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Article

Leishmanicidal Activity of *Brosimum glaziovii* (Moraceae) and Chemical Composition of the Bioactive Fractions by Using High-Resolution Gas Chromatography and GC-MS

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Dando continuidade às pesquisas de bioprospecção dos programas SisBiota CNPq/FAPESP e Biota-FAPESP, o presente artigo descreve os resultados de atividade leishmanicida e determinação da composição química das frações ativas de *Brosimum glaziovii* (Moraceae), por meio da cromatografia gasosa. Os extratos e frações das folhas e galhos foram testados *in vitro* em cepas de *Leishmania amazonensis*. As frações hexânicas das folhas e galhos apresentaram atividades potente e moderada, respectivamente (IC_{50} = 3,6 e 39,1 μg mL^{-1}). Por meio das análises de cromatografia gasosa foi possível identificar os esteróis: campesterol, β-sitosterol e estigmasterol e os triterpenos: α-amirina, β-amirina, acetato de β-amirina, e lupenona, além de outros constituintes apolares principalmente ácidos graxos e seus derivados. Este é o primeiro trabalho descrito sobre a atividade leishmanicida e composição química desta espécie.

As part of our ongoing SisBiota CNPq/FAPESP and Biota-FAPESP bioprospecting programs, this paper deals with the leishmanicidal properties of *Brosimum glaziovii* (Moraceae), and used gas chromatography analysis to determine the chemical composition of these fractions. The extracts and fractions from the leaves and branches of *B. glaziovii* were screened against *Leishmania amazonensis*. The hexane fractions from the leaves and branches displayed the highest leishmanicidal activities, with IC_{50} = 3.6 and 39.1 μg mL^{-1}. Using gas chromatography analysis it was possible to identify the sterols campesterol, stigmasterol, and β-sitosterol, as well as the triterpenes α-amyrin, β-amyrin, β-amyrin acetate, and lupenone, and other nonpolar components, mainly fatty acids and their derivatives. This is the first report on the leishmanicidal activity and the chemical composition of *B. glaziovii*.

Keywords: *Brosimum glaziovii*, leishmanicidal, sterol, triterpene, gas chromatography

Introduction

Neglected tropical diseases (NTDs) are a pathologically diverse group of infections caused by a variety of pathogens, such as viruses, bacteria, protozoa, and helminthes. According to the World Health Organization (WHO), 17 neglected tropical diseases exist. They are endemic in 149 countries and affect more than 1 billion people worldwide.[1] The tropical and subtropical regions of the globe concentrate the main countries with the lowest human development index (HDI) and the highest NTDs burden.[2]

Brazil ranks as the 70th country in terms of HDI; nine of the 10 major NTDs occur in this country. Leishmaniasis, tuberculosis, dengue, and leprosy exist in almost all the Brazilian regions. Most cases arise in poor regions, being the northern and northeastern Brazilian regions the most affected.[2]

Leishmaniasis threatens approximately 350 million people around the world. It is believed that as many as 12 million people are currently infected with this pathogen. Roughly 1-2 million new cases appear every year.[3] The

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human immunodeficiency virus (HIV) pandemic has contributed to raising the number of leishmaniasis cases in endemic areas.[4] In Brazil, more than 26,000 cases of cutaneous leishmaniasis (CL) emerged between 1988 and 2009;[5] more than 70,000 cases of visceral leishmaniasis (VL) arose between 1980 and 2008, with 3,800 deaths.[6]

Currently, leishmaniasis treatment relies on chemotherapy, which poses limitations such as toxicity, difficult administration route, and lack of efficacy in endemic areas. Despite the efforts devoted to finding new drugs that can fight against *Leishmania* spp., leishmaniasis treatment still depends on the use of pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), first developed over 60 years ago. Unfortunately, the emergence of resistant strains has limited the usefulness of these antimonials. Amphotericin B (and its liposomal formulation, Ambisome), pentamidine isethionate, miltefosine, and paromomycin constitute alternative medications, but their toxicity and high prices have limited their use.[7]

Brazilian biodiversity is among the richest in the world. Indeed, Brazil is home to many plant species with unknown potential. These plants could find appplication in the search for natural products to treat health problems, like NTDs.

Plants belonging to the family Moraceae - *Cecropia pachystachya* Mart.,[8] *Ficus mucusa*,[9] and *Pourouma guianensis*,[10] among others, possess antiprotozoal activities. In particular, *Brosimum glaziovii* (Moraceae) is endemic in Brazil, but habitat loss threatens this species. The absence of reports on the chemical composition and biological potential of this plant has motivated the phytochemical and biological investigation that is the subject of this article.

