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

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ABSTRACT

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-norcyclolaudenone and 24-methylene-cicloartanol 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-norcyclolaudenone, 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. (Cham-Bacab and Peña-Rodríguez 2001). *Annona muricata* acetogenins annonacinone and corossolone, and the *Platymiscium floribundum* coumarin scoparone 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 leishamanicidal compounds *M. paradisiaca* fruit peel, which is used in folk medicine to combat leishmaniasis (Weigel 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.

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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 solvent ethanol 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 vaniline/H_2SO_4 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 $CDCl_3$.

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 IOCL2272 (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 IOCL2272 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 1×10^6 cells/well. Promastigotes were added at a ratio 10:1 parasites/cell. Cells were cultured in Dulbecco's medium (Cultilab®) with 5% FCS, sodium bicarbonate, and 40 mg/ml gentamicin. Bottles were left open and cultivated under glass with 5% CO_2 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* IOCL2272 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 placed in the Schneider (Sigma®) medium supplemented (gentamicin 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 10^6 parasites/well in logarithmic phase. The reference medicine used was pentamidina. Negative controls were performed with 10^6 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 immunoassay to determine the EC_{50} 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®) was diluted 1:10,000 in PBSLT and after further washing, orthophenylenediamine chromogen (OPD) (Sigma–Aldrich®) was added. In order to stop the reaction 4 N chloric 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®).

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