

Effect of *Musa* spp. extract on eggs and larvae of gastrointestinal nematodes from infected sheep

Efeito do extrato de *Musa* spp. sobre ovos e larvas de nematódeos gastrintestinais provenientes de ovinos infectados

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Resumo

As helmintoses figuram como um dos principais entraves para o desenvolvimento da caprino-ovinocultura. *Haemonchus contortus* é a espécie que causa maior impacto nesta atividade pecuária. A resistência às drogas antiparasitárias e a procura por alimentos de origem animal livre de resíduos tem elevado a importância de tratamentos fitoterápicos. O objetivo desse trabalho foi desenvolver um extrato de *Musa* spp. e avaliar através de testes *in vitro* o efeito anti-helmíntico sobre ovos e larvas de nematódeos gastrintestinais de ovinos. Foram colhidas amostras de fezes de ovinos, naturalmente infectados, para a obtenção de ovos e larvas, seguida pela realização do teste da eclodibilidade de ovos e o teste de inibição da migração larval. Nos testes *in vitro* foram observados a inibição da eclodibilidade larval nas concentrações de 160 e 180mg mL⁻¹ de extrato e a inibição da migração larval nas concentrações de 800 e 1000 mg mL⁻¹. Os resultados indicam que o uso da folha de bananeira tem efeito anti-helmíntico e estudos *in vivo* acerca da aplicabilidade dessa tecnologia a campo devem ser feitos a complementar e trazer maiores informações ao que já foi revelado no presente estudo.

Palavras-chave: Fitoterapia, *Musa* spp., clínica de ruminantes, resistência parasitária

Abstract

Helminthes are listed as one of the main problems facing the development of goat and sheep production. *Haemonchus contortus* is the specie that causes greatest negative impact in ranching. Resistance to anti-parasitic drugs and demand for residue-free animal-derived food products has elevated the importance of herbal treatments. The aim of this study was to develop an extract of *Musa* spp. and assess by *in vitro* testing, the anthelmintic effect on eggs and larvae in the gastrointestinal nematodes in sheep. Stool samples from sheep naturally infected were used to obtain eggs and larvae and was then followed by a test of hatchability and a larval migration inhibition test. *In vitro* tests on the inhibition of larval hatchability at concentrations of 160 and 180 mg mL⁻¹ of larval extracts and inhibition of migration at concentrations of 800 and 1000 mg mL⁻¹ were observed. The results indicate that the use of banana leaf has an anthelmintic effect and that *in vivo* studies on the applicability of this technology to the field should be made to further understanding and bring more information to what has already been revealed in this study.

Key words: Phytotherapy, *Musa* spp., ruminant clinics, parasitary resistance

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Introduction

With the intense and inappropriate use of anthelmintic in breeding, ranchers often miss waiting for the proper period of time between treatments, resistant helminth strains have been selected (FONSECA et al., 2014). The lack of epidemiological features, the relatively low cost of treatment and the massive use of antiparasitics by producers (BORGES et al., 2011) are increasing the cases of resistance.

The growth interest in herbal drugs with potential to be used as anthelmintic can be seen in the raise of studies about possible vegetable sources of active principles. Furtado et al. (2005), testing the action of tannin using *C. papaya* extracts and *Musa paradisiaca* (papaya and banana), concluded that there could be different outcomes in relation to other works depending on methodology.

According to Hoste et al. (2006), therapies with mineral or plant components, particularly the tannin, are options for future study of the strategic control of worms in ruminants. There are two theories that explain tannins efficiency. The first one focuses on the direct effect on the life cycle of helminthes and the second one on an indirect effect: the protein intake is protected from degradation in the rumen and increases its availability in the lower gastrointestinal tract (CEZAR et al., 2008). Human and Veterinary Medicine have used tannic acid for various therapeutic purposes and its effects are still being studied (AYRES; ALMEIDA, 2010). A literature review in 2007 (OLIVO et al., 2007) pointed to a lack of data about the use of plants as anthelmintic (particularly *Musa* spp.). In this literature review, the authors addressed the need for further multidisciplinary investigations in order to gather information on the palatability and consumption of the banana plant and also information related to species and animal category, and the degree of helminths control.

The interest in studying the herbal use of this plant for some authors has originated from

recommendations of popular knowledge. Studying the medicinal use of plants from Cerrado, Bessa et al. (2013) used tannins as selection criteria and its properties were examined, although the paper did not specifically consider its anthelmintic potential. Furtado (2006) chose *Genipa americana* for *in vitro* tests, successfully using as criterion the fact that it was a plant rich in tannins. Schmahl et al. (2010) observed that *Musa* spp. influenced on motility of nematode larvae (*Trichuris muris*) when in contact with the fruit extract.

