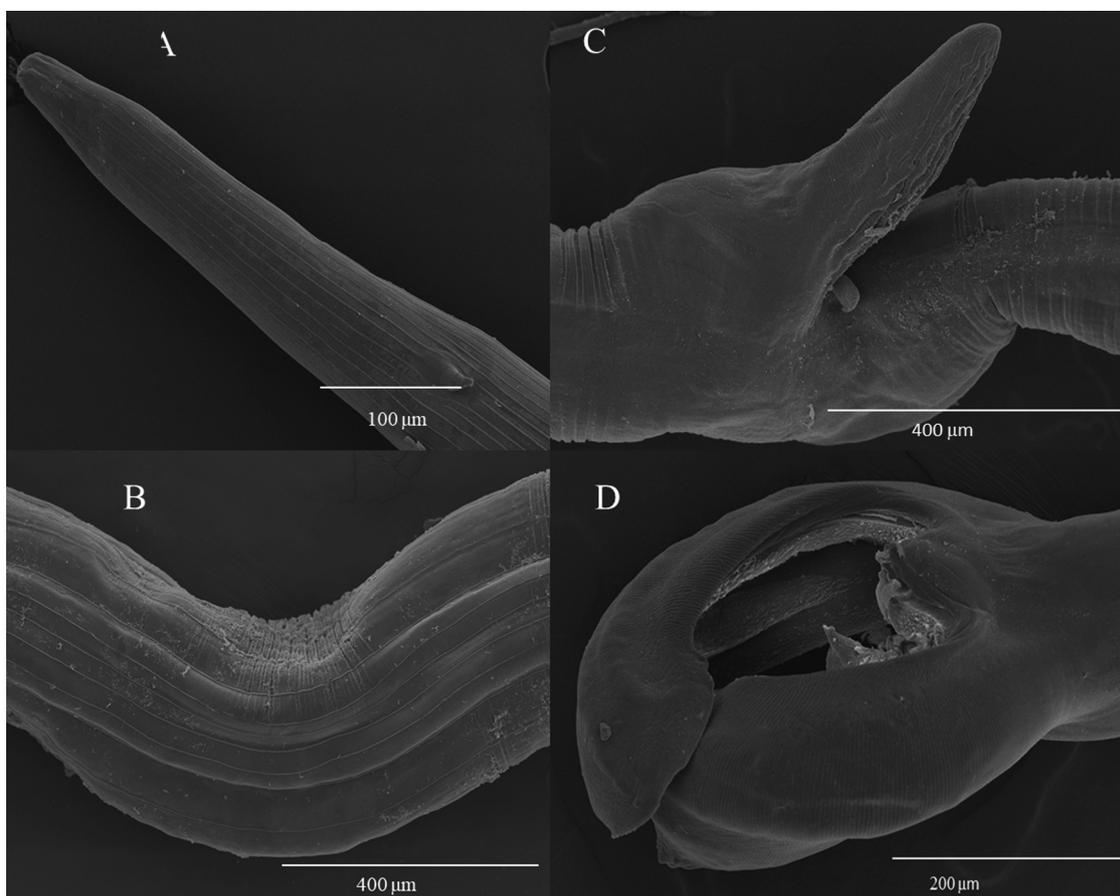


Fig. 2. Normal structure of adults of *Haemonchus contortus*. Control treatment at 24 h, cephalic region (A); middle region (B); vulva of a female (C), caudal region of a male (D).

in the cuticle of *Ascaris* genus worms. Katiki et al. (2013) used hydrolysable tannins extracted from plants, against *C. elegans* and identified mortality after 24 h of exposition with doses of 20 and 25 mg/mL. In the current experiment, the immobility and damage of *H. contortus* adults was observed after 24 h with a lower dose, 8 mg HTE/mL, and with comparable dosage (25 mg/mL), the mortality observed in our research was 100%. The lethal dose differs as the parasite specie and tannins origin differs, being evident in experiments conducted by Katiki et al. (2013).

Regarding to the safety of the effective doses of THE as potential anti-*Haemonchus* treatment, it is important to mention that the LD₅₀ obtained in the current study were previously probed *in vivo* (Corona-Palazuelos et al., 2016). Applying the predicted formula of NASEM (2016), the dose calculated according to Corona-Palazuelos et al. (2016) was 1 g of TE by kg of DM, the daily TE intake was 83.85 g by bull-calf. That is, daily water intake of calves was calculate with the formula: $DWI, L/day = -6.0716 + (0.70866 \times \text{Maximum Temperature in } ^\circ\text{C}) + (2.432 \times \text{DMI in kg/day}) - (3.87 \times \text{Pluvial precipitation in mm}) - (4.437 \times \text{Salt in diet as } \%)$ suggested by NASEM (2016). The calculated water intake for cattle weighting 231.6 kg at 22 °C, and 0.3% of salt in its diet was 22.39 L/day. Then, dividing 83.85 g of TE between 22.39 L, the mean concentration expected was 3.74 g of TE by L of water; this value was round to four, and concentration of 4 mg TE/mL was established as a base for the current experiment. Where 0 mg/ml was the Control treatment, 2 mg/mL symbolized a half of the reference doses, and 8 mg/ml represented the double of reference dose. Additionally, the highest dose of 100 mg/mL was included looking for a very high dosage clearly rebasing the higher practical dose in which tannins extract could have effective activity against *H. contortus*, and serves as upper limit to complete the exploration curve to determine the

L₅₀ dose; while 25 mg/mL dose represent a quarter of it.

