Antileishmanial Activity of a Calophyllum brasiliense Leaf Extract

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Bibliography
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Introduction

Leishmaniasis is among the most important neglected diseases, affecting more than 12 million people in 88 countries; about 350 million more are at risk of infection [1,2]. In Brazil, the incidence of American cutaneous leishmaniasis (ACL) has increased in recent decades, affecting all age groups and both sexes [3]. In addition to its high incidence and wide geographical distribution, cutaneous leishmaniasis is important because, depending on the infecting species, it can cause destructive, disfiguring, and disabling injuries, with a great impact on the psychosocial adjustment of patients [4].

Unfortunately, the drugs presently utilized in the treatment of leishmaniasis (pentavalent antimonials, amphotericin B, and pentamidine) are limited to some extent by their toxicity to the patients, requirements for intravenous administration and long-term treatment, lack of efficacy, and high cost, and are prone to stimulate drug resistance [5,6]. Numerous studies have shown that these drugs induce apoptosis in Leishmania. Sudhandiran and Shaha [7] showed that intracellular amastigotes of Leishmania donovani treated with potassium antimony tartrate, as assessed by nuclear DNA fragmentation and externalization of phosphatidylserine, showed characteristics of apoptosis.

Apoptosis is a type of programmed cell death characterized by morphological changes such as cell shrinkage, nuclear chromatin condensation, projections of the plasma membrane, and formation of apoptotic bodies. Other features involved in the apoptotic process include externalization of phosphatidylserine, oxidative stress, loss of mitochondrial membrane potential, release of mitochondrial proteins, and protease activity (caspases). These characteristics distinguish it from necrosis, which is considered a passive, rapid, and severe death, in which leakage from the intracellular environment and a consequent inflammatory reaction occur [8].

Calophyllum brasiliense Camb. (Clusiaceae) is a large tree that grows mainly in some areas of the Atlantic Forest in Brazil. Its leaves possess antibacterial activity, cytotoxic activity against certain tumor cell lines, and antimicrobial activity in BALB/c mice infected with Leishmania (Leishmania) amazonensis.

Aiming to identify ultrastructural changes and DNA fragmentation in Leishmania (Viannia) braziliensis, promastigotes were treated with a concentration of the dichloromethane extract and coumarin (−) mammea A/BB from C. brasiliense leaves that inhibited 50% of the parasites (IC₅₀), and were evaluated by transmission and scanning electron microscopy. Ultrastructural changes showed different levels of mitochondrial alterations, including mitochondrial swelling and a reduction in the density of the mitochondrial matrix. Induced DNA fragmentation, as observed by TUNEL, suggested that the extract and coumarin (−) mammea A/BB induced apoptosis-like cell death. These results suggest that the combination of C. brasiliense extract and coumarin (−) mammea A/BB can be considered a promising candidate for the development of new antiprotozoal agents, because of its significant leishmanicidal activity.
fected with Leishmania (Leishmania) amazonensis, and induced healing of lesions in topically treated animals. Brenzan et al. [15] showed that a methylene chloride extract and coumarin (-) from leaves of C. brasiliense had activity against promastigotes of Leishmania (Viannia) braziliensis, with IC50s of 60 and 23.2 µg/mL, respectively. Dichloromethane, aqueous extracts, and coumarins showed anti-Mycobacterium tuberculosis H37Rv activity [16]. The purpose of this study was to evaluate the ultrastructure of promastigotes of L. (V.) braziliensis treated with C. brasiliense and coumarin (-) mammea A/BB, as observed by different microscopic techniques and DNA fragmentation.

Results

The compound coumarin (-) mammea A/BB was identified and the spectral data were described in our previous study [15,17,18] (Fig. 1).

Transmission electron microscopy was used to compare ultrastructural changes in promastigotes of L. (V.) braziliensis after treatment with C. brasiliense, with untreated parasites (Fig. 2). Parasites treated with the dichloromethane extract and isolated coumarin (-) mammea A/BB from C. brasiliense showed significant ultrastructural changes (Fig. 2B,D,F,H) including intense mitochondrial swelling, reducing the density of the mitochondrial matrix (Fig. 2B,D) to C. brasiliense extract and isolated coumarin (-) mammea A/BB. Other important ultrastructural alterations were intense cytoplasmic vacuolization and atypical autophagic vacuoles (Fig. 2D), abnormal chromatin condensation, the presence of many lipid vacuoles (Fig. 2B,D,G,H), and blebs on the cell surface (Fig. 2H).

