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Antileishmanial activity and trypanothione reductase effects of terpenes from the Amazonian species *Croton cajucara* Benth (Euphorbiaceae)

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ABSTRACT

Background: Leishmaniasis comprises several infectious diseases caused by protozoa parasites of *Leishmania* genus. In recent years, there has been a growing interest in the therapeutic use of natural products to treat parasitic diseases. Among them *Croton cajucara* Benth. (Euphorbiaceae) is a plant found in the Amazonian region with a history of safe use in folk medicine.
Purpose: The purpose of this study was to investigate the effects of clerodane diterpenes, *trans*-dehydrocrotonin (DCTN), *trans*-crotonin (CTN) and acetylaleuritolic acid (AAA) obtained from powdered bark of *C. cajucara* against promastigotes, axenic and intracellular amastigotes of *Leishmania amazonensis*. Furthermore, the effects of DCTN and CTN on the trypanotiona reductase enzyme were also investigated. The extraction of the terpenes was carried out as previously reported (Maciel et al., 1998; 2003).
Methods: The effect of the isolated compounds (DCTN, CTN and AAA) from the bark of *C. cajucara* was assessed *in vitro* against promastigotes, axenic amastigotes and intracellular amastigotes of *L. amazonensis* by counting of remaining parasites in a Neubauer chamber in comparison to pentamidine used as standard drug. The action of natural products on trypanothione reductase was assessed using soluble protein fraction of promastigotes. The assays were performed by incubation with HEPES, EDTA, NADPH and trypanothione disulfide to quantify the NAPH consumption by TryR.
Results: The results showed very high efficacy, especially of the diterpene DCTN, against promastigotes (IC_{50} = 6.30 ± 0.06 μg/ml) and axenic amastigotes (IC_{50} = 19.98 ± 0.05 μg/ml) of *L. amazonenesis*. The cytotoxic effect of the best active natural product was evaluated on mouse peritoneal infected macrophages (IC_{50} = 0.47 ± 0.03 μg/ml in 24 h of culture), and the treatment revealed that DCTN never reaches toxic concentrations while reducing the infection and, most importantly, with no toxicity (>100 μg/ml with 0% of macrophage kill) when compared to pentamidine (37.5 μg/ml with 100% of macrophage kill). Furthermore, all of the natural products assayed on the trypanothione reductase enzyme inhibited the enzyme activity compared to the control.
Conclusion: Clerodane diterpenes from *C. cajucara* showed promising *in vitro* antileishmanial effects against *L. amazonensis*, specially the DCTN with no macrophage toxicity up to the assayed concentration. In addition, the action on trypanothione reductase enzyme revealed a possible mechanism of action.

Introduction

Leishmaniasis comprises several diseases caused by protozoan parasites of the *Leishmania* genus, which are transmitted by sandflies. This parasite has been endemic in 88 countries of four continents (Paloque et al., 2012) and has caused serious public health problems. The infection, manifests as cutaneous, mucocutaneous or visceral leishmaniasis (Vendrametto et al., 2010). The World Health Organization (WHO) considers leishmaniasis to be one of the most

Abbreviations: Anal. calc., analysis calculated; FCS, fetal calf serum; IC_{50}, half-maximal inhibitory concentration; IC_{50} ± SD, half-maximal inhibitory concentration ± standard deviation; BALB/c, albino mouse laboratory-bred strain of the house mouse; IF, infection index; SF, soluble fraction; TryR, trypanothione reductase; $T(S)_2$, trypanothione disulfide.

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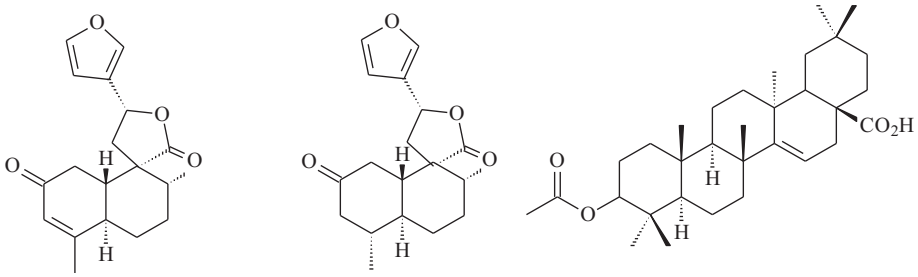

Fig. 1. *Nor*-clerodane diterpenes structures of *trans*-dehydrocrotonin (DCTN) and crotonin (CTN), and triterpene structure of acetylaleuritolic acid (AAA) isolated from bark of *Croton cajucara*.

serious and most neglected diseases worldwide and recommends meglumine antimoniate as the first-choice treatment. This drug requires a long treatment period (Croft et al., 2006; WHO, 2015), is highly toxic and can cause serious side effects (Rodrigues et al., 2009). Second-line drugs include pentamidine and amphotericin B; however have also showed highly toxic effects. Recently, miltefosine, an alkylphosphocholine compound, was approved for visceral *Leishmania* infections, but teratogenic and gastrointestinal side effects have been reported (Porwal et al., 2009). Thus, there is an urgent need for safer and more efficient compounds for the treatment of leishmaniasis.