The dramatic damage on body structure of parasites observed by electronic microscopy is similar to that reported for condensed tannins. There was alteration on nematode cuticle with loss of its normal structure, being evident the effect mainly in the mouth region, in the anum and vulva of female, concordant with the observations reported by Martínez-Ortiz de Montellano et al. (2010). Moreover, in our experiment, alteration in the bursa copulatrix of males was observed.

Damage in the female vulva and in the bursa copulatrix of male affect the reproductive function of parasites, due to mechanical obstruction of expulsion of gametes or eggs (Martínez-Ortiz de Montellano et al., 2010).

According with electronic microscopy images, apparently, the hydrolysable tannins induce damage in the nematode body by two mechanisms: In one-way, the exposition of parasite to the hydrolysable tannins induces lesions and damage in the external cuticle. This alteration could be due to ability of tannins forming complexes with proteins, the formation of collagen-tannin complex it is very probable due the high content of proline in the collagen (Athanasiadou et al., 2001; Olivas-Aguirre et al., 2015). In another way, the ingested hydrolysable tannins can bind protein of the internal mucosa causing autolysis, inducing internal rupture and expulsion of viscera, as reported with condensed tannins (Athanasiadou et al., 2001). The findings of the current experiment suggest that anthelmintic activity of hydrolysable tannins against *Haemonchus contortus* is a combined effect of motility inhibition, disablement for feeding and impairment of its reproductive activity. So that, in close agreement with those exposed by Hoste et al. (2012), tannins could be considered as an emerging alternative to contribute in the nematode control in ruminants. In particular, it has been demonstrated that the HT studied in this work have the

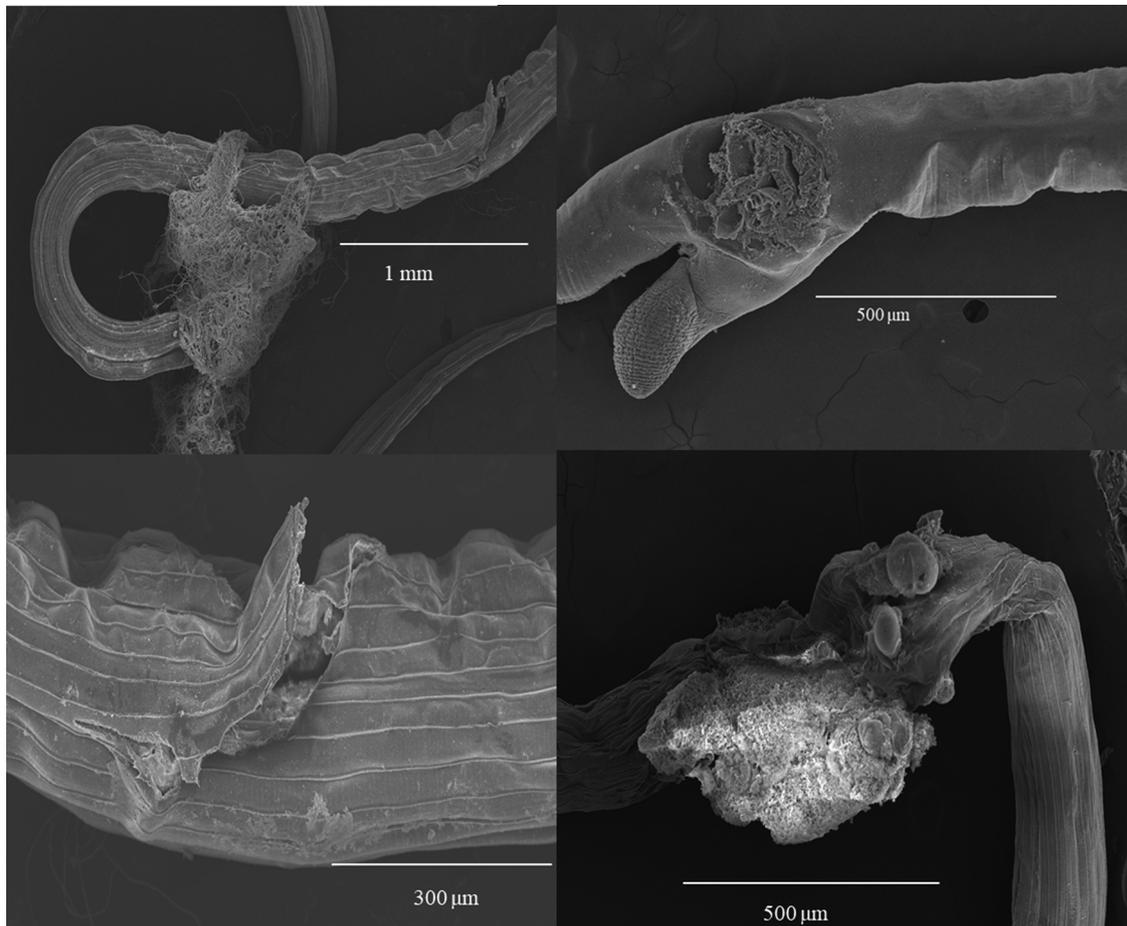


Fig. 3. Damaged structure of *Haemonchus contortus* exposed to 8 mg of HTE/mL for 24 h. Middle region of a female with rupture of the cuticle (A), and vulvar region with evisceration, exposing the intestine, uterus and eggs (B).