After promastigotes of L. (V.) braziliensis were treated with the dichloromethane extract and coumarin (-) mammea A/BB from C. brasiliense (60 µg/mL and 23.2 µg/mL, respectively) for 24 h, dramatic changes in the size, shape, and cell surface of the promastigotes were observed by SEM, compared with untreated parasites (Fig. 3). In the untreated group, the promastigotes of L. (V.) braziliensis had a typical elongated form (Fig. 3A). In parasites treated with C. brasiliense, cell shrinkage was observed, with rounded cells. Changes in the cell surface of the parasites included retraction of the membrane and the generation of promastigotes...
with aberrant cell bodies, with the presence of multi-flagellated cells (Fig. 3C,D). Projections of the plasma membrane were also observed in the treated parasites (Fig. 3E).

The assay of genomic DNA fragmentation by agarose gel electrophoresis was performed with promastigotes of L. (V.) braziliensis that were treated with the dichloromethane extract and coumarin (−) mammea A/BB from C. brasiliense (60 µg/mL and 23.2 µg/mL, respectively). After 24 h, nucleosome-sized DNA fragments were observed, with a DNA ladder-like pattern in the agarose electrophoresis gel and varying degrees of smearing, only with the dichloromethane extract (Fig. 4). In the control group, no fragmentation was observed.

Labeled nuclei were used to characterize DNA fragmentation. Fig. 5 shows TdT-labeled nuclei in promastigotes treated with the dichloromethane extract and coumarin (−) mammea A/BB from C. brasiliense (Fig. 5D,F) after 24 h, compared to the control groups. The treated parasites have intensely fluorescent nuclei, indicating DNA fragmentation (Fig. 5B).

Discussion

Drugs commonly used for the treatment of leishmaniasis pose different problems, including high toxicity, side effects, need for hospitalization and long-term administration, and the emergence of resistant strains. In addition to these problems, the high cost of the compounds prevents many patients from obtaining appropriate treatment [5]. Because all of the antileishmanial drugs have severe side effects, in recent years, the use of natural products, especially those derived from plants, has generated interest in finding alternative therapies with higher activity and lower toxicity [19].

In the present study, we evaluated ultrastructural changes as observed by different microscopy techniques and DNA fragmentation in L. (V.) braziliensis after treatment with extracts and, mainly, coumarin (−) mammea A/BB isolated from leaves of C. brasiliense. Promastigotes that were treated with C. brasiliense showed different levels of mitochondrial changes, including mitochondrial swelling and a reduction in the density of the mitochondrial matrix.

Other studies have demonstrated ultrastructural changes in the mitochondrial morphology of promastigote forms of L. amazonensis treated with natural leishmanicidal agents, such as dihydroxy-methoxychalcone from Piper aduncum L. (Piperaceae) inflorescences [20]. Leishmania infantum promastigotes exposed to essential oil and citral underwent considerable ultrastructural alterations, namely mitochondrial swelling [21]. In this study, the treatment with these substances induced ultrastructural changes in the promastigotes of L. (V.) braziliensis, which suggests that mitochondria are involved in the action of these compounds.

Mitochondria are essential organelles that are involved in the regulation of programmed cell death. One of the primary roles of mitochondria is the production of cellular energy by oxidative phosphorylation [22]. Several investigators have suggested that mitochondrial alterations might be caused by a decrease in endogenous sterols, which are essential for cell maintenance and

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the normal structural organization of the mitochondrial membrane of trypanosomatids [23]. These are important targets in the treatment of leishmaniasis.

Chromatin condensation is typically found in cell death that occurs through apoptosis. Atypical intense vacuolization with cytoplasmic disorganization in the parasites that were treated with the dichloromethane extract and coumarin (−) mammea A/BB from *C. brasiliense* leaves induced several ultrastructural changes in the promastigotes of *L. (V.) braziliensis*. Mitochondrial ultrastructural changes, cytoplasmic vacuolization, cell shrinkage, cytoplasmic condensation, nuclear condensation, no change in membrane permeability, fragmentation of genomic DNA, and a rounded shape of the parasites were observed. The results of this study suggest that the *C. brasiliense* extract and coumarin (−) mammea A/BB can be considered promising candidates for the development of new antiprotozoal agents because of their significant leishmanicidal activity. However, further biochemical studies are needed to reveal the detailed mechanism of action of *C. brasiliense* on the promastigotes of *L. (V.) braziliensis*, and to develop less-toxic drugs for the treatment of cutaneous leishmaniasis. Future experiments will focus on the determination of caspase activation, occurrence of the externalization of phosphatidylserine, and depolarization of the mitochondrial membrane potential of cells.