To our knowledge, only three species of the same genus as *B. glaziovii* have been phytochemically examined: *B. gaudichaudii*, whose roots contain coumarins, chalcones, cinnamic and dihydrocinnamic acid derivatives, triterpenes, and sterols;[11-13] *B. acutifolium*, which possesses flavonoids and lignoids;[14-18] and *B. rubescens* Taubert, whose heartwood accumulates coumarins.[19]

As part of the SisBiota CNPq/FAPESP and Biota-FAPESP biodiscovery programs, which aim to search for new hits and lead compounds from the Brazilian flora, this study evaluated the leishmanicidal activity of *Brozimum glaziovii* (Moraceae) and used high-resolution gas chromatography (HRGC) and gas chromatography-mass spectrometry (GC-MS) to analyze the chemical composition of the bioactive extracts. HRGC constitutes a promising tool to identify the chemical profile of complex nonpolar extracts and fractions. It is an alternative methodology to aid the construction of the inventory and the characterization of the biodiversity of São Paulo state, which should be useful when exploring mechanisms for its conservation and sustainable use.

Experimental

Plant material

The leaves and branches of *Brosimum glaziovii* were collected from the Botanic Garden of São Paulo (São Paulo, Brazil) in October 2006 and identified by Dr Inês Cordeiro (IBt-SMA). A voucher specimen (col. S. Romaniuc Neto 1298) was deposited at the State Herbarium "Maria Eneida P. Kaufman" of the Institute of Botany (São Paulo, Brazil).

Preparation of the extracts

Air-dried and powdered leaves (0.85 kg) and branches (2.5 kg) were exhaustively extracted by maceration with ethanol at room temperature. After filtration, the solvent was evaporated at low temperature (< 40 °C) and under reduced pressure, to yield the crude extract from the leaves (35.3 g) and branches (57.3 g). Part of the crude extract from the leaves (10 g) and branches (17 g) was dispersed in water/methanol 4:1 (600 mL) and successively partitioned with hexane (200 mL, 3×), ethyl acetate (200 mL, 3×), and *n*-butanol (200 mL, 3×), to afford four fractions from the leaves, namely the hexane (1.5 g), ethyl acetate (1.2 g), *n*-butanol (0.5 g), and the lyophilized aqueous methanol (0.7 g) fractions, as well as four fractions from the branches - the hexane (2.5 g), ethyl acetate (6.1 g), *n*-butanol (2.1 g), and the lyophilized aqueous methanol (2.2 g) fractions. Samples of the ethanol extracts and of the hexane, ethyl acetate, *n*-butanol, and the lyophilized aqueous methanol fractions were further assayed for their leishmanicidal action. The hexane fractions were analyzed by gas chromatography coupled to a flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS).

Sample cleanup for HRGC analysis (GC-FID)

Samples of the hexane fraction from the leaves and branches (10.0 mg) were dissolved in chloroform (3.0 mL) and percolated through a chromatographic column consisting of celite/norit (1:1, 100.0 mg) + silica gel (200.0 mg); the fractions were eluted with chloroform (10.0 mL). After evaporation to dryness at room temperature, the eluate was dissolved in hexane/ethyl acetate (7:3) and analyzed by HRGC, in triplicate.

GC-FID conditions

The hexane fractions were analyzed by HRGC on a Varian model CP-3800 gas chromatograph equipped with split injector [initial splitless; 0.75 min (1:50); 2.00 min (1:20)] at 260 ºC. The flame ionization detector was set at 310 and 290 ºC for the SPB-5 and SPB-50 columns, respectively. The injected volume was 2.0 µL. SPB-50 (cross-linked 50% phenylmethylsiloxane, 30 m × 0.25 mm × 0.25 µm) and SPB-5 (cross-linked 5% phenylmethylsiloxan 30 m × 0.25 mm × 0.25 µm) capillary columns were employed. In the case of SPB-50, the column temperature was 280 ºC (isotherm). For SPB-5, a column temperature program was used - the initial temperature was 250 ºC, maintained for 12 min, followed by a temperature rise to 280 ºC at 6 ºC min^{-1}. Then, the temperature was kept at 280 ºC for additional 30 min. Nitrogen was employed as the carrier gas at an average linear velocity of 1 mL min^{-1}. The triterpenes and sterols were identified by comparison of the relative retention (RR) of the samples with the RR of authentic standards. Cholesterol was used as internal standard.

