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In vitro Anthelmintic effect of *Melia azedarach* L. and *Trichilia claussenii* C. against sheep gastrointestinal nematodes

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ABSTRACT

The control of parasitic diseases in small ruminants is mainly done with the use of synthetic anthelmintics. However, incorrect and indiscriminate use of these products has caused the emergence of parasite resistance. Plants with anthelmintic activity are used in folk veterinary medicine, but it is necessary to investigate and scientifically validate low-cost phytotherapeutic alternatives for future use to control gastrointestinal nematodes in small ruminants by family farmers. Thus, the aim of this study was to evaluate the *in vitro* anthelmintic effect of plant extracts from *Melia azedarach* and *Trichilia claussenii* by the egg hatch test (EHT) and larval development test (LDT) against sheep gastrointestinal nematodes. The hexane extract of *M. azedarach* fruits was extracted through cold percolation and the methanol extract of *T. claussenii* leaves was obtained by extraction at room temperature in solvents in order of increasing polarity. The efficacy results were analyzed using the Probit program of SAS. The *M. azedarach* extract showed a LC_{50} of 572.2 μg/mL and LC_{99} of 1137.8 μg/mL in the EHT, and LC_{50} of 0.7 μg/mL and LC_{99} of 60.8 μg/mL in the LDT. In turn, the *T. claussenii* extract presented a LC_{50} of 263.8 μg/mL and LC_{99} of 522.5 μg/mL in the EHT and LC_{50} of 1.1 μg/mL and LC_{99} of 26.4 μg/mL in the LDT. Comparing the extracts of the species from the Meliaceae family, *T. claussenii* showed greater anti-parasite potential *in vitro* than *M. azedarach*. However, studies on the isolated compounds, toxicity and administration forms to animals are also needed to validate low-cost alternative herbal remedies for use to control gastrointestinal nematodes by family farmers.

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1. Introduction

Farmers throughout the world raise small ruminants, in areas with a wide range of agro-ecological characteristics. The distribution of small ruminants in Mozambique is related, not only to favorable agro-climatic factors, but also to its socio–economic role in society. However, mismanagement, poor hygiene and precarious housing conditions all contributed to the incidence of disease and high mortality (van Niekerk and Pimentel, 2004).

Parasitic diseases are among the factors that limit small ruminant production worldwide, accounting for large economic losses due to retarded growth, weight loss, reduced food consumption, lower milk production, impaired fertility and, in cases of massive infections, high mortality rates (Cavalcante et al., 2009). Currently, nematode control programs in small ruminants seek not only to cure the clinical disease, which is characterized by high mortality rates, but mainly to reduce the losses caused by subclinical parasitism. This control is mainly through the use of anthelmintic chemotherapy. However, these drugs are not accessible to small farmers in rural African communities (Githiori et al., 2004).

A large number of medicinal plants have been used to treat parasitic infections in humans and animals (Akhtar et al., 2000). Despite the difficulties, smallholders resort to folk knowledge to protect their herds, through the use of medicinal plants to control various animal diseases, by trial and error (Githiori et al., 2004). The use of plants with anthelmintic properties seems to be an effective alternative, both from the standpoint of parasite control and their low environmental impact from residues in relation to commercial anthelmintics. Herbal medicine can increase profits, by reducing the use of conventional anthelmintics and extending the useful life of the limited number of anthelmintics available (Chagas et al., 2008).

Melia azedarach (Meliaceae), native of Asia which is widely distributed in almost all tropical and subtropical countries, is described as having several therapeutic properties (Burks, 1997).

