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 trypanothione 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 (IC50 = 6.30 ± 0.06 μg/ml) and axenic amastigotes (IC50 = 19.98 ± 0.05 μg/ml) of L. amazonensis. The cytotoxic effect of the best active natural product was evaluated on mouse peritoneal infected macrophages (IC50 = 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 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 sand-flies. 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...
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, they have also showed highly toxic effects. Recently, miltefosine, an alklyphosphocholine 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 dehydrocrotonin (DCTN) and crotonin (CTN), both the clerodane-type 19-nor-terpenes and the triterpene acetyl aleuritolic acid (AAA) isolated from bark of Croton cajucara.

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 submision to chromatography on a silica gel column eluted with mixtures of hexane-CH3Cl-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 1H and 13C 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 C20H26O4: C 72.79; H 7.93; found C 72.74; H 7.89. CTN anal. calc. for C19H24O4: C 72.26; H 8.49, found C 72.32; H 8.47. AAA anal. calc. for C32H50O4: 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 × 10^5/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 0.25 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 IC50 ± 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
assays were performed. The drug pentamidine isethionate (May & Baker Lab., England) was used as reference drug.

**Axenic amastigotes assays**

The axenic amastigotes were obtained by promastigote culture in the Schneider medium with a pH of 7.2. After 3 days, the culture was centrifuged for 10 min at 3000 g and was resuspended in the Schneider medium at pH 7.5, and after 5 days in the log phase, the medium was changed and centrifuged in the same conditions as above. The parasite concentration was adjusted at $5 \times 10^5$ parasites/ml using the Trypan blue dye (0.1% PBS) to determine the parasite viability, and the sample was then resuspended in the Schneider medium at pH 5.5 supplemented with 20% FCS. All procedures were completed in an ice bath and were incubated at 26°C for 10 days. At this point, the procedure was repeated in the same conditions as day 5 and was incubated at 32°C. The amastigote culture at the 16 day was ready to use and was then shocked for use in the appropriate assays (Castro-Pinto et al., 2004). The IC$_{50}$ ± SD values were calculated by linear regression from these percentages of inhibition $x$ log [dose] using statistical error limits up to 10%.

**Intracellular amastigotes assays**

Murine peritoneal macrophages were isolated from peritoneal space of BALB/c mouse with iced RPMI-1640 medium (Sigma-Aldrich), supplemented with 1 mM L-glutamine, 1 M HEPES, penicillin G (105 IU-I) and streptomycin sulfate (0.10 g L$^{-1}$). The concentration was adjusted to $2 \times 10^{-6}$ macrophage/ml and incubated in a LAB-TEK chamber at 37°C and 5% CO$_2$ for 1 h. Non-adherent cells were removed and stationary-phase *L. amazonensis* promastigotes were added at a 3:1 parasite/macrophage ratio. The cultures were incubated for further 4 h, and free parasites were removed. The chambers were washed, and the monolayers were incubated with DCTN for further 24, 48 and 72 h. The cultures were fixed in methanol and stained with Instant Prov (Newprov, Curitiba, Brazil) hemological dye, and examined under microscopy. The number of intracellular amastigotes was assessed by counting amastigotes in at least 100 macrophages per each sample and percentage of reduction of infected macrophages. The results were expressed by infection index (IF) using the following equation:

$$IF = \% \text{infected cells} \times \text{amastigotes number/total number of macrophages}$$

The IC$_{50}$ ± SD values were obtained by linear regression using GraphPad Prism 5.0 using statistical error limits up to 10%. All tests were conducted in duplicate for each concentration, and two independent assays were performed.

