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Major Article

Screening of the *in vitro* antileishmanial activities of compounds and secondary metabolites isolated from *Maytenus guianensis* Klotzsch ex Reissek (Celastraceae) chichuá Amazon

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Abstract

Introduction: *Maytenus guianensis* is a member of the Celastraceae family that is used in traditional medicine, particularly for its anti-parasitic and anti-cancer effects. To explore the ethnopharmacological potential of this plant, the present study was designed to screen the *in vitro* antileishmanial activities of extracts and compounds isolated from *M. guianensis*. **Methods**: *Maytenus guianensis* stems and leaves were extracted in acetone, followed by the preparation of eluates and isolation of secondary metabolites using chromatography on a glass column with silica gel as the fixed phase. The chemical components were identified using spectroscopic methods, including one- and two-dimensional nuclear magnetic resonance of hydrogen-1 and carbon-13, mass spectroscopy, and infrared spectroscopy. The anti-*Leishmania amazonensis* activities of these eluates and compounds were evaluated by direct promastigote counting and viability assays. **Results**: It was found that the hexane bark eluate produced the strongest anti-*L. amazonensis* effect, with 90-100% inhibition of the promastigote form. The isolated metabolite that produced the best result was tingenone B, followed by a compound formed by the union of tingenone and tingenone B (80-90% inhibition). **Conclusions**: *Maytenus guianensis* shows anti-parasite activity that warrants further investigation to determine the mechanisms underlying this antileishmanial effect and to evaluate the pharmacological potential of these eluates and isolated secondary metabolites, while minimizing any adverse effects.

Keywords: Triterpenes. *Leishmania amazonensis*. Chemotherapy. Ethnopharmacology.

INTRODUCTION

Previous studies have indicated the popularity of plant-based treatments for a range of parasitic diseases, including leishmaniasis. Many compounds isolated from plants have shown promising anti-protozoan activities; these include chalcones, alkaloids, naphthoquinones, lignans, neolignans, and terpenes[1] [2] [3] [4] [5]. However, a large number of medicinal plants remain to be scientifically evaluated and studies have indicated that the active constituents of more than 95% of endemic Brazilian species have not yet been identified[6] [7] [8]. This represents a potentially exploitable pharmacological and economic resource, particularly for Amazonian plant species[8] [9], many of which have not even been cataloged[10].

Numerous species of Amazonian plants have medicinal properties, including those belonging to the therapeutically important Celastraceae family; these have a range of pharmacological activities such as anti-ulcer, insecticide, immunosuppressant, anti-rheumatic, antibacterial, anti-

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parasitic, and anti-cancer effects[11] [12] [13]. In Brazil, this family is represented by four genera: *Austroplenckia*, *Franhofera*, *Maytenus*, and *Salacia*[14]. The *Maytenus* genus is the largest of these and approximately 200 species have been identified in Brazil[15], representing almost 40% of the known *Maytenus* species[16]. In the Northern Amazon region, the *Maytenus guianensis* species in particular has been used in traditional medicine for the treatment of various diseases and for its anti-parasitic effects[12] [17]. The present study therefore aimed to conduct an *in vitro* screening of the antileishmanial activities of extracts and compounds isolated from *M. guianensis*.

METHODS

Collection and identification of plant material

Maytenus guianensis stem bark and leaves were collected in the Adolpho Ducke Forest Reserve, located at Km 26 of the Manaus-Itacoatiara Road (AM-010) (latitude 02º53'S, longitude 59º58'W). The identification of this species was carried out in the Herbarium of the National Institute for Amazonian Research *[Instituto Nacional de Pesquisas da Amazônia* (INPA)], voucher specimen number 188.485.