### Materials and Methods

#### Plant material

Leaves of *C. brasiliense* were collected in December 2010 on Car- doso Island in the state of São Paulo, Brazil, and identified by Prof. Dr. Maria Claudia M. Young. A voucher specimen (SP 363818) is deposited and authenticated at the Herbarium of the Instituto de Botânica de São Paulo, São Paulo, Brazil.

#### Preparation of extract of *Calophyllum brasiliense*

Leaf powder (1.354 kg) was dried at room temperature and then macerated in ethanol:water (9:1) with successive extractions until all active compounds were retrieved [27]. The extract was filtered and evaporated in a vacuum evaporator at 35–40°C to remove the organic solvent. The EtOH/H₂O extract dark-green, water-insoluble residue produced was solubilized in dichloromethane and the organic solvent was completely removed at room temperature, yielding a dichloromethane extract (77.14 g) that was stored at −10°C, in the dark, until use.

Subsequently, the dichloromethane extract was chromatographed in a vacuum silica gel column (40 × 8.0 cm) with hexane (1000 mL), hexane/dichloromethane (50:50), dichloromethane; dichloromethane/ethyl acetate (90:10 to 50:50); ethyl acetate, methanol, and, finally, methanol:water (90:10).

#### Purification of coumarin (−) mammea A/BB standard

The procedure for purification and identification of the coumarin (−) mammea A/BB was described in previous studies [17, 18].
Sample preparation
The coumarin (−) mammea A/BB was quantified using a calibration graph with five points. The calibration graph for HPLC was recorded with coumarin (−) mammea A/BB amounts ranging from 15 to 250 µg/mL, and was used in the C. brasiliense extract for quantitative purposes. To obtain the stock solutions, coumarin (−) mammea A/BB was weighed and dissolved in methanol at a concentration of 1000 µg/mL. The solution was filtered through a 0.45-mm membrane filter (Millipore). The samples were prepared in triplicate.

The crude extract from C. brasiliense leaves was weighed and dissolved in methanol at a concentration of 6000 µg/mL and the resulting solution was filtered through a 0.45-mm membrane filter (Millipore). The samples were prepared in triplicate.

Instrumentation and chromatographic conditions
The analyses were carried out using a Waters 1525 liquid chromatograph equipped with a binary pump (LC-10 AD), automatic injection 135 valve (Rheodyne) with a 20-µL loop, CTO10Avp column oven, and a UV/visible light detector (Waters 2489), controlled by Breeze 2 Software. The amount of coumarin (−) mammea A/BB was determined in the extract from C. brasiliense leaves. The HPLC analyses were carried out on a Metasil ODS column at 25°C using acetonitrile-water 55:45 v/v for 80:20 (0-25 min), 80:20 v/v for 100% (10-20 min), 100% acetonitrile (20-25 min), and 55:45 v/v (26-30 min) as the mobile phase, flow rate of 0.6 mL/min, and detection at 336 nm. The run time was 30 min. The sample injection volume was 20 µL. Three determinations were carried out for each sample. The data were analyzed using Statistica 6.0 software (Statsoft, Inc.).

Culture and maintenance of Leishmania
L. (V.) braziliensis (MHOM/BR/1987/M11272) was maintained by the inoculation of 1 × 10⁷ parasites into the hind paws of hamsters. After 30 days, the animals were killed by intraperitoneal administration of ketamine (160 mg/kg) and xylazine (50 mg/kg), and fragments of popliteal lymph nodes were inoculated into 199 culture medium (Gibco®) supplemented with 10% FBS, 1% human urine, 20 mM L-glutamine, and antibiotics (100 IU/mL penicillin and 0.1 mg/mL streptomycin), and incubated at 25°C, then maintained by weekly subculturing of the parasites. The experimental protocol was approved by the Ethics Committee on the Use of Experimental Animals of the State University of Maringá (protocol 051/2010).