**Soluble fraction preparation of *L. amazonensis***

The soluble fraction (SF) was obtained from infective promastigotes of the *L. amazonensis* culture (Schneider medium with FCS 10%). The parasites were removed from the medium by centrifugation at 500 g/10 min (Sorvall Biofuge Stratos, Loughborough, LE, UK). The pellet was resuspended in PBS at a pH of 7.2 and was then centrifuged two more times under the same conditions. Finally, 40 mM HEPES (Sigma-Aldrich) and 1 mM EDTA (Sigma-Aldrich) was added to the final buffer. The material was lysed in a Dounce-type homogenizer and centrifuged at 12,500 g/15 min. The supernatant was considered to be the soluble fraction containing trypanothione reductase (TryR) (Castro-Pinto et al., 2007). The whole preparation of FS was carried out at 8–12°C to avoid damage to the enzyme. The protein concentration of FS was assessed using a µ-Quant spectrophotometer (Biokit Instrument Inc., Winooski) at 260 and 280 nm. The concentration was expressed according to the following equation (Johnstone and Thorpe, 1982):

$$\text{Protein concentration (mg/ml)} = (\text{optical density at 280 nm} \times 1.5) - (\text{optical density at 280 nm} 	imes 0.75).$$

All samples was stored at −70°C until the assays were performed.

**Trypanothione reductase assay**

The ability of terpenes (AAA, DCTN and CTN) to inhibit TryR activity was assessed using the equivalent of 1 mg/ml of soluble protein fraction. The terpenes, at 1 mM, were incubated with the soluble fraction for 6 min, and after 40 mM HEPES pH 7.5, 1 mM EDTA, and 100 µM NADPH were added, plus 100 mM trypanothione disulfide (T(S)$_2$), to optimize and direct the reaction to NADPH consumption by TryR. The control contained all reagents with no T(S)$_2$ addition. The assay was initiated in the spectrophotometer (Shimadzu Corporation, Japan) at 340 nm to check the NADPH consumption. All reactions were performed at 25°C in a total volume of 300 µl. The inhibition percentage was calculated based on optical density decrease (González et al., 2005; Castro-Pinto et al., 2004, 2007; Castro et al., 2008).

**Statistical analysis**

Each experiment was repeated thrice, each time in quadruplicate. Significance was determined using non-paired Student’s t test and one-way ANOVA. The differences were considered to be significant when $p < 0.05$.

**Results and discussion**

The *C. caujaca* clerodane diterpenes DCTN, CTN and triterpene AAA were evaluated for anti-leishmanial effects against *L. amazonensis*. Promastigotes showed a higher sensitivity to clerodane diterpene DCTN with IC$_{50} = 12.07 \pm 0.06$ µg/ml at 24 h of culture (Table 1). The three terpenes were then assayed with *L. amazonensis* axenic amastigotes in 24 h of culture, showing a similar activity than against promastigotes. The most active compound was the DCTN with IC$_{50} = 19.98 \pm 0.05$ µg/ml. The assays with axenic amastigotes showed a higher activity of DCTN with IC$_{50} = 12.50 \pm 0.08$ and 5.63 ± 0.07 µg/ml in culture for 48 and 72 h, respectively. Thus, after these promising results, the cytotoxicity and activity of *nor*-clerodane diterpene DCTN against amastigotes was evaluated in mouse peritoneal macrophage cells. Pentamidine, an anti-parasitic agent, was used as positive control and was also assayed in the same culture conditions. The macrophage cells in the presence of DCTN were not killed up to concentration of 100 µg/ml after 24, 48 and 72 h of culture, in accordance with previous reports for *T. cruzi* infected macrophage cells (Campos et al., 2010). Additionally, DCTN has been shown to have no genotoxic or cytotoxic effects (Agner et al., 1999). The treatment of macrophage infected culture revealed that

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>Promastigotes</td>
<td>Axenic amastigotes</td>
</tr>
<tr>
<td>DCTN</td>
<td>12.07 ± 0.06</td>
</tr>
<tr>
<td>AAA</td>
<td>41.7 ± 0.02</td>
</tr>
<tr>
<td>CTN</td>
<td>48.0 ± 0.09</td>
</tr>
<tr>
<td>pentamidine$^b$</td>
<td>23.1 ± 0.04</td>
</tr>
</tbody>
</table>

$^a$ SD = standard deviation.