Preparation of extracts and eluates

The stem bark samples were dried in an oven (ET-394/2, Tecnal) with forced ventilation at 50°C for 48h prior to scraping them to remove the bark and increase the contact surface. This produced 1.9kg of material, which was extracted three times with acetone (3L × 3) at room temperature for 72h per extraction. The extracts were filtered and the solvent was evaporated using a rotaevaporator (ET-211, Tecnal) to produce 200g gross acetone stem bark extract (ASB). Eluates were prepared from 50g EBC and fractionated using column chromatography with silica gel and elution using hexane and ethyl acetate. This produced 14.42g hexane eluate of bark (HEB) and 25.81 g ethyl acetate eluate of bark (EAEB).

The leaf samples were dried in an oven (ET-394/2, Tecnal) with forced ventilation at 50°C for 48h and subsequently crushed to generate a larger contact surface with the solvent. This material (1.2kg) was extracted three times with each solvent (1.5L × 3) in the following order of polarity: hexane, chloroform, ethyl acetate, and ethanol. This produced 8.11g hexane eluate of leaves (HEL), 10.77 g chloroform eluate of leaves (CEL), 3.50g ethyl acetate eluate of leaves (EAEL), and 4.61g ethanol eluate of leaves (EEL).

Isolation of secondary metabolites

The chemical constituents of HEB and HEL were isolated and purified because these eluates produced the best screening results (data shown in the Results section).

The isolation was performed by chromatography on a glass column, using silica gel from Merck and Vetec (63-200µm) as the fixed phase. The length and diameter of the column varied according to the amount of sample and the type of silica employed. For thin-layer chromatography (TLC), silica gel 60 (2-25µM) on Polyester T - 6145 was used (Sigma Chemical Co.) and fluorescence was detected at 250nm. The solvents used for these chromatographic elutions were hexane, ethyl acetate, and methanol, either pure or combined to produce a polarity gradient. The separated components were revealed by exposing the analytical TLC plates to ultraviolet light at a wavelength of 254nm and by spraying them with universal revealer (ethanol:acetic acid:sulfuric acid at 80:10:10), followed by heating in an oven at 100°C for about five minutes.

Mass spectra were obtained by electron impact (70 Ev) using gas chromatography-mass spectrometry (Hewlett - Packard 5971) with a capillary column (30m × 0.25mm) of dimethylpolysiloxane BD-1, with He as the carrier gas. The temperatures employed were 250°C in the injector and 200°C in the detector, while the column changed from 35-180ºC by 1º/min and from 180-250ºC by 10°C/min.

The structural identification of the isolated chemical constituents of *M. guianensis* was carried out by spectroscopic methods such as one- and two-dimensional nuclear magnetic resonance of hydrogen-1 (NMR-^{1}H) and carbon-13 (NMR-^{13}C), mass spectroscopy (MS), and spectroscopy in the infrared region (IR).

Evaluation of antileishmanial activity

These tests were performed at the Laboratory of Biotechnology Applied to Health, Oswaldo Cruz Foundation Porto Velho Unit, [*Fundação Oswaldo Cruz, Unidade de Porto Velho*, (FIOCRUZ-RO)], Rondônia. Male mice were obtained from the FIOCRUZ-RO vivarium at 8-10 weeks old. The animals were kept under standard vivarium conditions.

The present study used promastigotes of *Leishmania amazonensis* (IFLA/BR/67/PH8). Cultures were established from mice that had previously been inoculated subcutaneously with 10^5 promastigotes of *L. amazonensis* in the right hind paw. Two months after infection, the animals were euthanized by cervical dislocation and the legs, with the lesion and necrotic skin, were removed under sterile conditions. The tissue taken from the infected paws was macerated in Roswell Park Memorial Institute medium (RPMI) to obtain amastigotes of *L. amazonensis*. The resultant material was centrifuged at 1000rpm for 10 min at 4°C. The supernatant was carefully removed and the pellet was suspended in RPMI supplemented with 10% fetal bovine serum. This culture was maintained at 24°C for about five days until the amastigotes had progressed to promastigotes.

