Activity of cycloartane-type triterpenes and sterols isolated from *Musa paradisiaca* fruit peel against *Leishmania infantum* chagasi


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**A B S T R A C T**

The aim of the study was to evaluate in vitro the antileishmanial activity of triterpenes and sterols isolated from *Musa paradisiaca* (banana) fruit peel used traditionally to treat leishmaniasis. The compounds were isolated from the ethanolic extract of the peel of the banana fruit by column chromatography. The chemical structure of compounds was determined by $^1$H and $^{13}$C – nuclear magnetic resonance spectroscopy. The cytotoxicity was measured in RAW 264.7 cells and LLC-MK2. Leishmanicidal activity against *L. infantum* chagasi promastigotes was performed by the MTT colorimetric method and activity against amastigotes was assayed in mammalian cells using in situ ELISA method. Five compounds were identified, consisting of three triterpenes: cycloeucalenone, 31-norcycloaudenone and 24-methylene-cycloartenol and a mixture of two sterols: beta-sitosterol and stigmasterol. With the exception of cycloeucalenone, all compounds showed statistically similar activity against promastigote to pentamidine. While, acting against amastigotes, excluding 31-norcycloaudenone, other compounds showed activity similar to amphotericin B. All compounds showed low cytotoxicity in mammalian cells.

**Conclusion**: This study partially confirms the use of *Musa paradisiaca* in folk medicine against leishmaniasis. Further in vivo studies are necessary to evaluate the efficacy.

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**Introduction**

In Northeastern Brazil there are many places where leishmaniasis is endemic with several lethal cases among humans and dogs. A change was observed in relation to the urbanization of this disease, which in the past occurred only in the countryside but nowadays affects large urban centers (Lindoso and Goto 2006). The search for new leishmanicidal compounds is constant since the drug arsenal used to treat this illness is not sufficient and displays undesirable side effects.

Several compounds isolated from plants, such as terpenoids, sterols, flavonoids, alkaloids, phenolics and naphthoquinones have been studied to evaluate their effects on *Leishmania* spp. (Chamb-Bacab and Peña-Rodriguez 2001). *Annona muricata* acetogenins annonacinone and corosollone, and the *Platymiscium floribundum* coumarin scoporane caused different susceptibilities on *Leishmania* spp. promastigotes (Vila-Nova et al. 2013). Some cycloartane-type triterpene glycosides, isolated from the genus *Astragalus*, have exhibited antitumor, immunodepressant, antiviral, and leishmanicidal activities (Ozipek et al. 2005). Cycloartane-type triterpenes can be found in the fruit peel of *Musa* spp. (Imam and Akter 2011).

In our continuous search for leishmanicidal compounds *M. paradisiaca* fruit peel, which is used in folk medicine to combat leishmaniasis (Weigl et al. 1994), was selected to isolate cycloartane-type triterpenes and tested against *L. infantum* chagasi, in attempt to confirm the popular use of this dischargeable material.

**Materials and methods**

**Plant material**

A bunch of unripe “silver” bananas (*M. paradisiaca*) was acquired in the local market in Fortaleza, Ceará, Brazil.
Preparation of ethanol extract of banana peel

The peel of unripe banana fruit was removed, minced and placed to dry at 40 °C for a period of one week. Subsequently, this material was ground and placed in commercial ethanol (70%) for one week. Then, the solution was filtered and the ethanol solvent was removed in a rotary evaporator, yielding the ethanol extract of banana peel (EEBP). Subsequently, the EEBP was subjected to a filtering column using solvents of increasing polarity level, hexane, chloroform, ethyl acetate, methanol and water. The solvent of each organic phase was evaporated to obtain the extracts (Pizzolatti et al. 2003).

Isolation of the compounds by chromatographic methods

The EEBP was subjected to column chromatography on silica gel (δ 0.063–0.200 mm; 70–230 mesh). The columns had varying lengths and sizes in proportion to the sample and eluents of P.A. grade were used in order of increasing polarity: hexane, chloroform, ethyl acetate and methanol. Thin layer chromatography (TLC) was performed using GF 254 silica gel 60 on glass slides, forming the chromatographic plate. The development of the TLC plates was made by spraying with vanillin 2.5% solution of perchloric acid in ethanol (1:1) followed by heating in an oven at about 100 °C.