Authentic triterpene and sterol standards

The triterpenes and sterols used as standards were previously isolated from Lauraceae and Rubiaceae species, including *Alibertia macrophylla*, *A. sessilis*, *Licaria rodriguesii*, *L. subbulata*, and *Aniba parviflora*. The molecular structures of these compounds were identified by the ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and the MS techniques.[20,21]

GC-MS conditions

Coupled gas chromatography-mass spectrometry (GC-MS) analysis was performed on an Agilent Technologies 7890A gas chromatograph coupled to a 5975C mass selective detector (Agilent Technologies) under the following instrumental conditions: DB-5 capillary column (cross-linked 5% diphenyl/95% dimethyl polysiloxane, 30 m × 0.25 mm × 0.25 µm, Agilent Technologies Inc., USA). Helium was used as the carrier gas at a flow rate of 1.00 mL min^{-1}. The injection volume was 1 µL; the split ratio was 20:1. The oven temperature was increased to 50 °C and held at this temperature for 2 min. Then, the temperature was raised to 250 °C at a rate of 8 °C min^{-1}, to 300 ºC at a rate of 3 °C min^{-1}, and to 310 °C at a rate of 3 °C min^{-1}. The total run time was 47 min. The mass spectra were obtained by electron ionization (EI) at 70 eV. All registered peaks were identified by comparison of the mass spectra with those available in the NIST library.

Parasite cultures

Promastigotes of the *Leishmania amazonensis* MPRO/BR/1972/M1841-LV-79 strain were maintained at 28 ºC in liver-infusion tryptose medium (LIT) supplemented with 10% heat-inactivated fetal calf serum (FCS). LIT medium was prepared by mixing 10 mg mL^{-1} hemin (bovine, type I) (1 mL) with a solution containing NaCl (4.0 g), KCl (0.4 g), Na_2PO_4 (8.0 g), glucose (2.0 g), liver infusion broth (5.0 g), and tryptose (5.0 g) at pH 7.0 (900 mL).

Evaluation of the leishmanicidal activity

An antileishmanial assay using promastigote forms of the *Leishmania amazonensis* MPRO/BR/1972/M1841-LV-79 strain was performed by employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) colorimetric method described by Santos *et al.*[22] At the end of the exponential growth phase (4-day-old culture forms), the cultured promastigotes were seeded in 96-well microplates at 8×10^6 parasites mL^{-1}. The *B. glaziovii* extracts and fractions were dissolved in dimethyl sulfoxide (DMSO; the highest concentration was 1.4%, which was not hazardous to the parasites, as assessed previously), added to the parasite suspension to final concentrations between 1.6 and 100 µg mL^{-1}, and incubated at 28 ºC for 72 h. The assays were carried out in triplicate. The reference drug was pentamidine isethionate purchased from Sigma-Aldrich (St Louis, MO, USA). To this end, a 16.7 mg mL^{-1} stock solution of the reference drug was prepared in sterile deionized water and added to the parasite suspension, to obtain final pentamidine isethionate concentrations between 1.6 and 100 µg mL^{-1}. Leishmanicidal effects were assessed by the MTT method with modifications.[22] The 50% mean inhibitory concentration (IC_{50}) of the tested compounds and the positive control were determined by calculating the percentage of cytotoxicity.

Evaluation of the cytotoxicity on J774 macrophages

To evaluate the cytotoxicity of the *B. glaziovii* extracts and fractions on J774 macrophage lineages, the latter cells were grown in 25 cm^2 culture flasks containing 5 mL of Roswell Park Memorial Institute (RPMI) medium supplemented with 10% bovine fetal serum, kept in an incubator at 37 °C and 5% CO_2 atmosphere, and transferred to a new medium on a weekly basis. After removal of the medium, the cell monolayer was removed from the flask with a scraper and transferred to a 15 mL conical tube containing 1 mL of RPMI medium (RPMI 1640 medium consisting

of Gibco® powder, distilled water, sodium bicarbonate, Hepes buffer, bovine fetal serum, and antibiotics); the concentration of the cells was set to 1×10^5 cells mL^{-1}. Cell viability was evaluated by addition of the vital dye trypan blue (0.02%). The cell suspension (200 µL) was distributed in TPP 96-well plates and incubated at 37 °C and 5% CO_2 for 24 h. The supernatant was removed from the plate; then, 97 µL of RPMI and 3 µL of the extracts and fractions that had activity < 100 µg mL^{-1} were diluted with sterile water and added in decreasing concentrations (250 to 1.5 µg mL^{-1}), in triplicate. Next, the cells were incubated at 37 °C and 5% CO_2 for 24 h. Pentamidine was used as the reference drug. After incubation, 10 µL of MTT/phenazine methosulfate (PMS) solution was added, and the cells were incubated at 37 °C and 5% CO_2 for 75 min. Then, 100 µL of sodium dodecyl sulfate (SDS) was added, which was followed by incubation at room temperature for 30 min. The absorbance at 540 nm was measured in a microplate reader (Elisa reader, Readwell Touch/Robonik). The results were expressed in LC_{50} (lethal concentration that killed 50% of the cells).