For the *in vitro* propagation of promastigotes, stationary phase parasites were diluted in erythrosine B (0.04%) and counted in a Neubauer hemocytometer chamber using an optical microscope at 400× magnification. The red-colored parasites were considered to be dead, while those that were birefringent and mobile were considered to be alive. The average number of live parasites in 1mL was calculated using the following formula: number of parasites = average of four quadrants × the dilution × 10^4[18]. After these calculations, 5×10^5 promastigotes/mL were placed in RPMI with 10% fetal bovine serum and the parasites were maintained at 24°C; these were passed every five days, up to a maximum of 15 days.

The viability of the promastigotes exposed to the compounds was assessed by two methods: direct counting

and colorimetric assay using MTT ((3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). For both methods, the promastigotes were counted using a Neubauer chamber and approximately 5×10^5 early stationary phase *L. amazonensis* promastigotes were incubated with 100µg/mL of test compound at different concentrations. For both methods, promastigotes incubated in culture medium were used as the negative control and those incubated with pentamidine at 100µg/mL acted as the positive control. The counting method was conducted using Eppendorf microtubes and the MTT assays were carried out in 96-well cell culture plates. The promastigotes were incubated with the indicated compounds at 25°C for 48h. For the direct counting assay, 10µL of each sample was diluted using erythrosine B (0.04%) and counted using the Neubauer chamber hemocytometer, as described above. The results were expressed as percentages. For the MTT assay, the 96-well plate was centrifuged at 1500rpm for 15 min at 4°C prior to removal of 80µL from each well. The same volume of RPMI medium was then added to each well, followed by 10µL MTT solution (5mg/mL). This was incubated for a further 4h in the same incubator. The same centrifugation procedure was then performed and 150µL dimethyl sulfoxide (DMSO) was added to the plate. After a further incubation for 1h at room temperature, the plates were gently shaken and read spectrophotometrically at 570nm. The optical density results were expressed as a percentage of the mean optical density of the negative control cells to represent cytotoxicity.

Statistical analyses of the data were performed using one-way analysis of variance (ANOVA) and multiple comparison post hoc Tukey tests. The level of significance considered in all the tests was 0.01. Graph pad Prism 5.0 software was used to perform these statistical analyses.

Ethicals considerations

The experiments were performed in accordance with the standards established by the Ethics Committee for Animal Use (CEUA) and the research project was approved by the CEUA of FIOCRUZ-RO under the protocol number: 2013/12.

RESULTS

Compounds isolated

Six secondary metabolites were isolated from the HEB: friedelin (**Figure 1A**), friedelanol (**Figure 1B**), 16β-hydroxyfriedelin (**Figure 1C**), 29-hydroxyfriedelin (**Figure 1D**), 22β-hydroxytingenone (tingenone B; **Figure 1E**), and 22β-hydroxypristimerin (**Figure 1F**). A seventh compound was formed by the union of two metabolites: tingenone (**Figure 1G**) and tingenone B.

From HEL, we isolated β-sitosterol (**Figure 1H**) and a compound formed by the union of α-amyrin (**Figure 1I**) and β-amyrin (**Figure 1J**). All of the metabolites isolated in this study had previously been described as constituents of *M. guianensis* by our group[19]. β-Sitosterol and 22β-hydroxypristimerin were not tested for antileishmanial activity because they showed poor solubility in the relevant solvents.

Antileishmanial activity

In the direct counting method, the HEB and EEF eluates solubilized in ethanol showed the best results; these produced 100% and > 80% inhibition, respectively (**Table 1**). Using DMSO as the solvent, the best results were observed using the EAEL eluate (> 60% inhibition), CEL, and EEL (both > 50% inhibition; **Table 1**). The HEB also produced the best results in the MTT assay, with > 90% cytotoxicity, followed by HEL (> 60%; **Table 1**).

The secondary metabolites that showed the best antileishmanial results were tingenone B (solubilized in ethanol) and the compound formed by the union of tingenone + tingenone B (solubilized in DMSO); both produced 100% promastigote inhibition in the direct counting method, while the MTT assay indicated > 90% cytotoxicity for tingenone B and 80% for tingenone + tingenone B (**Table 2**).