A silica gel column chromatography of ethanol extract (60 g) of the unripe banana peel was performed. The solvents were used in mixtures of increasing polarity, starting with hexane then hexane/chloroform (1:1), chloroform, chloroform/ethyl acetate (1:1), ethyl acetate/methanol (1:1) and methanol. The fractions (500 ml) were collected and solvents were eliminated in a rotary evaporator. TLC plates sprayed with vanillic/H₃SO₄ reagent were used to compare the obtained fractions and compounds were isolated. The hexane/chloroform fraction (6.6 g), which contained triterpenes by Liberman–Buchard test (Matos 2009), was rechromatographed using the same mixtures of solvents, collecting 70 ml × 10 ml fractions and a compound C1 was obtained from hexane, with hexane/chloroform compound C2 was obtained, with chloroform/ethyl acetate C3 and from ethyl acetate/methanol, compound C4 was separated.

Structural determination of the isolated compounds

The chemical structures of the purified compounds were confirmed by spectroscopic analysis of the nuclear magnetic resonance spectra recorded on a Bruker Avance DRX-300 spectrometer, in CDCl₃.

HPLC-Finger print of hexane:chloroform (1:1) extract and constituents of M. paradisiaca

HPLC instrument: Shimadzu system with a SPD-10A VP detector, SCL-10A VP controller; LC-10A VP bomb, software LC solution, column: CLC ODS (m) 15 cm, running time: 30 min, flow rate: 1 ml/min, detector: 212 nm, mobile phase: 100% methanol grade HPLC, loop of injection: 20 μl.

Cultivation of L. infantum chagasi

Promastigotes of L. infantum chagasi strain I0CL2272 (Department of Pathology/UFC) were grown in Schneider (Sigma®) medium supplemented with gentamicin 40 mg/ml, 5% human male sterile urine and 10% fetal calf serum (FCS) (Cultilab®). Cultures were maintained in a BOD incubator at 23.6 °C and transplanting was done every three to four days.

Amastigotes of L. infantum chagasi strain I0CL2272 were cultured with the murine monocyte cell line RAW 264.7 (Sigma–Aldrich®) in 96 well microplates. Cells were counted in a Neubauer chamber and plated at a density of 1 × 10⁶ cells/well. Promastigotes were added at a ratio 10:1 parasites/cell. Cells were cultured in Dulbecco’s medium (Cultilab®) with 10% FCS, sodium bicarbonate, and 40 mg/ml gentamicin. Bottles were left open and cultivated under glass with 5% CO₂ at 37 °C. After 24 h, the amastigotes inside the monocyte cells were observed under inverted microscope.

Assays on L. infantum chagasi promastigotes

The methodology used to evaluate the leishmanicidal activity of sterols from M. paradisiaca against promastigote and amastigote of L. infantum chagasi I0CL2272 was based on Tempone et al. (2005). The sterols and triterpenes were dissolved in dimethylsulfoxide (DMSO) and diluted in Schneider medium (Sigma®). The final concentration of DMSO did not exceed 1%. L. infantum chagasi were plated in the Schneider (Sigma®) medium supplemented with 40 mg/ml, 5% human male sterile urine and 10% fetal calf serum (FCS) (Cultilab®) and 10% PBS with different concentrations of the drug and the parasite in the absence of the drug (control). The experiments were performed in 96-well plates with compounds at concentrations of 100, 50, 25, 12.5 and 6.25 μg/ml. Promastigotes of L. infantum chagasi were used at 10⁶ parasites/well in logarithmic phase. The reference medicine used was pentamidina. Negative controls were performed with 10⁶ parasites in Schneider (Sigma®) medium supplemented without compounds. The promastigotes were incubated at 26 °C for 48 h. Afterwards cell viability was examined based on the conversion of soluble tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) (Sigma®) into insoluble formazan by mitochondrial enzymes. Twenty μl of MTT [5 mg/ml] per well were added to the culture, and then kept for 4 h at 26 °C. Thereafter, 100 μl of a solution of sodium dodecyl sulfate (SDS) 10%: isopropyl alcohol (1:1) was added. After 15 min stirring a reading was made of the optical density in a spectrophotometer at 570 nm. Assays were performed in triplicate.