Transmission electron microscopy
Promastigotes of L. (V.) braziliensis in log-phase growth at a concentration of 1 × 10⁶ promastigotes/mL were treated with the IC₅₀ of the dichloromethane extract and coumarin (−) mammea A/BB isolated from C. brasiliense (60 µg/mL and 23.2 µg/mL, respectively) for 24 h at 25°C. After this period, the parasites were centrifuged (2000 rpm, 10 min), washed, and the samples were fixed with 2.5% glutaraldehyde in PBS (phosphate buffered saline) at 4°C and postfixed with 2% osmium tetroxide, dehydrated in acetone, and embedded in Epon 812 (Luft 1961). Ultrathin sections were contrasted with 2% uranyl acetate and lead acetate (Reynolds 1963; Watson 1958). Transmission electron microscopy (TEM) was performed with a JEOL 1200EX II transmission electron microscope.

Scanning electron microscopy
For scanning electron microscopy (SEM), promastigotes of L. (V.) braziliensis were challenged with the IC₅₀ of the dichloromethane extract and coumarin (−) mammea A/BB from C. brasiliense (60 µg/mL and 23.2 µg/mL, respectively). After 24 h incubation, the parasites were fixed in 2.5% glutaraldehyde, dehydrated in an acetone series, critical-point dried in a Bal-Tec CPD-030, and gold-coated in a Balzers SCD-030. SEM was performed with a JEOL JSM-6360 LV scanning electron microscope.

DNA fragmentation induced by Calophyllum brasiliense
To detect DNA laddering induced by C. brasiliense, the presence of DNA fragmentation was evaluated according to the method of Mukherjee et al. [28]. L. (V.) braziliensis (1 × 10⁶ promastigotes/mL) was treated with the dichloromethane extract and coumarin (−) mammea A/BB from C. brasiliense (60 µg/mL and 23.2 µg/mL, respectively) for 24 h. Pellets were resuspended in digestion buffer [10 mM ethylenediamine tetraacetic acid (EDTA), 50 mM Tris-HCl (pH 8.0), and 0.5% sodium lauryl sulfate], to which 0.5 mg/mL proteinase K was added, and incubated for 3 h in an ice bath. RNase A (0.1 mg/mL) was added to the mixture and incubated for 1 h at 55°C. The extraction was performed with 1:1 phenol:chloroform, and DNA was precipitated with 3 M sodium acetate and ice-cold ethanol (100%). Following overnight incubation at −20°C, the material was centrifuged at 14000 × g for 30 min, and 2.5 volumes of ethanol (70%) were added to the pellet. The material was again centrifuged at 14000 × g for 15 min. The pellet was air-dried and resuspended in Tris-EDTA buffer (50 µL, pH 8.0). DNA aliquots (10 µg) were electrophoresed on 2% agarose gel that contained ethidium bromide (0.5 µg/mL), using Tris-acetate-EDTA buffer (pH 8.0) for 45 min at 100 V, and photographed under ultraviolet light.

DNA fragmentation analysis by TUNEL
In situ, DNA strand breaks were detected using the APO-BrdU TUNEL Assay Kit™ (Invitrogen) according to the manufacturer’s specifications. Briefly, promastigotes of L. (V.) braziliensis (2 × 10⁶) are incubated for 24 h with or without the IC₅₀ of the dichloromethane extract and coumarin (−) mammea A/BB from C. brasiliense (60 µg/mL and 23.2 µg/mL, respectively), washed, and resuspended in 0.5 mL PBS. The parasite suspension is incubated with 5 µL of 1% paraformaldehyde diluted in PBS and kept on ice for 15 min. Then the cells are centrifuged at 300 × g for 5 min, rinsed in 5 mL PBS, and resuspended in 0.5 mL PBS. Five mL of the solution containing the parasites are added to cold 70% ethanol and incubated overnight for 15 h at −20°C. Then the parasites are centrifuged to remove the ethanol, resuspended in 1 mL PBS, and again centrifuged at 300 × g for 5 min. Subsequently, the parasites are resuspended in 50 µL of solution for labeling DNA [10 mL reaction buffer, 0.75 mL of TdT (deoxynucleotidyl terminal transferase), 8 µL of BrdU (5-bromo-2′-deoxyuridine-5′-triphosphate), and the final volume is completed to 31.25 mL with deionized water]. Incubation is carried out for 1 h at 37°C in a water bath. Every 15 min, the parasites are agitated to keep them in sus-
pension. At the end of incubation, 1 mL of rinse buffer (Triton X-100 and 0.1% BSA 5 mg/mL diluted in PBS) is added and the mixture is centrifuged for 5 min. The supernatant is removed and the parasites resuspended in 100 µL of a solution containing anti-BrdU antibody. Then the mixture is incubated for 30 min at room temperature and afterward analyzed by a fluorescence microscope.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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