In the last years, there has been a growing interest in the therapeutic use of medicinal plants and natural products for the prevention and treatment of parasitic diseases (Ibrahim et al., 2014; Izumi et al., 2012; Batista et al., 2009; Camacho et al., 2003). Among them is *Croton cajucara* Benth. (Euphorbiaceae) popularly known as "*sacaca*" which is a plant found in the Amazonian region with a safe history of use in folk medicine. Both the bark and the leaves of *C. cajucara* are popularly used in teas and pills for the treatment of several diseases, including diabetes, diarrhea, stomachaches, fevers, hepatitis and malaria (Maciel et al., 2007). *C. cajucara* has been shown to possess anti-genotoxicity, anti-atherogenic, anti-tumor, anti-ulcerogenic, hypoglycemic, hypolipidemic, anti-estrogen, anti-inflammatory and anti-nociceptive activities (Maciel et al., 2000; 2006). The leaves of *C. cajucara* contain steroids and flavonoids, both as major compounds and its bark is a rich source of terpenes, such as *trans*-dehydrocrotonin (DCTN) and crotonin (CTN), both the clerodane-type 19-*nor*-diterpenes and the triterpene acetyl aleuritolic acid (AAA) (Maciel et al., 1998; 2000; 2003).

Crude methanol extract of the bark of *C. cajucara* and its isolated terpenes, DCTN, CTN and AAA (Fig. 1) were conducted against *Trypanosoma cruzi*. In these assays, the crude extract was more effective than the isolated clerodanes DCTN or CTN on trypomastigote while the trypanocidal effect of the triterpene AAA was against epimastigotes as well as on intracellular amastigotes (Campos et al., 2010).

These early results indicating the effectiveness of terpenes against *T. cruzi* led us to investigate the anti-leishmanial activity of *C. cajucara* terpenes (DCTN, CTN and AAA) obtained from the bark of this *Croton* species, against promastigotes, axenic and intracellular amastigotes of *Leishmania amazonensis*. Furthermore, the effects of DCTN and CTN on the trypanothione reductase enzyme were also investigated.

Material and methods

Plant material

The stem bark of *C. cajucara* was collected in the Pará state (Amazonian region of Brazil) and identified by Nelson A. Rosa. A voucher specimen (no. 247) has been deposited in Herbarium of the Museu Paraense Emílio Goeldi (Belém- Brazil). In addition, the isolated tested samples were compared with voucher specimens.

Preparation of extracts. Terpenes isolation

The extraction of the powdered bark was carried out as previously reported (Maciel et al., 1998; 2003). Hexane followed by MeOH was used for extraction in a Soxhlet apparatus. After evaporation of the solvent, the hexane extract was filtered over a silica gel chromatography column, affording three fractions: A, B and C. According to the previously reported methodologies fractions B and C after submission to chromatography on a silica gel column eluted with mixtures of hexane-CH_2Cl_2-MeOH with increasing polarity give the terpenes AAA (0.06%), CTN (0.02%) and the major DCTN (0.7%). The MeOH extract was to the filtered over a silica gel chromatography column and eluted with hexane-EtOAc at different ratios of increasing polarity, led also isolation of the compounds AAA (0.02%), and DCTN (0.2%). Quantitative purity of the tested isolated compounds was assessed by ^{1}H and ^{13}C 1D and 2D-NMR analyses and also by comparison with data previously reported (Maciel et al., 1998; 2003) and elemental analysis (DCTN anal. calc. for $C_{20}H_{26}O_4$: C 72.79; H 7.93, found C 72.74; H 7.89. CTN anal. calc. for $C_{20}H_{28}O_4$: C 72.26; H 8.49, found C 72.32; H 8.47. AAA anal. calc. for $C_{32}H_{50}O_4$: C 77.06; H 10.10, found C 77.01; H 10.16).

Parasite culture

L. amazonensis promastigotes MHOM/BR/77/LTB0016 strains were grown at 25°C in the Schneider medium from Sigma-Aldrich (St. Louis, MO, USA) supplemented with 20% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM of L-glutamine, penicillin at 100 U/ml and streptomycin at 100 mg/ml from Sigma-Aldrich, at pH 7.2. Cells were harvested in the late log phase, resuspended in fresh medium, counted in Neubauer's chamber, and adjusted to a final concentration of 4×10^6/ml. This strain has been characterized by molecular and immunological techniques (Temporal et al., 2002).

Promastigotes assays

The assays were carried out in 96-well plates in a volume of 180 μl/well. The terpenes were added to a parasite culture in a concentration ranging from 150 to 9.38 μg/ml solubilized in DMSO (the highest percentage used was 1.6%, v/v, which was not hazardous to the parasites). After 24 h incubation at 26°C, the remaining parasites were counted in a Neubauer chamber, and the percentage of inhibition was calculated and compared to the controls (DMSO without the drugs and with the parasites alone). The $IC_{50} \pm SD$ values were calculated by linear regression from these percentages of inhibition x log [dose] using statistical error limits up to 10%. All tests were conducted in triplicate for each concentration, and three independent