The selectivity index (SI), an important characteristic to define hit compounds, was calculated by dividing the values obtained for the cytotoxic activity against macrophages J774 by the results achieved during leishmanicidal actions ($SI = LC_{50} / IC_{50}$) for the most active extracts and fractions.

Results and Discussion

To expand the current knowledge about the plants of the Moraceae family, in this work we evaluated the antileishmanial potential of extracts and fractions from the leaves and branches of *B. glaziovii* (Moraceae) and investigated the chemical profile of the active fractions by high-resolution gas chromatography (HRGC) and gas chromatography-mass spectrometry (GC-MS). Antileishmanial assays of the extracts and fractions from the leaves and branches of *B. glaziovii* against the promastigote form of *L. amazonensis* helped us to achieve our goals. Table 1 summarizes the main results.

Among all the tested fractions and extracts, the nonpolar hexane fraction from the leaves of *B. glaziovii* displayed the highest activity against *L. amazonensis*, with IC_{50} of 3.6 ± 0.34 µg mL^{-1}. This result was close to that obtained with the positive control pentamidine ($IC_{50} = 4.0 \pm 0.26$ µg mL^{-1}), the drug of choice to treat cutaneous leishmaniasis in South America.[23] The activity of the hexane fraction from the leaves of *B. glaziovii* was higher than that of the corresponding ethanol extract ($IC_{50} = 82.9 \pm 0.48$ µg mL^{-1}). This evidenced that fractionation potentiated the activity by concentrating the most active compound(s) in the hexane fraction. The ethyl acetate fraction from the leaves of *B. glaziovii* had lower biological activity than the hexane fraction (51.8 ± 2.26 µg mL^{-1}). Nevertheless, these data indicated that *B. glaziovii* contains antileishmanial compounds with different polarities.

As for the leishmanicidal action of the branches of *B. glaziovii*, fractionation also potentiated the activity, as verified by comparison of the activities of the hexane and ethyl acetate fractions (IC_{50} = 39.1 ± 1.15 and 48.7 ± 1.87 µg mL^{-1}, respectively) with that of the crude ethanol extract (IC_{50} > 100 µg mL^{-1}), considered inactive. Hence, the branches of *B. glaziovii* also presented leishmanicidal activity, albeit almost 10-fold lower than the leaves.

Regarding cytotoxicity evaluation, we calculated the selectivity index (SI), a relevant characteristic to define hit compounds, for the ethanol extracts and the hexane

Table 1. Leishmanicidal and cytotoxic activities of the extracts and fractions from *B. glaziovii* in µg mL^{-1}

Extracts and fractions	Antiprotozoal activity (IC_{50}) *Leishmania amazonensis*	Cytotoxicity (LC_{50}) Macrophage JJ74	Selectivity index (SI)
Leaves			
Ethanol	82.9 ± 0.48	266.50 ± 4.95	3.21
Hexane	3.6 ± 0.34	129.25 ± 1.06	35.90
Ethyl acetate	51.8 ± 2.26	200.75 ± 7.42	3.87
n-Butanol	> 100	–	–
Aqueous methanol	> 100	–	–
Branches			
Ethanol	> 100	–	–
Hexane	39.1 ± 1.15	127.25 ± 1.06	3.25
Ethyl acetate	48.7 ± 1.87	200.50 ± 2.12	4.12
n-Butanol	> 100	–	–
Aqueous methanol	> 100	–	–
Pentamidine[a]	4 ± 0.26	7.5 ± 0.35	1.87

[a]Positive control.

and ethyl acetate fractions from the leaves and branches of *B. glaziovii*. To this end, we divided the cytotoxic activity of the extract or fraction against J774 macrophages (LC_{50}) by its leishmanicidal activity (SI = LC_{50} / IC_{50}). We employed macrophages to determine SI, because they are the main host cells for *Leishmania* (Table 1). Using the LC_{50} value of 129.3 μg mL^{-1} found for J774 macrophages treated with the hexane fraction from the leaves of *B. glaziovii* (which was the most active fraction), we calculated an SI of 35.9 for *L. amazonensis*, which is almost twenty times higher than that of the control drug pentamidine (SI = 1.9, Table 1). Therefore, this fraction possesses good selectivity for *L. amazonensis* promastigote forms rather than macrophages. The SI values determined for the other active extracts and fractions (ethanol extract and ethyl acetate fraction from the leaves and the hexane and ethyl acetate fractions from the branches) indicated that they were less selective than the hexane fraction from the leaves, but they still were twice more selective for the parasite than the control drug pentamidine (SI = 1.9). Hence, these extracts and fractions are also attractive for further chemical characterization.