DISCUSSION

Hexane eluates of several plant species from the Amazon region have shown anti-parasitic activities against *Leishmania* promastigotes; these include *Annona foetida*[20], *Garcinia brasiliensis*[21], and *Lacistema pubescens*[22] against *L. amazonensis*, *Gustavia elliptica* against *L. braziliensis*[23], and *Guarea kunthiana* and *Casearia sylvestris* against *L. donovani*[24].

One possible explanation for the antileishmanial activities of these extracts and hexane eluates is the presence of terpenoids. These compounds have shown anti-parasitic efficacy in several studies[3][4][5][13][25][26][27][28][29][30][31][32][33][34][35] and are also found in several *Maytenus* species[19][36][37][38][39][40][41][42][43][44][45].

Tingenone B showed the best results in the present study and this compound was previously reported to show anti-parasitic activity against *Trypanosoma cruzi* (IC < 0.25µg/mL), *Trypanosoma brucei* (IC < 0.25µg/mL), and *Leishmania infantum* (IC < 0.51µg/mL)[46]. Torres-Santos et al.[26] and Teles et al.[29] investigated the effects of isolated triterpenes on promastigotes of *L. amazonensis* and found that the presence of the carboxylic acid grouping was associated with antiprotozoal activity. Pentacyclic triterpenes, with more hydroxyl groups, had greater effects on these promastigotes and this chemical characteristic is also present in tingenone B, which is a pentacyclic triterpene with a quinonamethide skeleton. Other compounds with the same skeleton have shown great potential for the production of potent drugs against leishmaniasis[30] and Chagas disease[30][47]. Previous studies have shown that triterpenoids stimulate granulocytosis and phagocytosis, thus helping to fight infection[48], causing cytoplasmic extravasation, corpuscle formation, and mitochondrial swelling[49].

Another possible explanation for the anti-parasitic activities of these terpenoids is their activation of programmed cell death within the parasites[50] and interference with the parasite cell differentiation process in the host. This is an extremely complex process involving fine regulation of gene expression[51]. However, the mechanism of action by which triterpenoids inhibit the growth of *Leishmania* promastigotes has not yet been fully elucidated[52].