Assays on L. infantum chagasi amastigotes

The medium was removed by aspiration, and the drugs were dissolved in DMSO, diluted in RPMI and were added at concentrations of 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 μg/ml in the microtiter plate containing confluent layer of cells with amastigotes. The in situ immunonassay to determine the ECoE ELISA was performed according to Piazza et al. (1994). Before starting the tests, the infected cells were stained and parasites observed under a microscope. When more than 50% of infected cells in a microscopic field were observed, the parasite load was considered appropriate to perform the test. Briefly, after 18 h incubation with the compounds, the microplate wells were incubated with 0.01% saponin (Sigma–Aldrich®) in 1 × PBS plus 1% bovine serum albumin (Sigma–Aldrich®) for 30 min at 37 °C. The blocking reaction was made with 1 × PBS plus 5% skimmed milk (Nestle®) for 30 min at 37 °C. The microplates were washed and dried three times, then the serum from immunized rabbits diluted 1:500 in 1 × PBS was added, followed by 3% skimmed milk 0.05% in Tween 20 (PBSLT) plus 10% FCS, and the plates were kept at 37 °C overnight. The conjugated anti-rabbit IgG (Sigma–Aldrich®) diluted 1:10,000 in PBSLT and after further washing, orthophenylene diamine chromogen (OPD) (Sigma–Aldrich®) was added. In order to stop the reaction 4 N hydrochloric acid (Novaquímica®) was added, and the reading was performed using a 570 nm filter in a microplate reader. The positive control for this assay was 40 μg/ml amphotericin B (Sigma–Aldrich®) and the negative control was the Dulbecco medium alone (Cultilab®).
Cytotoxicity to mammalian cells

RAW 264.7 and LLC-MK2 cell were cultured in 96-well plates at a concentration of 10^5 cells per well in RPMI medium containing 10% of modified PBS at 37 °C and 5% CO₂. After 48 h, the medium was changed and incubated with the extracts at different concentrations under the same conditions. After 24 h, cell viability was determined by MTT assay. 20 μl of MTT solution (5 mg/ml in PBS) was added to each well and incubated for 3 h at 37 °C and 5% CO₂. Subsequently, the medium was aspirated and the culture was added 100 μl of DMSO. The absorbance was determined with a spectrophotometer at a wavelength of 570 nm.

Statistical analysis

The EC₅₀ values, drug concentration able to inhibit 50% of parasites, with a confidence interval of 95% were calculated using a non-linear regression curve using the statistical software GraphPad Prism 5.0. The entire experiment was performed in triplicate.

Results

The structures of isolated compounds were elucidated by ¹H and ¹³C NMR, including uni- and bidimensional techniques, and also compared with literature data.

The chemical composition of the lipophilic extracts of unripe pulp and peel of banana fruit ‘Dwarf Cavendish’ (Mus a cuminiata) was determined by gas chromatography–mass spectrometry. In banana peel, sterols represent about 49–71% of the lipophilic extract with two triterpene ketones (31-norcycloclaudenone and cycloeucalenone) as the major components (Oliveira et al. 2008). Previous studies reported the isolation of several triterpenes such as cyclomusalenol, cyclomusalenone, cyclartanol, stigmaster-7-en-3-ol, 24-methylene cycloartanol, stigmaster-7-methylene, lanosterol and beta-amyrin. Cycloartane triterpenes such as 3-epicyclo-eucalenol, 3-epicyclo-musalenol, 24-methylene polinastanone, 28-nor cyclo musalenone, 24-oxo-29-norcycloartanol have been also isolated from the fruit peel of M. sapientum (Imam and Akter 2011).