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To investigate the anthelmintic activity of *M. azedarach*, *in vivo* studies have been performed with aqueous, methanolic and ethanolic extracts of the fruits in chickens (Akhtar and Riffat, 1985), of the seeds in sheep (Pervez et al., 1994) and of the fruits (Falbo et al., 2008) and seeds and leaves *in vitro* against *Haemonchus contortus* (Maciel et al., 2006). The genus *Trichilia* (Meliaceae) has about 70 species, distributed mostly in lowland areas of tropical America, with 14 species in Africa and two in the Indo-Malay peninsula. It also occurs in the forests of the lower Amazon (Reitz, 1984). Surveys of the Meliaceae family have identified the genus *Trichilia* as a potential source of substances with insecticidal action (Matos et al., 2009), similar to *Azadirachta indica*, the best known species within the family in this respect. However, no studies have been reported of the effects of *Trichilia* spp. on gastrointestinal nematodes of small ruminants.

Despite not being compatible with true high-throughput screening, significant screening can be conducted against parasites *in vitro* using directed methodologies (Woods and Knauer, 2010). Among *in vitro* techniques, the egg hatch test (EHT) and larval development test (LDT) are the most widely employed. Both have shown comparable and reliable results with regard to detection of anthelmintic resistance (Várady et al., 2009). Currently these methods have been adapted and others, such as the larval feeding test (LFT), larval exsheathment test (LET) and *in vitro* direct challenge test (IVDCT) have been developed, all being used for screening of active substances in plant extracts (Brunet et al., 2008).

This study aimed to evaluate *in vitro* the anthelmintic effect of *M. azedarach* and *T. claussenii*, both plant genera widely found in Brazil and Mozambique. This was achieved through scientifically validated techniques such as EHT and LDT on gastrointestinal nematodes of sheep.

2. Materials and methods

2.1. Preparation of extracts

Green *M. azedarach* fruits were collected and dried for seven days at 40 °C with circulation and air renewal and then ground in a rotating-knife mill. The dry matter obtained was submitted to extraction by cold percolation in a Soxhlet apparatus using hexane as solvent. Then the solvent was evaporated in a rotary evaporator until its total elimination, following which the extract was passed through a vacuum pump to remove any residual solvent. The extract was stored, refrigerated at +4 °C until use. *T. claussenii* leaves were collected and dried at room temperature. The extraction also occurred at room temperature and at rest with solvents in order of increasing polarity (hexane, MeOH and MeOH/H2O 1:1) for 7 days. The solvents were then evaporated to obtain the methanol extract, which was also stored at ±4 °C until use. The active ingredients of this material were determined beforehand by liquid–liquid chromatography, according to Matos (2006).

2.2. In vitro tests

2.2.1. Determination of extract concentrations

Five concentrations were determined following the ratio of 2: 150, 75, 37.5, 18.75 and 9.375 μg/mL for the EHT and 18.7, 9.35, 4.67, 2.34, 1.17 and 0.58 μg/mL for the LDT, with at least in six replicates for each concentration. In the EHT, the lowest concentration was determined when the hatching was similar to the control and in the LDT by the quantity of the extract available for use. The highest concentration in both tests was based on the turbidity of the concentration, since the higher the turbidity, the harder it was to read the results.

2.2.2. Egg hatch test (EHT)

Feces were collected directly from the rectum of two previously selected Santa Ines donor sheep. They both had an eggs per gram count (EPG) above 2000 and a natural infection of 95% *H. contortus* and 5% *Trichostrongylus* sp. whose L_3 larvae from feces culture were previously identified according to Ueno and Gonçalves (1998). Between 10 g and 20 g of feces was processed according to the technique of Coles et al. (1992) for egg recovery. Approximately 100 eggs in 25 μL of distilled water were placed into each well in a 24-well plate. Plant extract were prepared in such a way that the final volume in each well was 1 mL with six replicates being prepared for each extract and each concentration. For a better extract dilution, we added Tween 80 at 3% and completed the final volume with distilled water. The negative control was with distilled water and the vehicle control with the emulsifier Tween 80 at 3%. The plates were identified and placed for 24 h in an acclimatization chamber (±27 °C and RH > 80%). After this period, a drop of Lugol's iodine was added and then the eggs and larvae that had hatched (L_1) were counted using an inverted microscope. The percentage efficiency (E) per well was calculated according to the formula below:

$$E = \frac{(\text{egg} + L_1) - L_1}{\text{egg} + L_1} \times 100$$

2.2.3. Larval development test (LDT)

Following the method described by Hubert and Kerboeuf (1992), 25 μL of suspension containing approximately 100 eggs was added to each well of the 24-well plates, along with 80 μL of nutrient medium containing *Escherichia coli* and distilled water up to a volume of 200 μL. Six replicates for each extract and each concentration was prepared. The plates were identified, wrapped in PVC film and kept for 24 h in an acclimatization chamber (±27 °C and RH > 80%). After this period, the same inverted microscope was used to observe whether the larvae had hatched. Then the plant treatments were prepared to a final volume of 500 μL in each well. The emulsifier dimethyl sulfoxide (DMSO) at 1% was used to dilute the extracts. The negative control was prepared with distilled water and the vehicle control with the emulsifier DMSO at 1%. After the addition of the plant extracts in the wells, the plates were wrapped in PVC film and kept in the chamber under the same conditions for four days. After this period, the number of L_1, L_2 and L_3 in each well was counted with the inverted microscope and the percentage efficiency (E) was calculated according to the formula below:

$$E = \frac{(L_1 + L_2 + L_3) - L_3}{L_1 + L_2 + L_3} \times 100$$

2.2.4. Statistical analysis of data

The calculation of the lethal concentration of the extracts in the *in vitro* tests was performed by fitting a regression using normal and logistic distributions, with the parameter estimates of these equations obtained by maximum likelihood. The procedure used the SAS Probit to estimate the LC_{50} and LC_{99}, with the independent variables (dose), transformed by natural logarithm (log dose). The models' goodness-of-fit was evaluated using Pearson's chi-square test and the maximum likelihood ratio.

3. Results

The inhibition percentages of egg hatching and larval development increased with increasing concentrations of the extracts, demonstrating a dose-dependent response, except with *T. claussenii* extracts in EHT. In the EHT, the highest tested concentrations

did not reach 50% hatching inhibition and higher concentrations could not be assessed because the plates were dark, preventing readings (Fig. 1). The extracts showed greater efficacy in the LDT, where even the lowest concentration caused more than 50% development inhibition by the extracts from both plant species (Fig. 2). Unfortunately, it was not possible to test the plant extracts in the LDT at the lower concentrations due to the small amount of extract available. In the EHT, the vehicle control, consisting of Tween 80, caused 6.2% inhibition, while in the LDT the inhibition by DMSO was 10.9%.

The LC_{50} and LC_{99} calculation (Table 1) showed that the extracts have a poor ovicidal activity, but strong larvicidal action. In EHT, *T. claussenii* showed lower LC_{50} and LC_{99} than *M. azedarach*, therefore proving to be more efficient. The same happened for the LC_{99} in the LDT, but LC_{50} of *M. azedarach* was lower. Because of the good results in the LDT, the concentration–response curve (log 10) were calculated and are shown in Figs. 3 and 4.

4. Discussion

The aim of this study was to establish the effects of *M. azedarach* and *T. claussenii* extracts on egg hatching and larval development (L_1 to L_3) of gastrointestinal nematodes (95% *H. contortus* and 5% *Trichostrongylus* sp.). An analogous method has been used to test the ovicidal and larvicidal effects of plant extracts (Lorimer et al., 1996; Molan et al., 2003; Bizimenyera et al., 2006).

The inhibition percentages in EHT were not dose-dependent response for *T. claussenii* at the concentrations tested, as shown in Fig. 1. Sometimes there are problems in achieving a linear correlation between concentration and efficacy because, in an extract, the bioactive substances may not be distributed homogeneously within the material or it may be affected by the technique or process used to produce the extract (Chagas and Vieira, 2007). Nevertheless, *T. claussenii* showed better effect on the eggs than *M. azedarach* since it achieved lower LC_{50} and LC_{99} (Table 1). Maciel et al. (2006) using ethanol extract of seeds of *M. azedarach*, showed lower LC_{50} by the EHT (360 μg/mL) than the results of the present study, but in the LDT, the ethanol extract of leaves showed higher LC_{50} (9180 μg/mL). Taken together these results show the variation of *in vitro* anthelmintic activity for different *M. azedarach* extracts, i.e., due to different methods of extraction or separation of the active ingredients and plant part used.