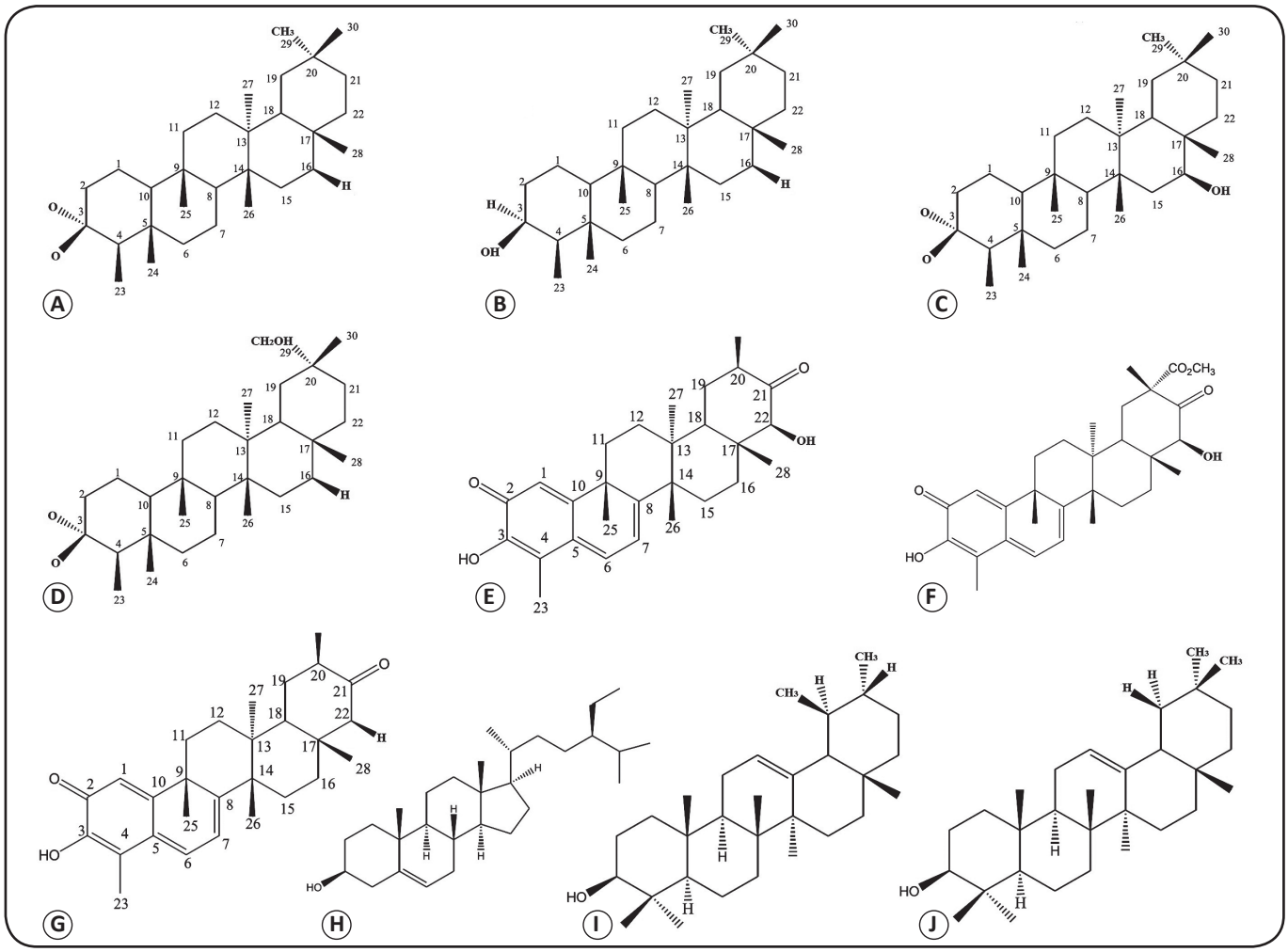

FIGURE 1. Chemical constituents of *Maytenus guianensis*. **(A):** friedelin; **(B):** friedelanol; **(C):** 16β-hydroxyfriedelin; **(D):** 29-hydroxyfriedelin; **(E):** tingenone; **(F):** 22β- hydroxytingenone (tingenone B); **(G):** 22β-hydroxypristimerin; **(H):** α-amyrin; **(I):** β-amyrin; **(J):** β-sitosterol.

TABLE 1

Antileishmanial activities of the isolated secondary metabolites of *Maytenus guianensis*.

Compounds	Solvent	Inhibition (%) mean (SD)	Cytotoxicity (%) mean (SD)
Control	-	0	Not applicable
SDS	-	Not applicable	100
Pentamidine (5µg/mL)	-	100[a]	92.1 ± 1.3
Ethanol (1%)	-	44.8 ± 2.8[a,b]	42.2 ± 4.1
DMSO (1.5%)	-	-1.2 ± 9.8[a]	71.1 ± 1.2
HEB (100µg/mL)	Ethanol (1%)	99.8 ± 0.2[a,c]	96.3 ± 3.1
EAEB (100µg/mL)	Ethanol (1%)	31.2 ± 0.6[a,b,c]	47.9 ± 3.8
EEL (100µg/mL)	Ethanol (1%)	85.2 ± 0.9[a,b,c]	10.1 ± 7.0
CEL (100µg/mL)	DMSO (1.5%)	54.3 ± 7.1[a,b,c]	38.7 ± 1.9
EAEL (100µg/mL)	DMSO (1.5%)	63.8 ± 3.2[a,b,c]	39.2 ± 0.8
HEL (100µg/mL)	DMSO (1.5%)	55.1 ± 3.1[a,b,c]	62.2 ± 9.1

