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Anthelmintic activity of *Jatropha curcas* L. seeds on *Haemonchus contortus*

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ABSTRACT

The aim of this study was to evaluate the anthelmintic activity of hexane (HE), ethyl acetate (EA) and ethanol (EE) extracts obtained from the seeds of *Jatropha curcas* using the egg hatch inhibition assay (EHA) and the artificial larval exsheathment inhibition assay (LEIA). For the egg hatch assay, HE, EA and EE were used in concentrations of 3.12, 6.25, 12.5, 25 and 50 mg ml^{-1}, accompanied by a negative control (5% Tween 80) and a positive control (0.025 g ml^{-1} thiabendazole). In LEIA, the extracts were tested at a concentration of 1000 μg ml^{-1}, accompanied by a negative control (PBS). To evaluate the effect of tannins, the extract with the greatest effect was incubated with polyvinyl polypyrrolidone (PVPP). The EE (50 mg ml^{-1}) inhibited 99.8% of egg hatching. After the addition of PVPP, the ovicidal effectiveness of EE was reduced to 91.9%. Using the HE and EA, inhibition of egg hatching was 15.3% and 32.2%, respectively. In the LEIA, 18.9% of L3 incubated with EE were exsheathed ($p < 0.01$). The addition of PVPP to EE reversed the inhibitory effect on larval exsheathment. The percentage of exsheathment of L3 incubated with HE (99.6%) and EA (97.8%) did not differ from the control group ($p > 0.05$). The results show that the effects of EE on eggs are not solely due to the tannins. However, these secondary metabolites are implicated in blocking the larval exsheathment.

1. Introduction

Livestock are an important source of income and food for small producers, and gastrointestinal nematodiasis remains a major cause of reduced production and impaired animal health (Githiori et al., 2005). Conventionally, gastrointestinal nematode infection is treated with synthetic anthelmintics. However, the development of resistant gastrointestinal nematode populations to anthelmintics is a reality in worldwide (Kaplan, 2004) and it is one of the most important and current problems in animal husbandry. Anthelmintic resistance has stimulated the search for new alternatives of treatment, including the use of medicinal plants (Jackson and Miller, 2006).

Jatropha curcas L. belongs to the family Euphorbiaceae. It seeds are used in folk medicine to treat several human and animal diseases (Kumar and Sharma, 2008). The predominant constituents in the seeds are toxoalbumin curcin, phorbol esters, terpenes, polyphenols and tannins (Llelaboye and Pikuda, 2009). Despite reports of toxicity (Abdel Gadir et al., 2003), the seeds of this plant are used in

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ethnoveterinary medicine as purgatives (McGaw and Eloff, 2008), molluscicides (Gübitz et al., 1999) and anthelmintics (Ketzis and Brown, 2000).

The objective of this study was to evaluate the anthelmintic action *in vitro* of hexane, ethyl acetate and ethanol extracts obtained from seeds of *J. curcas* on *Haemonchus contortus* using the egg hatching assay (EHA) and larval artificial exsheathment assay (LEIA). In addition, we aimed at examining the possible role of tannins in this antiparasitic activity.

2. Materials and methods

Seeds of *J. curcas* were obtained in an experimental field at Embrapa Eastern Amazon in the municipality of Don Eliseu, which is located in the state of Pará, Brazil. Plant samples were collected and sent to the Herbarium of Embrapa Eastern Amazon for botanical identification (voucher-184097).

Approximately 4 kg of *J. curcas* seeds were crushed and soaked for seven days in organic solvents in order of increasing polarity: hexane, ethyl acetate and ethanol. The solvent was evaporated using a rotary evaporator to obtain hexane (HE), ethyl acetate (EA) and ethanol (EE) extracts.

Phytochemical tests were performed as previously described by Matos (2009). Briefly, in the Lieberman–Burchard test, a chloroform solution of the extract is mixed with acetic anhydride and three drops of sulfuric acid. The development of a blue to green color indicates the presence of phytosteroids and red to brown color is indicative triterpenoids. Alcoholic extracts in presence of $FeCl_3$ solution produce a dark blue precipitate in presence of hydrolysable tannins and a green precipitate in the presence of condensed tannins or catechins. To alkaloids, the extracts were mixed with NH_4OH (pH 11) and the bases were extracted with diethyl ether-chloroform solution. In a separation funnel the organic layer was washed with HCl solution (0.1 M). The aqueous acid solution is divided into three portions for adding the reagents which precipitate alkaloids: Hager, Meyer and Dragendorff.