The biological activities of nonpolar extracts have often been attributed to complex mixtures of triterpenoid and/or steroid compounds.[24,25]

Gas chromatography coupled with mass spectrometry (GC-MS) is the most powerful technique to characterize nonpolar compounds in a mixture, so it constitutes an alternative to the preliminary screening of complex plant extracts. However, this hyphenated analytical technique is still inaccessible to many research groups. On the other hand, GC-FID analysis is rapid, simple, relatively cheap, and more readily available, so it is potentially applicable as a preliminary procedure.[21]

Aiming to identify the steroids and triterpenes present in the most active fractions from the leaves and branches of *B. glaziovii*, we used high-resolution gas chromatography (HRGC), a fast method that does not require pre-derivatization. The GC-FID method we employed in this study involved two different columns: SPB-5 and SPB-50. The low polarity, inertness toward organic compounds, and high temperature limit of these columns make them ideal for analysis of underivatized semi-volatile plant extracts.

By using the SPB-5 and SPB-50 columns to analyze the hexane fraction from the leaves of *B. glaziovii*, it was possible to obtain chromatograms with well-resolved peaks, which enabled proper identification of some components by RR values and comparison of these values with those of authentic samples (Figures 1A, 1B, 1C, and 1D). The RR values were calculated by dividing the retention time