Regarding the efficiency of tanniniferous plants in combating helminths, there are studies tending to be developed around molecular and biochemical aspects of tannins. It has been proven that there are more effective combinations of monomers (BRUNET et al., 2008). One of the factors that can lead to different results is adhering to the methodology and source of active-principle. Nogueira et al. (2012) concluded in their *in vitro* tests that *Musa* spp was highly effective inhibiting the outbreak of *Haemonchus contortus* eggs.

As the great potential of using herbal as anthelmintic in livestock production, this study aimed to conduct the *in vitro* evaluation of *Musa* spp efficiency on larvae and eggs of gastrointestinal nematodes from naturally infected sheep.

Material and Methods

The alcohol extract of Musa spp., eggs and larvae acquisition

The process for making the alcohol extract started with fresh leaves harvested in properties in the city of São Paulo and stored covered in open spaces. Leaves were chopped into strips manually in the direction of the fibers, and placed in a laboratory oven at 40 °C (since higher temperatures would alter the viability of tannin, according to Silvana Gorniák⁴.) After one day in the oven, the dried

⁴ Informação fornecida pela Professora Doutora Silvana Gorniák em 2009.

leaves were ground in a medium-sized machine and the powder was placed immersed in absolute alcohol (1: 3 parts). Two days later, the resulting tincture was filtered through a paper filter, and then processed by rota-evaporator to concentrate. The resultant solution was stored in open containers back in the oven at 40 °C to complete the extract concentration process. Upon reaching the requisite pasty consistency, the extract pools were stored in the refrigerator.

The eggs and larvae utilized in the *in vitro* test were collected from a naturally infected sheep from the region of Londrina, which was kept in the isolation ward of the Londrina State University Veterinarian Hospital, receiving *Musa*-free diet. They received water and food (forage) *ad libitum*. The worms were monitored by examination of OPG and properly controlled for the preservation of health and animals welfare.

Feces were collected from the rectum. Eggs and fecal cultures were isolated to obtain third-stage larvae (L3). The recovery of the eggs was performed according to the methodology described by Coles et al. (1992) adapted by Bizimenyera et al. (2006). The feces were homogenized in distilled water and filtered through a set of sieves. Eggs were retained in the 25mm sieve, washed with distilled water and centrifuged at 1,100 x g for 5 min in 50 mL tubes supplemented with water. The supernatant was discarded and saturated saline was added for resuspend the pellet. After further centrifugation under the same conditions, the supernatant was washed in a 25 um sieve. The collected eggs were stored in a sedimentation beaker for 2 h. After syphoning, they were counted in five aliquots of 100 mL.

Egg hatch test

No teste eclodibilidade de ovos, utilizou-se uma suspensão de ovos diluída em água destilada. Soluções do extrato foram preparadas utilizando-

se DMSO e água destilada para diluição. As concentrações finais dos extratos foram 20; 40; 60; 80; 100; 120; 140; 160 e 180mg mL⁻¹.

In the Egg Hatch Test, the eggs were suspended in an extract solution containing DMSO and distilled water for dilution. The final concentrations of the extracts were 20, 40, 60, 80, 100, 120, 140, 160 and 180 mg mL⁻¹. As a positive control, albendazole sulfoxide (50 µg mL⁻¹, Ricofarm 10®, Biofarm) was used. Distilled water plus DMSO was used as negative control.

In performing the test, 100 µL of suspension containing about 150 eggs was added in the wells of the plate for cell cultivation with three replicates for each treatment and control. 400 µL of extracts at different concentrations were added. The plates were manually homogenized and put in BOD stove (25 °C and RH> 80%) for 48 h. A drop of Lugol was added to each well, and eggs and L₁ larvae from hatched eggs were quantified to calculate the percentage of inhibition of larval hatchability, according to the technique described by Coles et al. (1992).

Larval migration inhibition assay

The larval migration inhibition assay was conducted according to the methodology described by Rabel et al. (1994). Third stage larvae (L₃) were collected from fecal cultures obtained by naturally infected sheep. Approximately 150 L₃ were incubated at 27 °C in Eppendorf tubes containing 1 mL of the herbal extract diluted in distilled water and DMSO concentrations of 50, 100, 200, 400, 600, 800 and 1000 mg mL⁻¹. The test was carried out with a negative (distilled water) and a positive (Levamisole Phosphate – 40 µg mL⁻¹, Ripercol®, Fort Dodge) control. After 3 hours of incubation, the tubes were centrifuged at 1,100 xg for 2 min. and the supernatant discarded leaving 200 µL. 1800 µL of the diluted extract was added in concentrations above the wells of the cell culture plate of 24 wells,

the test was carried out in sextuplicate. Then, the 22 μm mesh was placed into the wells and in the upper part of the mesh, 200 μL of larval suspension was added using the respective concentrations tested. The plate was covered and placed in incubator chamber type BOD for 2 h at 27 °C.