The dose–response LDT curves with log-transformed inhibition results clearly show that *T. claussenii* had a narrower range of response (from 1.213 to −1.152 μg/mL) (Fig. 4), than *M. azedarach* (from 1.978 to −2.581 μg/mL) (Fig. 3). First-stage larvae were more sensitive to the active substances in the extracts than were eggs, which has also been reported in studies where the same plant extract was evaluated (Macedo et al., 2010).

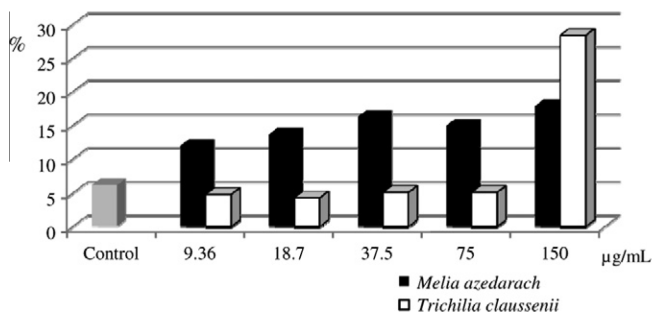

Fig. 1. Percentages of hatching inhibition in the egg hatch test for gastrointestinal nematodes of sheep submitted to five concentrations of hexane extract of *M. azedarach* fruits and methanol extract of *T. claussenii* leaves (μg/mL).

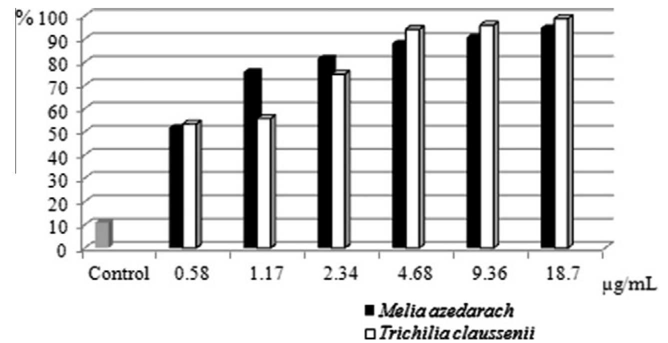

Fig. 2. Percentages of development inhibition in the larval development test for gastrointestinal nematodes of sheep submitted to five concentrations of hexane extract of *M. azedarach* fruits and methanol extract of *T. claussenii* leaves (μg/mL).

Table 1
Inhibition of egg hatching and larval development (LC_{50}, LC_{99} and *P*) of gastrointestinal nematodes of sheep subjected to treatment with hexane extract of *M. azedarach* fruits and methanol extract of *T. claussenii* leaves (μg/mL).

Plant extract	Egg hatch test			Larval development test		
	LC_{50}	LC_{99}	*P*	LC_{50}	LC_{99}	*P*
M. azedarach	572.2	1137.8	0.0033	0.7	60.8	0.0001
T. claussenii	263.8	522.5	0.0001	1.1	26.4	0.0001

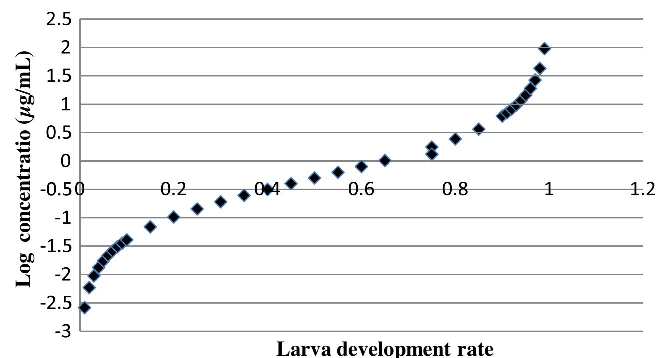

Fig. 3. Larval development test concentration–response curve of hexane extract of *M. azedarach* fruits against grastrointestinal nematodes of sheep.