$^b$ Positive control.
the terpenes showed that these compounds interfere in the system trypanothione/trypanothione reductase (Fig. 2). The tested C. cajucara natural products were able to inhibit the enzyme activity \( (p < 0.01) \) when compared to control and to control with addition of the substrate trypanothione; however, the terpene DCTN was more effective. Future investigations of these terpenes on glutathione/glutathione reductase system present in mammal cells should be performed.

Conclusion

Clerodane diterpenes from C. cajucara showed promising in vitro antileishmanial effects against L. amazonensis. DCTN clerodane diterpene presented the best profile against promastigotes, axenic amastigotes and intracellular amastigotes; furthermore, this diterpene did not present macrophage toxicity up to the assayed concentration. In addition, the action on trypanothione reductase enzyme revealed a possible mechanism of action. This is an important finding that could lead to the development of a new therapeutic agent against leishmaniasis.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support and fellowships.

References


Table 2

IC\(_{50}\) values (compound concentration required to kill 50% ± SD of the intracellular amastigotes of Leishmania amazonensis) of DCTN (trans-dehydrocrotonin) and pentamidine, and toxic concentration to kill all macrophages in 24, 48 and 72 h of culture.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment (h)</th>
<th>IC(_{50}) (μg/ml) Intracellular amastigotes</th>
<th>IC(_{50}) (μg/ml) Macrophages</th>
<th>% Macrophage killed SI (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCTN</td>
<td>24</td>
<td>0.47 ± 0.03</td>
<td>&gt;100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.28 ± 0.06</td>
<td>&gt;100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.16 ± 0.01</td>
<td>&gt;100</td>
<td>0</td>
</tr>
<tr>
<td>pentamidine</td>
<td>24</td>
<td>0.34 ± 0.03</td>
<td>37.50 ± 0.02</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.25 ± 0.01</td>
<td>18.75 ± 0.02</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.21 ± 0.01</td>
<td>9.38 ± 0.01</td>
<td>100</td>
</tr>
</tbody>
</table>

\( ^a \) SI = selectivity index (LC\(_{50}\) (macrophages)/IC\(_{50}\) (intracellular amastigotes).

Fig. 2. Effect of Croton cajucara nor-clerodane diterpenes, trans-dehydrocrotonin (DCTN) and crotonin (CTN), and triterpene acetylateduric acid (AAA) on the activity of trypanothione reductase in soluble fractions of infective Leishmania amazonensis promastigotes. The enzyme activity was evaluated by measuring the NADPH consumption compared to two different controls: one without substrate and drugs (control) and another one containing only the substrate trypanothione disulphide (T(S)\(_2\)).

Several natural products have showed to be as selective trypanothione reductase inhibitors in previous studies involving parasites of trypanosomatid class (Gallo et al., 2008; Campos et al., 2010; Macari et al., 2011). The enzyme trypanothione reductase is an important drug target in trypanosomatids because it is essential to the survival of these parasites and is involved in oxidative stress protection (Fairlamb, et al., 1985; Schmidt et al., 2002; Khan, 2007). This enzyme is NADPH dependent and catalyzes a thiol metabolism based on trypanothione, which has a part in regulated redox balance (Schmidt et al., 2002). Thus, in the present study we evaluated the effect of DCTN, CTN and AAA on the trypanothione reductase of L. amazonensis. The trypanothione reductase inhibitory activity was assessed by NADPH consumption in 240 nm spectrophotomeric lecture that works as trypanothione reductase co-factor of fraction soluble from L. amazonensis promastigotes culture (Castro-Pinto et al., 2004, 2007; Castro et al., 2008). The obtained results of trypanothione reductase assays with the terpenes showed that these compounds interfere in the system trypanothione/trypanothione reductase (Fig. 2). The tested C. cajucara natural products were able to inhibit the enzyme activity \( (p < 0.01) \) when compared to control and to control with addition of the substrate trypanothione; however, the terpene DCTN was more effective. Future investigations of these terpenes on glutathione/glutathione reductase system present in mammal cells should be performed.