Results and Discussion

The result of the Egg Hatch Test of gastrointestinal nematode eggs from sheep naturally infected, using alcoholic extract of *Musa* spp., is showed in Table 1, and the result of the Larval Migration Inhibition Assay of gastrointestinal nematodes eggs from sheep naturally infected, using alcoholic extract of *Musa* spp., in Table 2.

Table 1. Percentages of inhibition of hatching of gastrointestinal nematodes eggs from sheep per alcoholic extract concentration *Musa* spp. The significant inhibition was above 95% (160 and 180 mg mL⁻¹).

Concentrations	% inhibition
Negative control (distilled water)	9,89
20 mg mL ⁻¹	26,93
40 mg mL ⁻¹	30,75
60 mg mL ⁻¹	41,33
80 mg mL ⁻¹	48,90
100 mg mL ⁻¹	58,63
120 mg mL ⁻¹	81,84
140 mg mL ⁻¹	89,43
160mg mL ⁻¹	98,55
180 mg mL ⁻¹	100
Positive control	100

For both hatchability and larval migration, the limit considered was 95% of inhibition. Hatchability of eggs and larval motility inhibition were successful using 160mg mL⁻¹ and 180 mg mL⁻¹ and 800 mg mL⁻¹ and 1000 mg mL⁻¹ of extract, respectively.

The use of anthelmintics in under doses is an important problem associated with wrong management. As a result, strains of helminths (*Haemonchus* spp, *Trichostrongylus* spp and *Ostertagia* spp.) have been selected, as they are resistant to many commercial products (RAMOS et al., 2002). Thus, plant sources appear to be a good alternative for commercial anthelmintic. However, there is a lack of sufficient data to validate their use.

The present research proved that the *in vitro* use is efficient in both: Larval Migration Inhibition and Egg Hatch, for sheep worms. Thus, the tannin contained in *Musa* spp. would be suitable. Furtado et al. (2005) observed that there was no effectiveness against gastrointestinal nematodes of sheep, contradicting the results of the present work. This occurrence could be explained by the fact that the extracts were obtained from different sources: from *Musa paradise* flowers in the 2005 study and from a leaf pool (from the *Musa* spp.), in the current study. Furthermore, the plant processing in the current study did not exceed 40 °C, while Furtado et al. (2005) reached a temperature up to 70 °C, in which tannins would be degraded. This information demonstrates an important difference in methodology, as the author himself warned about the different results.

Table 2. Percentage of L₃ larvae (gastrointestinal nematodes from naturally infected sheep) migration by each alcoholic *Musa* spp extract concentration. The inhibition was considered significant when the inhibition of migration was above 95% (800 and 1000 mg mL⁻¹).

Concentration	% migration
Negative control (distilled water)	97,62281
50 mg mL ⁻¹	97,93229
100 mg mL ⁻¹	97,27106
200 mg mL ⁻¹	87,04955
400 mg mL ⁻¹	54,18734
600 mg mL ⁻¹	19,60784
800 mg mL ⁻¹	0
1000 mg mL ⁻¹	0
Positive control	0

In contrast, Nogueira et al. (2012) succeeded on finding inhibition in the hatchability of eggs in its three types of extract, using leaves, inflorescence and pseudostem; even surpassing the 40 °C in the processing of the extract. The major methodological difference was that the extract used was aqueous and non-alcoholic. In this mentioned work, inhibitions above 95% occurred for the three types of extracts at 2.5 mg mL⁻¹; 5.0 mg mL⁻¹ and 10.0 mg mL⁻¹, obtained from the standardized extract: 100 mg mL⁻¹. Such concentrations showed themselves lower than the concentrations that succeeded in this study: 160 mg mL⁻¹ and 180 mg mL⁻¹.

The proven effectiveness of tannin in this work contributes to the validation of the test compound *in vitro* for the helminth control in small ruminants.

Conclusions

This research concludes that alcoholic extract of *Musa* spp leaves has an effective action on the eggs and larvae of gastrointestinal nematodes from sheep, suggesting the anthelmintic effect of tannins from banana leaves and that they can be processed in the future for commercial application and being able to replace or act together with other anthelmintics.

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