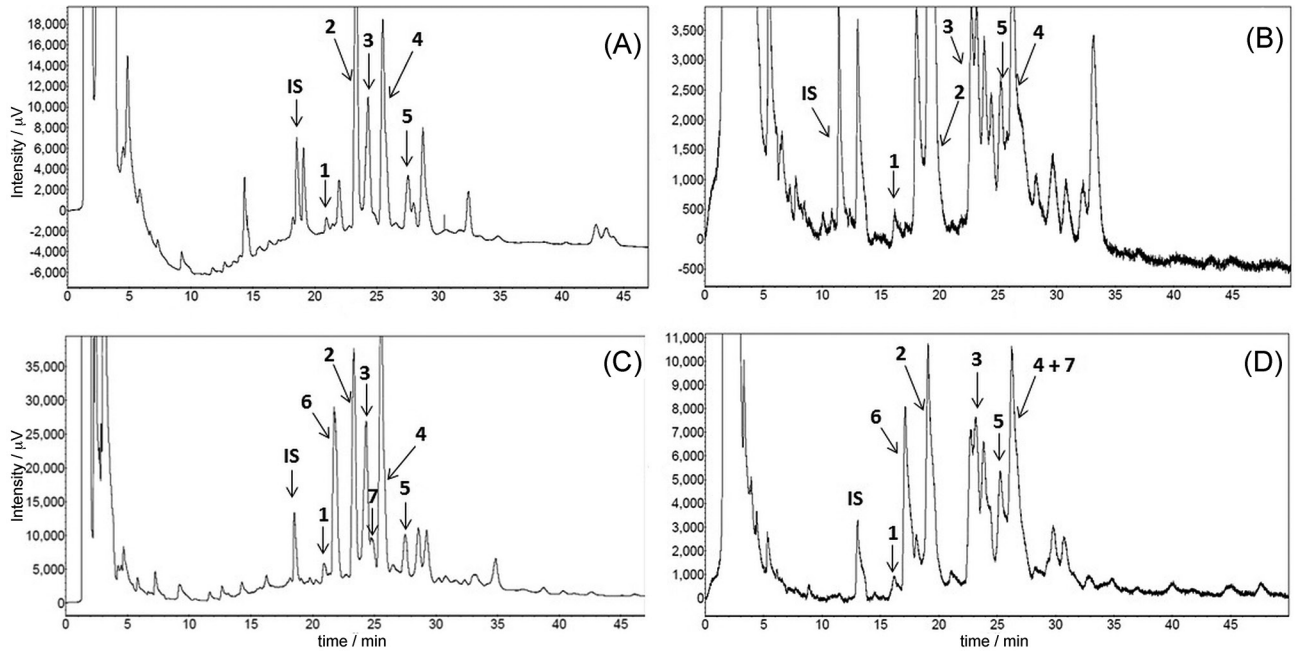

Figure 1. Gas chromatogram (HRGC) of *B. glaziovii*. (A) Leaves; SPB-5 column [**IS**: internal standard (cholesterol) (t_R = 18.60 min); (**1**) campesterol (t_R = 20.95 min); (**2**) β-sitosterol (t_R = 23.35 min); (**3**) β-amyrin (t_R = 24.32 min), (**4**) α-amyrin (t_R = 25.51 min), and (**5**) β-amyrin acetate (t_R = 27.53 min)]. (B) Leaves; SPB-50 column [**IS**: internal standard (cholesterol) (t_R = 13.06 min); (**1**) campesterol (t_R = 16.19 min); (**2**) β-sitosterol (t_R = 19.09 min); (**3**) β-amyrin (t_R = 23.26 min), (**5**) β-amyrin acetate (t_R = 25.22 min), and (**4**) α-amyrin (t_R = 26.22 min)]. (C) Branches; SPB-5 column [**IS**: internal standard (cholesterol) (t_R = 18.53 min); (**1**) campesterol (t_R = 20.90 min); (**6**) stigmasterol (t_R = 21.75 min); (**2**) β-sitosterol (t_R = 23.35 min); (**3**) β-amyrin (t_R = 24.33 min); (**7**) lupenone (t_R = 24.92 min); (**4**) α-amyrin (t_R = 25.56 mim), and (**5**) β-amyrin acetate (t_R = 27.50 min)]. (D) Branches; SPB-50 column [**IS**: internal standard (cholesterol) (t_R = 13.05 min); (**1**) campesterol (t_R = 16.15 min); (**6**) stigmasterol (t_R = 17.13 min); (**2**) β-sitosterol (t_R = 19.06 min); (**3**) β-amyrin (t_R = 23.20 min); (**5**) β-amyrin acetate (t_R = 25.23 min), and (**4**) α-amyrin (t_R = 26.26 mim), and (**7**) lupenone (t_R = 26.26 min)].

(t_R) of the compound by the retention time of the standard for both columns. Co-injection with authentic standards additionally confirmed the presence of each triterpene or sterol and avoided misclassifications due to overlapping of the retention zones.

The HRGC results (Table 2) attested to the major occurrence of the sterols campesterol (**1**), and β-sitosterol (**2**) and of the triterpenes β-amyrin (**3**), α-amyrin (**4**) and β-amyrin acetate (**5**) in the hexane fractions from the leaves and branches of *B. glaziovii*; besides these the sterol stigmasterol (**6**), and the pentacyclic triterpene lupenone (**7**) were also present in the hexane fraction from the branches of *B. glaziovii*.

Table 2. Chemical composition of the hexane fractions from the leaves and branches of *B. glaziovii* as assessed by HRGC analysis (SPB-5 and SPB-50 columns)

Standard compounds	Leaves	Branches	RR[a]	RR[b]
α-Amyrin	+	+	1.381	2.015
β-Amyrin	+	+	1.318	1.799
Lupeol	–	–	1.389	1.859
Taraxerol	–	–	1.285	1.750
α-Amyrin acetate	–	–	1.572	2.246
β-Amyrin acetate	+	+	1.486	1.927
Lupeol acetate	–	–	1.582	2.265
Bauerenyl acetate	–	–	–	2.536
Taraxerol acetate	–	–	1.464	1.928
Friedelanoyl acetate	–	–	1.823	2.949
Friedelin	–	–	1.580	2.776
Lupenone	–	+	1.341	2.011
α-Amyrenone	–	–	1.341	1.940
β-Amyrenone	–	–	1.279	1.727
Germanicone	–	–	1.264	1.658
Campesterol	+	+	1.131	1.231
Stigmasterol	–	+	1.175	1.302
β-Sitosterol	+	+	1.263	1.482

[a]SPB-5 column; [b]SPB-50 column. RR: relative retention related to the internal standard (cholesterol); (+): presence of the compound; (–): absence of the compound.

The mixture of α/β-amyrin triterpenes exists in many medicinal plants and accounts for many bioactive properties, including analgesic, antimicrobial, and anti-inflammatory actions.[26] These compounds can underlie the activity exhibited by the hexane fractions from *B. glaziovii*. Interestingly, although these substances occur in the hexane fractions from both the leaves and branches, the fraction from the leaves showed stronger activity, suggesting that other compounds also contribute to the antileishmanial properties.

To prove the identity of the substances identified by GC-FID (by comparison with the authentic compounds) and to obtain the full profile of the hexane fractions from the leaves and branches of *B. glaziovii*, we also performed the GC-MS analysis for the hexane fractions (Supplementary Information). We identified the substances on the basis of the NIST library and by comparison with literature data. Only the compounds with 90% of similarities with the database or higher than that were taken into account for our analysis. The GC-MS analysis of the hexane fractions of leaves and branches are given in the Table 3. Besides the confirmation of the triterpenes and sterols previously identified by HRGC, the GC-MS allowed us to detect a further series of compounds constituted mainly of long chain fatty acids and its derivatives.