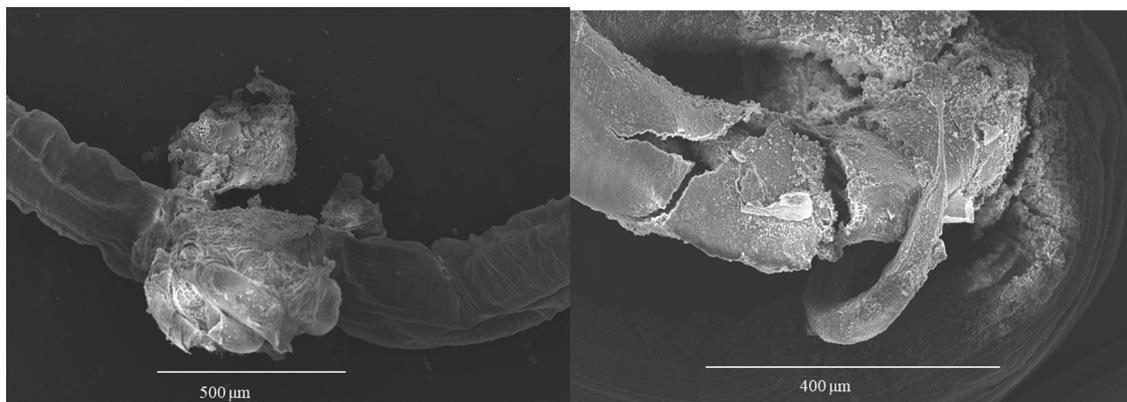


Fig. 4. Damaged structure of *Haemonchus contortus* exposed to 25 mg of HTE/mL 24 h. Middle region of a female with cuticle alteration, rupture and evisceration (A) and a magnification (B).

capacity to improve digestion in ruminants and nutrient absorption (Frutos et al., 2004) using doses within the range tested in our work, without reporting adverse effects. In such a manner that, the standardized commercial HTE tested in this work can be proposed as a nutraceutical or a functional food.

5. Conclusions

Hydrolysable tannins extract from chestnut at 25 mg/mL after 24 h is lethal for adults of *Haemonchus contortus*, a nematode parasite of ovines and bovines. The anthelmintic activity is based both in external

and internal structural damage of the parasite. Main injuries observed are destruction around the mouth, anum, vulva and bursa copulatrix, as well as loss of cuticle structure integrity in the middle of the body coupled with expulsion of digestive tract components. Thus, hydrolysable tannins emerge as an alternative to contribute in the nematode control in ruminants.

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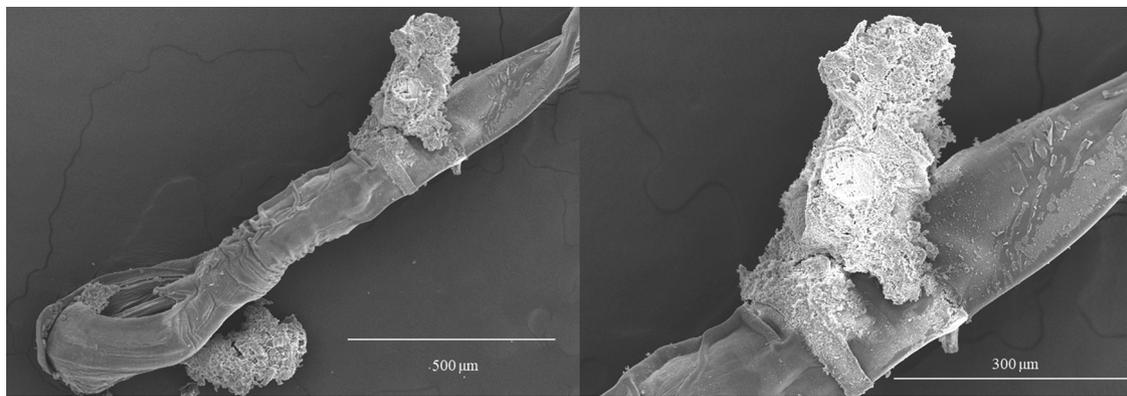


Fig. 5. Damaged structure of *Haemonchus contortus* exposed to 50 mg of HTE/mL for 24 h. Caudal region of a male with flaking cuticle in layers, breaking and fragmentation of the cuticle with evisceration (A) and a magnification (B).

Conflicts of interest

None.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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