Total phenols were quantified using the methodology of Souza et al. (2007), which uses the Folin–Ciocalteu reagent with a calibration curve prepared with gallic acid. Total tannins were analyzed according to Pansera et al. (2003) using the Folin Denis reagent with a calibration curve prepared with tannic acid. Both measurements were performed on a spectrophotometer at 515 and 720 nm for total phenols and tannins, respectively. The results for total phenols were expressed as mg g^{-1} (equivalent to gallic acid) and tannins as mg g^{-1} (equivalent to tannic acid).

One sheep was maintained in a metabolic cage and treated on alternate days with three anthelmintics (Fenbendazole, Panacur®, Intervet, 8.25 mg/kg; Levamisole, Ripercol®, Fort Dodge, 5 mg/kg and Ivermectin, Ivomec®, Merial, 0.2 mg/kg) to eliminate natural infections. In order to use the animal as a source of fresh *H. contortus*, 5000 *H. contortus* third-stage larvae (L3) were orally administered to the sheep. After 21 days, approximately 10 g of feces was collected directly from the rectum of the sheep experimentally infected, and the sample was processed according to the technique described by Hubert and Kerboeuf (1992) for egg recovery. The L3 were collected according to Roberts and O'Sullivan (1950).

The EHA was based on the method described by Coles et al. (1992). A 0.25 ml suspension of eggs (approximately 100 fresh eggs) were distributed in 5 ml tubes and mixed with the same volume of HE, EA or EE extract at concentrations of 3.12, 6.25, 12.5, 25 and 50 mg ml^{-1}. The HE, EA and EE were dissolved in 5% Tween 80. The mixtures were incubated for 48 h at room temperature. Next, Lugol drops were added to stop the eggs from hatching, and all eggs and first stage larvae were then counted. Each concentration of HE, EA and EE extract was accompanied by a negative (5% Tween 80) and a positive control (0.025 mg ml^{-1} thiabendazole). Five replicates were performed for each extract concentration.

The LEIA was performed according to Alonso-Diaz et al. (2008). One thousand ensheathed *H. contortus* larvae were incubated for 3 h with HE, EA or EE at concentrations of 1000 μg ml^{-1}. After incubation, the larvae were washed three times in PBS (pH 7.2). Larvae were subjected to an artificial exsheathment process through contact with a solution of sodium hypochloride (2%) diluted 1:300 in PBS (pH 7.2). The kinetics of larval exsheathment in the different experimental treatments were then monitored, and exsheathed larvae were counted at 0, 10, 20, 30, 40, 50 and 60 min. Each extract was accompanied by a negative control (PBS). Six replicates were performed for each treatment and negative control.

To confirm the action of tannins on the observed anthelmintic effect, the extracts with the greatest effect were incubated with 50 mg ml^{-1} polyvinyl polypyrrolidone (PVPP) as described by Alonso-Diaz et al. (2008).

2.1. Statistical analyses

Results of the EHT were compared by ANOVA and Tukey's test using Graph Pad Prism 3.0. The mean effective concentration (EC_{50}) to inhibit egg hatching was calculated by the probit method (SPSS 8.0 for Windows). LEIA results were analyzed by the Kruskal–Wallis test using the SAS program. The results were expressed as a mean percentage ± standard deviation, and the differences were considered significant when $p < 0.05$.

3. Results

Phytochemical analysis of *J. curcas* seeds showed the presence of phytosteroids in HE and EA extracts. The extracts were negative for the other secondary metabolites investigated. The EE was positive for tannins, catechins and triterpenes. The concentrations of total phenols for HE, EA and EE extracts were 67, 87 and 108 mg g^{-1} (gallic acid equivalent), respectively. Tannins were not detected in HE and EA while the EE concentration was 3.2 mg^{-1} g (tannic acid equivalent).

The test results are described in Table 1. The EE at concentrations of 50 mg ml^{-1} and 25 mg ml^{-1} inhibited egg hatching by 99.8% and 99.6%, respectively, which was not statistically different from the positive control. The ovicidal effect of EE was dose-dependent, demonstrating an