The ¹H NMR spectrum of C1 showed a doublet at δ 4.69 (2H, J = 15.4 Hz, geminal coupling) characteristic of a terminal double bond. A double doublet at δ 0.4 and δ 0.6 (J = 3.75 Hz) corresponds to a cyclopropene ring present in cycloartane-type triterpenes, common compounds isolated from banana peel. The absence of signals characteristic of hydroxymethylene groups (~δ 3.2) and the presence of a signal in δ 213 in the ¹³C NMR spectrum suggests a C=O group at C-3 position. The terminal double bond was also confirmed due to peaks at 150.2 ppm (carbon without hydrogen) and 109.1 ppm (a terminal sp³ carbon linked to two hydrogens). These data in comparison with previous reports of Ragasi et al. (2007) characterize C1 as 31-norcycloclaudenone.

The ¹H NMR spectrum of C2 also showed a doublet at δ 4.69 (J = 15.81 Hz) of a terminal double bond, which is confirmed in ¹³C NMR spectrum by the peaks in δ 156.9 (carbon without hydrogen) and 106.0 ppm (a terminal sp² carbon). A cyclopropene methylene is represented by two double doublets at δ 0.4 and δ 0.6 (J = 2.25 Hz). Several methyl groups between δ 0.7 and δ 0.9 characterize a triterpene skeleton and ¹³C NMR spectrum confirms this information and the presence of a ketone group at δ 213. The comparison of overall peaks of ¹H and ¹³C NMR spectra with literature data led to identification of C2 as cycloeucalenone (Khuonghvu et al. 1975).

C3 fraction showed absorption peaks characteristics of a mixture of stigmasterol and β-sitosterol (Chaturvedula and Prakash, 2012), already mentioned in Musa species (Oliveira et al. 2008).

C4 also showed peaks of the cyclopropene moiety at δ 0.3 and δ 0.6, a terminal double bond represented by a doublet at δ 4.7 (J = 18 Hz) and several singlets between δ 0.8 and δ 1.0, which correspond to methyl groups of triterpenes. The analysis of ¹³C NMR spectrum of C4 suggested the structure of 24-methylene cycloartanol based also on the following peaks: C-1 (32.09), C-2 (29.86), C-3 (79.02), C-4 (39.64), C-5 (47.32), C-6 (21.07), C-7 (28.25), C-8 (48.04), C-9 (20.02), C-10 (26.13), C-11 (26.13), C-12 (34.09), C-13 (45.46), C-14 (48.97), C-15 (33.02), C-16 (26.61), C-17 (52.36), C-18 (18.20), C-19 (29.63), C-20 (35.69), C-21 (18.20), C-22 (34.09), C-23 (31.77), C-24 (156.80), C-25 (33.97), C-26 (22.02), C-27 (22.15), C-28 (19.46), C-29 (25.61), C-30 (15.38), C-31 (106.5). These data were similar to those of previous report (Barla et al. 2006).

The chemical structures of M. paradisiaca tested compounds are shown in Fig. 1.

The relative percentage of main constituents of the hexane:chloroform (1:1) extract was achieved by HPLC analysis of total extract and constituents (Fig. 2A). The major compound is 31-norcycloclaudenone (85.22%), followed by cycloeucalenone (9.38%), stigmasterol (3.74%), 24-methylene cycloartanol (1%) and under the conditions of HPLC chromatographic analysis beta-sitosterol appear with the same retention time of cycloeucalenone at 15.11 min. In the HPLC analysis of the mixture stigmasterol and
β-sitosterol (Fig. 2B), the relative percentage of these two steroids was established, then β-sitosterol percentage in relation to initial hexane:chloroform (1:1) extract was possible to measure as 0.66%.

In the leishmanicidal assays, cycloeucalenone showed no activity against the promastigote form of L. infantum chagasi, whereas 31-nor-cycloeucalenone, the mixture stigmasterol + β-sitosterol and 24-methylene-cycloartanol presented leishmanicidal activities with EC50 values of 39.29 µg/ml, 14.35 and 16.55 µg/ml, respectively (Table 1). These activities were similar to the control pentamidine with EC50 value of 23.71 µg/ml.

In relation to amastigote assay, the mixture stigmasterol + β-sitosterol, 24-methylene-cycloartanol and cycloeucalenone with EC50 values of 126.5, 98.75 and 185 µg/ml, respectively, had statistical similarity to the standard drug Amphotericin B with EC50 32.23 (14.30–72.62).

