Veterinary Parasitology 226 (2016) 10–16

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

In vitro anthelmintic activity of the *Zizyphus joazeiro* bark against gastrointestinal nematodes of goats and its cytotoxicity on Vero cells

Danilo Cavalcanti Gomes[a], Hélimar Gonçalves de Lima[a], Ariádne Vieira Vaz[a], Nathália Silva Santos[a], Francianne Oliveira Santos[a], Êuder Reis Dias[b], Mariana Borges Botura[c], Alexsandro Branco[b], Maria José Moreira Batatinha[a,*]

[a] *Laboratório de Toxicologia, Hospital de Medicina Veterinária, Universidade Federal da Bahia, Brazil*
[b] *Laboratório de Fitoquímica, Universidade Estadual de Feira de Santana, Brazil*
[c] *Laboratório de Toxicologia, Universidade Estadual de Feira de Santana, Brazil*

ARTICLE INFO

Article history:
Received 10 November 2015
Received in revised form 1 June 2016
Accepted 2 June 2016

Keywords:
Anthelmintic
Cytotoxicity
Goats
Larvae
Eggs
Saponin

ABSTRACT

This study examined the *in vitro* effect of the *Zizyphus joazeiro* bark against gastrointestinal nematodes of goats and its cytotoxicity on Vero cells. The ovicidal activity of the crude hydroethanolic extract (CE), its partitioned hexane (HE) and aqueous extract (AE) and saponins fraction (SF), including betulinic acid (BA), a biogenetic compound from this plant found in HE, were investigated using the inhibition of egg hatch assay (EHA). Thereafter, the extracts and the SF were evaluated through the larval motility assay (LMA) and larval migration inhibition assay (LMIA). The AE and SF promoted a complete inhibition of the egg hatch, and the effective concentration to inhibit 50% (EC_{50}) values was 1.9 and 1.3 mg/mL, respectively. The highest percentages of inhibition in EHA observed after treatments with CE, HE and BA corresponded to 79, 48 and 17%, respectively. The extracts and SF did not show larvicidal activity in LMA and LMIA. The AE and SF demonstrated cytotoxic effects in 3-4,5-dimethylthiazol-2-yl, 2,5diphenyltetrazolium bromide (MTT) and trypan blue tests; however, SF was more toxic (50% inhibitory concentration, IC_{50} = 0.20 mg/mL). The chemical characterization of the SF was made through Proton Nuclear Magnetic Resonance (^{1}H NMR) and Electrospray Ionization Mass Spectrometry (ESI–MS) analyses, which led to the identification of two saponins known as Joazeiroside B and Lotoside A. The results obtained from the research of this saponin content provide important information about the biological activity, especially the anthelmintic effect present in the plant investigated. That also suggests the types of bioactive compounds that may be responsible for this antiparasitic activity exhibited by the plant extracts.

1. Introduction

The gastrointestinal nematodes (GIN), especially the species *Haemonchus contortus*, are highly prevalent parasites in small ruminants; also, they are responsible for large economic losses in livestock (Sargison et al., 2012). The GIN infections results in anemia, submandibular edema, weight loss, decrease in milk production, lethargy and increased death rate (Molento et al., 2011). The use of synthetic anthelmintics is the most common form for controlling GIN (Molento et al., 2011). However, the continuous and exclusive use of these products has contributed to the development of resistance parasites and increased risks of residues in the meat and milk of these animals and the environment (Torres-Acosta et al., 2012). In this context, new alternatives for the control of these parasites have been investigated, including studies using plants with therapeutic potential, with greater efficacy and low toxic effect (Hoste and Torres-Acosta, 2011).

Zizyphus joazeiro, popularly known as juazeiro, is a native and widely distributed species in northeastern Brazil. This plant occurs naturally in semiarid regions, being an endemic species of the Caatinga biome. Its use is varied, both in folk medicine and in animal nutrition, rural buildings and also for obtaining firewood (Lucena et al., 2008). Saponins are secondary metabolites widely distributed in the plant kingdom that possess one to five sugar chains linked to hydrophobic aglycone, known as sapogenin (Podolak et al., 2010). These compounds occur chemically as glycoside of steroids or triterpenes, characterized according to the chemical character of aglycone, (Thakur et al., 2011); they have a wide range

* Corresponding author at: Escola de Medicina Veterinária e Zootecnia, Universidade Federal da Bahia, Av. Ademar de Barros, 500, Ondina, CEP: 40170-110, Salvador, Bahia, Brasil.
E-mail address: mjmb@ufba.br (M.J.M. Batatinha).

http://dx.doi.org/10.1016/j.vetpar.2016.06.004
0304-4017/

of pharmacological properties, including anti-inflammatory, antifungal, molluscicidal and antiparasitic (Podolak et al., 2010). The triterpenoids saponin derivatives are abundant in *Z. joazeiro* bark, from where saponins known as jujubosides (Kato et al., 1997) and joazeirosides (Schühly et al., 2000) have been isolated. There are no reports on the anthelmintic activity of both saponins of this plant. Another constituent found in the bark of *Zizyphus joazeiro* known as betulinic acid (BA) has showed a variety of biological activities ranging from the inhibition of the replication of the human immunodeficiency virus (HIV), the antioxidant (Yogeeswari and Sriram, 2005) and anthelmintic properties (Shai et al., 2009).

In the present study, we evaluated the *in vitro* efficacy of the extracts, SF and BA against the GIN of goats belonging to the Trichostrongylidae family and its cytotoxicity against cultured cells of the African green monkey kidney cell line (Vero).