The hexane fraction of leaves mainly contains palmitic acid (*n*-hexadecanoic acid) and its derivative (hexadecanoic acid, ethyl esther); stearic acid derivatives [(12-octadecadienoic acid (*Z,Z*)-; 9,12,15-octadecatrienoic acid, (*Z,Z,Z*)-] and octadecanoic acid, ethyl ester); linoleic acid, ethyl ester and the diterpene alcohol-phytol. For the hexane fraction of branches the most abundant fatty acids were the palmitic acid derivative (hexadecanoic acid, ethyl esther); the stearic acid derivatives [(octadecanoic acid, ethyl ester and 9,12,15-octadecatrienoic acid, (*Z,Z,Z*)-] and linoleic acid, ethyl ester. Some fatty acids, like palmitic acid,[27] among others[28] have been shown to have antiprotozoal activity.

These results demonstrated that, despite the similarities between the hexane fractions from the leaves and branches of *B. glaziovii*, these fractions exhibited different chemical profiles and antileishmanial activity. Taken together, these findings corroborated that plant tissues produce compounds in different ways, which can be relevant when considering the chemical interface between plants and the surrounding environment.[29]

The preliminary phytochemical screening by HRGC proved to be a useful tool to identify secondary metabolites, in this case, the triterpenes and sterols presented in complex extracts and fractions of *B. glaziovii*, in special the bioactive hexane fraction, which the chemical profile was successfully mapped (Figure 2).

Further bio-guided studies regarding the isolation and antileishmanial evaluation of the pure compounds are necessary to identify the bioactive compounds or a possible synergism between the molecules present in the active fractions of *B. glaziovii*.

Conclusions

Neglected tropical diseases are a recognized public health concern affecting mostly the impoverished regions of

Table 3. Compounds identified in the hexane fractions from the leaves and branches of *B. glaziovii* using gas chromatography coupled with electron ionization mass spectrometry (GC-MS)

Compounds	Molecular formula	Molecular weight	Leaves	Branches
Phenol, 4,4'-(1-methylethylidene)bis-; 95[a]; 95[b]	$C_{15}H_{16}O_2$	228	X	X
n-Hexadecanoic acid; 99[a]; 99[b]	$C_{16}H_{32}O_2$	256	X	X
Tetradecanoic acid, ethyl ester; 90[b]	$C_{16}H_{32}O_2$	256	–	X
Cyclohexene, 4-(4-ethylcyclohexyl)-1-pentyl-; 95[b]	$C_{19}H_{34}$	262	–	X
2-Pentadecanone, 6,10,14-trimethyl-; 91[a]	$C_{18}H_{36}O$	268	X	–
Pentadecanoic acid, ethyl ester; 99[b]	$C_{17}H_{34}O_2$	270	–	X
Hexadecanoic acid, methyl ester; 98[b]	$C_{17}H_{34}O_2$	270	–	X
Dibutyl phthalate; 90[b]	$C_{16}H_{22}O_4$	278	–	X
9,12,15-Octadecatrienoic acid, (*Z,Z,Z*)-; 99[a]; 96[b]	$C_{18}H_{30}O_2$	278	X	X
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester; 91[a]; 90[b]	$C_{16}H_{22}O_4$	278	X	X
9,12-Octadecadienoic acid (*Z,Z*)-; 99[a]; 99[b]	$C_{18}H_{32}O_2$	280	X	X
12-Methyl-*E,E*-2,13-octadecadien-1-ol; 93[a]	$C_{19}H_{36}O$	280	X	–
Cyclopropaneoctanal, 2-octyl-; 97[b]	$C_{19}H_{36}O$	280	–	X
2-Methyl-*Z,Z*-3,13-octadecadienol; 91[a]	$C_{19}H_{36}O$	280	X	–
Ethyl 9-hexadecenoate; 95[a]; 99[b]	$C_{18}H_{34}O_2$	282	X	X
Hexadecanoic acid, ethyl ester; 99[a]; 98[b]	$C_{18}H_{36}O_2$	284	X	X
Phytol; 91[a]; 95[b]	$C_{20}H_{40}O$	296	X	X
Heptadecanoic acid, ethyl ester; 98[a]; 99[b]	$C_{19}H_{38}O_2$	298	X	X
9,12,15-Octadecatrienoic acid, ethyl ester, (*Z,Z,Z*)-; 99[a]; 99[b]	$C_{20}H_{34}O_2$	306	X	X
Linoleic acid, ethyl ester; 99[a]; 99[b]	$C_{20}H_{36}O_2$	308	X	X
Ethyl oleate; 98[b]	$C_{20}H_{38}O_2$	310	–	X
Octadecanoic acid, ethyl ester; 99[a]; 99[b]	$C_{20}H_{40}O_2$	312	X	X
4,8,12,16-Tetramethylheptadecan-4-olide; 93[a]	$C_{21}H_{40}O_2$	324	X	–
Nonadecanoic acid, ethyl ester; 93[a]; 96[b]	$C_{21}H_{42}O_2$	326	X	X
Nonadecanoic acid, ethyl ester; 95[b]	$C_{21}H_{42}O_2$	326	–	X
Cyclodocosane, ethyl-; 96[a]	$C_{24}H_{48}$	336	X	X
4,4,6a,6b,8a,11,12,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14, 14a,14b-octadecahydro-2*H*-picen-3-one; 99[a]	$C_{30}H_{48}O$	424	X	–
Fern-7-en-3β-ol; 90[a]	$C_{30}H_{50}O$	426	X	–
Vitamin E; 99[a]	$C_{29}H_{50}O_2$	430	X	–
Ethyl tetracosanoate; 95[b]	$C_{26}H_{52}O_2$	396	–	X
Squalene; 99[b]	$C_{30}H_{50}$	410	–	X
Lanosterol; 93[b]	$C_{30}H_{50}O$	426	–	X