The sterols and triterpenes isolated from M. paradisiaca were tested against RAW 264.7 cells and LLC-MK2 (Table 2). Pentamidine, the control drug, presents higher toxicity than compounds from banana peel.

Table 1
Leishmanicidal activity of triterpenes and sterols isolated from the fruit peel of M. paradisiaca.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>EC50 (µg/ml)</th>
<th>Promastigote</th>
<th>Amastigote</th>
</tr>
</thead>
<tbody>
<tr>
<td>31-Norciclocladenone (C1)</td>
<td>39.29</td>
<td>34.44–44.82</td>
<td>100</td>
</tr>
<tr>
<td>Cycloeucalenone (C2)</td>
<td>&gt;100</td>
<td>185.0</td>
<td>610.5</td>
</tr>
<tr>
<td>Stigmasterol + β-sitosterol (C3)</td>
<td>14.35 (10.38–19.84)</td>
<td>126.5 (64.97–246.2)</td>
<td></td>
</tr>
<tr>
<td>24-Methylene-cycloartanol (C4)</td>
<td>16.55 (11.13–24.60)</td>
<td>98.75 (47.34–206.0)</td>
<td></td>
</tr>
<tr>
<td>Pentamidine</td>
<td>23.71</td>
<td>(18.44–30.50)</td>
<td>32.23</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>–</td>
<td>–</td>
<td>32.23</td>
</tr>
</tbody>
</table>

Table 2
Cytotoxicity in mammalian cell of sterols isolated from the fruit peel of M. paradisiaca.

<table>
<thead>
<tr>
<th>Drug</th>
<th>EC50 (µg/ml)</th>
<th>Cell RAW</th>
<th>Cell LLC-MK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>31-Norciclocladenone</td>
<td>197.7</td>
<td>697.5</td>
<td>359.0</td>
</tr>
<tr>
<td>Cycloeucalenone</td>
<td>241.8</td>
<td>610.5</td>
<td>344.1</td>
</tr>
<tr>
<td>Stigmasterol + β-sitosterol</td>
<td>124.5</td>
<td>377.3</td>
<td>199.9</td>
</tr>
<tr>
<td>24-Methylene-cycloartanol</td>
<td>505.6</td>
<td>2758</td>
<td>249.1</td>
</tr>
<tr>
<td>Pentamidine</td>
<td>17.9</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Similar letters means no statistical difference.

Discussion

The peel, sap and fruit of M. paradisiaca are used in folk medicine in the treatment of leishmaniasis in an endemic area of leishmaniasis in Northwestern Ecuador (Weigel et al. 1994). The main compounds present in Musa spp. are sterols and cycloartane-type triterpenes (Oliveira et al. 2008). Numerous benefits have been attributed to the phytosterols, such as being anti-inflammatory, anti-carcinogenic, and protective of immune systems (Quilez et al. 2003). Ozipek et al. (2005) reported the leishmanicidal and trypanocidal activity of cycloartane-type triterpene glycosides isolated from the lower stem parts of Astragalus oleifolius. These compounds showed notable growth inhibitory activity against Leishmania donovani with IC50 values ranging from 13.2 to 21.3 µg/ml.

A major obstacle in the treatment of leishmaniasis are the side effects caused by the drugs currently available that have high toxicity, including nephrotoxic potential of amphotericin B, second choice drug in Brazil (WHO 2010). Singh et al. (2008) considered compounds with EC50 values of mammalian cells above 250 µg/ml to be safe, so cycloartane-type triterpenes and sterols from M. paradisiaca are safe compounds against tested cells.

Banana peels are used in many recipes to prepare cakes, biscuits and are added in banana sweets preparations, so...
the population already uses it without suffering from any toxic effects (Oliveira et al. 2009). The banana peel due to its leishmanicidal constituents with low toxicity could be a useful source for the development of a product to be used in Leishmaniasis treatment.

Conclusion

The cycloartane-type triterpenes and the mixture of steroids sitosterol and stigmasterol display leishmanicidal action against *L. infantum chagasi* strain and low toxicity to RAW 264.7 and LLC-MK2 cells. The presence of leishmanicidal compounds in banana peel partially supports its ethnopharmacological use.

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