2. Materials and methods

2.1. Reagents and equipment

Buthanol, acetic acid, ethanol, methanol and *n*-hexane (analytical grade) were purchased from Vetec (Brazil). Thin-Layer Chromatography (TLC) analysis was performed on plates coated with silica gel (kieselgel 60 F_{254}, 0.20 mm, Merck). Column chromatography was run using silica gel 60 (70–230 mesh, Sigma-Aldrich) with different combinations of ethanol and water. Liquid–liquid partition was performed using *n*-hexane and water. The BA that was used as a standard and biological test was purchased from Sigma-Aldrich®. Proton Nuclear Magnetic (^{1}H NMR) spectra were recorded on a Brunker *DRX-300* spectrometer at 300 MHz; all spectra in DMSO; chemical shifts δ in ppm, *J* in Hz, using TMS as internal standard. Electrospray Ionization Mass Spectrometry (ESI–MS) analysis was measured on a Brunker Daltonics Esquire 3000 Plus spectrometer operating in positive mode. The sample was solubilized in methanol and water (1:9).

2.2. Plant material

Barks of *Zizyphus joazeiro* Mart., Rhamnaceae, were collected on January 10, 2008 in the city of Feira de Santana (Bahia, Brazil). A voucher specimen (HUEFS 61790) was stored in the herbarium of the State University of Feira de Santana, Brazil. The bark of this plant was selected due to the high concentration of saponins.

2.3. Extraction and fractionation

The barks of *Z. joazeiro* (2.0 kg) were dried in an oven at 50 °C for three days. After that, the material was powdered and macerated with hydroethanolic solution (80%) at room temperature for seven days. After this period, the sample was filtered through a vacuum filtration system and concentrated under reduced pressure to give the hydroethanolic extract (CE). The CE (9.0 g) was re-solubilized in water (300 mL) and subjected to liquid–liquid partition with hexane to provide the hexane extract (HE) and aqueous extract (AE). The BA was identified in HE by TLC comparison using a standard pattern (Cheok et al., 2014).

2.4. Recovery saponins fraction (SF)

The active AE was chromatographed on a column packed with silica gel and eluted with EtOH and H_2O as solvent (100/0; 90/10; 80/20; 70/30; 60/40; 50/50; 40/60; 30/70; 20/80; 10/90; 0/100%, 200 mL each) to provide 9 fractions, namely F1–F9. The fractions were monitored by means of TLC (eluent BuOH/AcOH/H_2O 4:5:1). Only fractions F1, F2 and F3 were positive to the presence of saponins, which were mixed and named as saponin fraction (SF) (Santos et al., 2015).

2.5. Parasitological tests

For parasitological assays, the eggs and larvae were obtained from the feces of two goats naturally infected with GIN, which are kept in the School of Veterinary Medicine, Federal University of Bahia (UFBA). Eggs were recovered from the feces according to the protocol described by Hubert and Kerboeuf (1992). For the Larval migration inhibition assay (LMIA) and Larval motility assay (LMA), infective larvae (L_3) were obtained by means of coproculture (Ueno and Gonçalves, 1998). The generic identification of the nematode population (*Haemonchus* spp., *Trichostrongylus* spp. and *Oesophagostomum* spp.) was determined according to Ueno and Gonçalves (1998). The parasite population was greater than 90% *Haemonchus* spp.

The procedures of this study were approved by the Ethics Committee for the use of animals of the UFBA (Protocol 17/2012).

2.5.1. Egg hatch assay

The test was performed according to Coles et al. (1992). The egg suspension was distributed in a 96-well microplate. Subsequently, the extracts, BA and the SF were added in the wells in different sequences. The determination of the concentrations used was based on results from pilot experiments, and were well-defined concentrations of 0.8; 1.2; 1.8; 2.7; and 4 mg/mL for extracts and fractions. The concentrations of BA (1.0, 0.75 and 0.5 mg/mL) were defined based on a study by Shai et al. (2009). In the 1 mg/mL of BA, the study showed action on larvae of *H. contortus*. Negative controls containing distilled water, DMSO and Tween 80 were also included, in addition to the positive control thiabendazole (0.025 mg/mL). After 48 h of incubation at 27 °C, egg hatching was stopped by adding Lugos's iodine solution and the eggs and larvae (L1) of each well were counted. Three independent experiments with four replicates of each concentration and controls were performed to ensure the validity of the results. The percentage of inhibition of the egg hatching was determined by the ratio: number of eggs/(number of eggs + number of L1) × 100.

2.5.2. Larval migration inhibition assay (LMIA)

Migration was performed according to Molento and Prichard (2001), in which infective larvae (L3) of GIN obtained from stool cultures are used. Approximately 400 larvae in 500 μL PBS were distributed to each well of a 48-well culture plate, and then were added to the extracts and SF in a concentration of 4 mg/mL. Two control groups, one with PBS and the other with levamisole (0.5 mg/mL), were used. The cultures of larvae were incubated at 27 °C for six hours. Thereafter, agar solution was added. The final solution was transferred to a Petri dish containing a special apparatus (plastic cylinder superimposed on two nylon screens containing frozen water). Later, these plates were incubated again at 27 °C, and were exposed to a light source for 18 h to stimulate the movement of the larvae that migrate outwards to the aqueous agar moiety present on the plate. This liquid portion containing the larvae was transferred to a falcon tube and centrifuged at 1500*g* for 5 min. The supernatant was removed, and a final volume of 2 mL was maintained. A homogenous aliquot (200 μL) was removed from this volume for the quantification of the larvae. The results of the larval counts for each aliquot were multiplied by 10. The whole experiment was carried out in three repetitions with five replicates.

The percent efficacy was determined according to the following formula: $E = [(Mc - Mtr)/Mc] \times 100$. Here, E represents the efficacy percentage, Mc represents the mean number of larvae counted in