SD: standard deviation; **SDS:** sodium dodecyl sulfate; **DMSO:** dimethyl sulfoxide**; HEB:** acetone stem bark extract**; EAEB:** ethyl acetate eluate of bark**; EEL:** ethanol eluate of leaves**; CEL:** chloroform eluate of leaves**; EAEL:** ethyl acetate eluate of leaves**; HEL:** hexane eluate of leaves**; MTT:** ((3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Inhibition was determined using the direct counting method. [a]$p < 0.01$, as compared to the negative control (*Leishmania amazonensis* in culture medium). [b]$p < 0.01$, as compared to the positive control (pentamidine). [c]$p < 0.01$, as compared to the relevant solvent control (ethanol or DMSO). Cytotoxicity was determined using the MTT method. SDS was employed as a non-specific positive control and pentamidine was used as the positive control. The solvent controls were ethanol or DMSO.

TABLE 2

Antileishmanial activities of isolated secondary metabolites of *Maytenus guianensis.*

Compounds	Solvent	Inhibition (%) mean (SD)	Cytotoxicity (%) mean (SD)
Control	-	0	Not applicable
SDS	-	Not applicable	100
Pentamidine (5µg/mL)	-	100[a]	92.1 ± 1.3
Ethanol (1%)	-	44.8 ± 2.8[a,b]	42.2 ± 4.1
DMSO (1.5%)	-	-1.2 ± 9.8[a]	71.1 ± 1.2
Tingenone B (100µg/mL)	Ethanol (1%)	100 ± 0.1[a,b]	81.9 ± 0.6
Tingenone B (100µg/mL)	DMSO (1.5%)	Not applicable	97.1 ± 1.7
α-Amyrin + β-amyrin (100µg/mL)	Ethanol (1%)	53.2 ± 0.4[a,b,c]	10.1 ± 4.8
29-Hydroxyfriedelin (100µg/mL)	DMSO (1.5%)	46.2 ± 4.0[a,b,c]	34.8 ± 5.9
16β-Hydroxyfriedelin (100µg/mL)	DMSO (1.5%)	50 ± 0.0[a,b,c]	55.2 ± 3.1
Friedelin (100µg/mL)	DMSO (1.5%)	45.3 ± 4.1[a,b,c]	76.3 ± 5.4
Friedelanol (100µg/mL)	DMSO (1.5%)	14.4 ± 2.7[a,b]	48.2 ± 0.1
Tingenone + tingenone B (100µg/mL)	DMSO (1.5%)	100 ± 0.0[a,c]	88.7 ± 1.2

SD: standard deviation; **SDS:** sodium dodecyl sulfate; **DMSO:** dimethyl sulfoxide**; MTT:** ((3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)**.** Inhibition was determined using the direct counting method. [a]$p < 0.01$, as compared to the negative control (*Leishmania amazonensis* in culture medium). [b]$p < 0.01$, as compared to the positive control (pentamidine). [c]$p < 0.01$, as compared to the relevant solvent control (ethanol or DMSO). Cytotoxicity was determined using the MTT method. SDS was employed as a non-specific positive control and pentamidine was used as the positive control. The solvent controls were ethanol or DMSO.

The present study found that the *M. guianensis* HEB showed the strongest anti-*L. amazonensis* activity, with 90-100% inhibition of the promastigotes. Among the secondary metabolites, tingenone B produced the best result, followed by tingenone + tingenone B (80-90% inhibition). These findings indicate that *M. guianensis* has anti-parasitic potential and warrants future studies to elucidate this antileishmanial mechanism of action and to evaluate the cytotoxic and genotoxic potential of eluates and isolated secondary metabolites from this plant, in order to fully exploit its pharmacological efficacy and minimize any adverse effects.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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