[a]Percentage of similarity of the compounds of leaves with NIST library; [b]percentage of similarity of the compounds of branches with NIST library.

the world. In the American continent, Brazil is the country that is most impacted by leishmaniasis. This country also relies on wide biodiversity, which constitutes a superb source of chemical diversity. By using rational screening, it is possible to identify hits and leads that will be essential for further research into the development of potential drugs. The chemical and pharmacological potential of many plants remains unexplored. HRGC proved to be a rapid, simple, and relatively cheap preliminary screening tool to characterize plant mixtures in a reliable way. This technique enabled the identification of some triterpenes and sterols present in the bioactive extracts from *Brosimum glaziovii*, traced as campesterol (**1**), β-sitosterol (**2**), β-amyrin (**3**), α-amyrin (**4**), β-amyrin acetate (**5**), stigmasterol (**6**) and lupenone (**7**). Although the identified metabolites have been known for some time, this is the first time that their presence has been detected in the species *B. glaziovii*. The GC-MS analysis allowed the confirmation of the substances

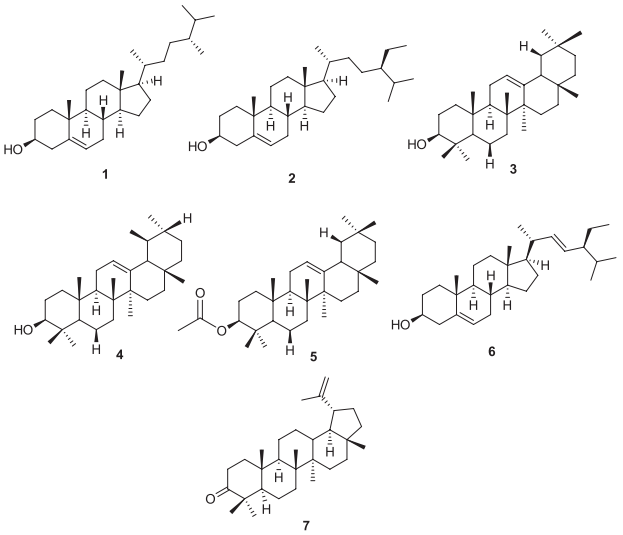

Figure 2. Structures of the triterpenes (**3**-**5** and **7**) and the sterols (**1**-**2** and **6**) present in *B. glaziovii.*

identified by HRGC and the identification of the major constituents of the bioactive fractions, including some long chain fatty acids and their derivatives.

This study demonstrated the strong leishmanicidal activity of the hexane fraction from the leaves of *B. glaziovii* (IC_{50} = 3.6 μg mL^{-1}) against the *L. amazonensis* strain as well as its low cytotoxicity (IC_{50} = 129.3 μg mL^{-1}) against macrophage J774 as compared with the control drug pentamidine (which has well-known strong activity, IC_{50} = 4.0 μg mL^{-1}, but high toxicity, IC_{50} = 7.5 μg mL^{-1}).

Also, the composition of the active fraction showed that *B. glaziovii* is a potential source of leishmanicidal compounds for further pharmacological and toxicological studies.

Supplementary Information

Supplementary information (Figures S1-S65) is available free of charge at http://jbcs.sbq.org.br as PDF file.

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