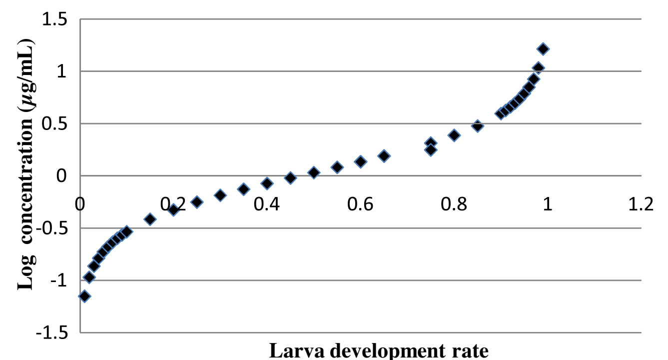

Fig. 4. Larval development test concentration–response curve of methanol extract of *T. claussenii* leaves against grastrointestinal nematodes of sheep.

Chemical analysis of the extracts from the *M. azedarach* fruits revealed the presence of tannins, phenolic compounds and steroids (Dantas et al., 2000; Maciel et al., 2006). Tannins are substances described as possessing anthelmintic activity. They can act through two types of mechanisms: connection to free proteins, thus

reducing the availability of nutrients, resulting in larval death by starvation; or connection to the larval cuticle, rich in glycoproteins, causing death. The last mechanism suggests that the tannins in the extracts may be the active ingredient on eggs and larvae of *H. contortus* (Athanasiadou et al., 2001).

The biological activity of *T. claussenii in vitro* was also observed by Matos et al. (2009), who obtained 100% larval mortality on *Spodoptera frugiperda* at 1000 mg/kg when delivered in an artificial diet. Besides mortality, the extract inhibited or delayed the larval development by 1–3 days. This was also observed in the LDT in the present study; when the extract did not cause mortality, it did delay development. Although few studies have been performed with the *Trichilia* genus, Matos (2006) cites some studies where antifungal, antibacterial and antiviral activity was reported. Both anti-inflammatory and antiparasitic activity was reported by Togola et al. (2005). According to Matos (2006), 24-methylen-3β,4β,22α-trihydroxy-colesterol and 3-β-*O*-β-D-lucopyranosilsitosterol were isolated from the methanol extract of *T. claussenii* leaves (as tested in the present study). The effects of *T. claussenii* on eggs and larvae may be associated with these or other organic compounds already isolated and identified in stems, leaves and fruits (Pupo et al., 1996, 1997, 1998). However, the anthelmintic activity of these compounds alone has not yet been evaluated.

In vivo anthelmintic activity of *M. azedarach* was demonstrated by Akhtar and Riffat (1985) on *Ascaridia galli* and by Falbo et al. (2008) on sheep gastrointestinal nematodes, with an efficiency of 33.2%. According to Squires et al. (2010), in small ruminants the rumen may serve as a reservoir, slowing the passage of the anthelmintic product, thus prolonging the exposure of *H. contortus* to the active substances. On the other hand, Pervez et al. (1994) observed inefficacy of *M. azedarach* and suggested this may be due to the destruction of the active substances by the ruminal flora and other aspects, such as ruminal pH. Antifungal (Carpinella et al., 1999), insecticidal (Gajmer et al., 2002) and acaricidal (Borges et al., 2005) effects of *M. azedarach* extracts from fruits and seeds have been reported. However, this plant's anthelmintic activity on gastrointestinal nematodes in small ruminants remains to be clarified by *in vivo* experiments.

Comparing the extracts of the two species, *T. claussenii* extract showed better results in the tests than did the *M. azedarach* extract. Both extracts showed anthelmintic activity, but studies of the isolated compounds, toxicity and administration to animals also are needed. In the future the results should be validated *in vivo* as low-cost alternative herbal remedies to control gastrointestinal nematodes by family farmers.

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