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Research Article

Activity of Cuban Plants Extracts against *Leishmania amazonensis*

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Natural products have long been providing important drug leads for infectious diseases. Leishmaniasis is a major health problem worldwide that affects millions of people especially in the developing nations. There is no immunoprophylaxis (vaccination) available for *Leishmania* infections, and conventional treatments are unsatisfactory; therefore, antileishmanial drugs are urgently needed. In this work, 48 alcoholic extracts from 46 Cuban plants were evaluated by an *in vitro* bioassay against *Leishmania amazonensis*. Furthermore, their toxicity was assayed against murine macrophage. The three most potent extracts against the amastigote stage of *Leishmania amazonensis* were from *Hura crepitans, Bambusa vulgaris*, and *Simarouba glauca*.

1. Introduction

Leishmaniasis is a protozoan parasitic disease found in 16 developed and 72 developing countries with 12 million cases [1]; it causes around 70000 deaths annually [2]. So far, no vaccine approved for human use is available [3]. Various antileishmanial agents are readily available in the market although none of these chemotherapy drugs are free from harmful side effects and toxicity [4–7]. Currently, the development of new drugs against leishmaniasis is a need.

The interest in plants products, specially in medicinal plants or their extracts, surfaced all over the world due to the belief that many herbal extracts have been extensively used by native populations to treat leishmaniasis [8, 9] and scientific reports have demonstrated their potential [10, 11]. In the present study, the antileishmanial activity of 48 extracts from 46 Cuban plants was tested to validate the antiprotozoal properties of Cuban plants.

2. Methods

2.1. Plant Materials. Vegetative samples of 46 species were used, and their general data are presented in Table 1. All plants were collected and authenticated according to the Cuban Flora by M. S. Ramón Scull and Dr. Pedro Herrera. Their voucher specimens or collector's numbers were assigned, and a sample was deposited in a herbarium (Table 1).

2.2. Preparation of Plant Extracts. The plant organs were dried in an oven with ventilation system at 30°C and crushed. The fluid extracts were prepared by maceration for seven days using 80% ethanol as solvent and 20% water, according to the Regulation Norm 309 (Regulation Norm, 1992). Solvent was evaporated, and the extracts were lyophilized and dissolved in dimethyl-sulfoxide (DMSO, BDH, England) at 20 mg/mL and stored at 4°C.

2.3. Reference Drug. Pentamidine (Richet, Buenos Aires, Argentina) was used as positive control and a stock solution prepared at a concentration of 10 mg/mL.

2.4. Parasites. The MHOM/77BR/LTB0016 strain of *Leishmania amazonensis* was kindly provided by the Department of Immunology, Oswaldo Cruz Foundation (FIOCRUZ), Brazil. Parasites were routinely isolated by aspiration with needle from mouse lesions and maintained as promastigotes

Table 1: List of plants used in this study and collections data.

Plants species	Family	Part used	Collection date	Geographical areas	Voucher specimen
Allium sativum L.	Alliaceae	Leaves	2006	NBG[a], Havana	HAJB-HFC[c] 87089
Aloe barbadensis L.	Asphodelaceae	Leaves	2010	"La Quiruvina," Artemisa	HAC[d] 42671
Alternanthera sessilis (L.) R. Br. ex DC.	Amaranthaceae	Leaves	2008	IPF[b], Havana	HAJB[e] 87100
Annona glabra L.	Annonaceae	Leaves	2006	NBG, Havana	HAJB 8300098
Argemone mexicana L.	Papaveraceae	Leaves	2009	IPF	HAJB-HFC 87090
Artemisia absinthium L.	Asteraceae	Leaves	2006	NBG, Havana	HAJB 9700183
Artemisia vulgaris L.	Asteraceae	Leaves	2010	"La Quiruvina," Artemisa	HAC 42673
Azadirachta indica A.Juss	Meliaceae	Leaves	2008	IPF[b], Havana	HAJB-HFC 87099
Bambusa vulgaris Schrad. Ex J. C. Wendl.	Bambusinae	Leaves	2006	NBG, Havana	HAJB 9500087
Bambusa vulgaris Schrad. Ex J. C. Wendl.	Bambusinae	Root	2010	NBG, Havana	HAJB 9500087
Bidens pilosa L.	Asteraceae	Leaves	2006	NBG, Havana	HAJB 9700169
Bursera simaruba (L.) Sarg.	Burseraceae	Leaves	2006	NBG, Havana	HAJB 8603305
Cajanus cajan (L.) Millsp.	Fabaceae	Leaves	2010	"La Quiruvina," Artemisa	HAJB-HFC 42670
Cassia grandis L.	Leguminosae	Leaves	2006	NBG, Havana	HAJB 8300069
Cecropia peltata L.	Urticaceae	Leaves	2010	NBG, Havana	HAJB 8603239
Chenopodium ambrosioides L.	Chenopodiaceae	Leaves	2010	"La Quiruvina," Artemisa	ROIG[e] 4639
Cissus sicyoides L.	Vitaceae	Leaves	2010	NBG, Havana	HAJB-HFC 87092
Citrus limetta Risso	Rutaceae	Leaves	2006	NBG, Havana	HAJB-HFC 87093
Cucurbita maxima Dutch.	Cucurbitaceae	Seeds	2006	NBG, Havana	HAJB-HFC 87091
Cupressus sempervirens L.	Cupressaceae	Leaves	2006	NBG, Havana	HAJB 8603788
Curcuma longa L.	Zingiberaceae	Rhizome	2006	NBG, Havana	HAJB 9700178
Cymbopogon citrate Stapf.	Poaceae	Leaves	2006	NBG, Havana	HAJB 8700008
Hura crepitans L.	Euphorbiaceae	Leaves	2006	NBG, Havana	HAJB 8600198
Indigofera suffruticosa Mill.	Fabaceae	Leaves	2006	NBG, Havana	HAJB 100079
Koanophyllon villosum (Sw.) King & H. Rob.	Asteraceae	Leaves	2010	NBG, Havana	HAJB-HFC 87094
Lepidium virginicum L.	Brassicaceae	Leaves	2006	NBG, Havana	HAJB 8700259
Luffa cylindrica L.	Cucurbitaceae	Leaves	2006	NBG, Havana	HAJB 8600366
Mangifera indica L.	Anacardiaceae	Leaves	2006	NBG, Havana	HAJB 9700183
Melaleuca leucadendron L.	Myrtaceae	Leaves	2006	NBG, Havana	HAJB 8501918
Melia azedarach L.	Meliaceae	Root	2006	NBG, Havana	HAJB 8402273
Momordica charantia L.	Cucurbitaceae	Leaves	2008	NBG, Havana	HAJB 9700180
Ocimum sanctum L.	Lamiaceae	Leaves	2006	NBG, Havana	HAJB 9200485
Parthenium hysterophorus L.	Asteraceae	Leaves	2006	NBG, Havana	HAJB 9700175
Parthenium hysterophorus L.	Asteraceae	Root	2010	NBG, Havana	HAJB 9700176
Petiveria alliaceae L.	Phytolaccaceae	Leaves	2006	NBG, Havana	HAJB 244
Picramnia pentandra Sw.	Simaroubaceae	Leaves	2006	NBG, Havana	HAJB 8303268
Punica granatum L.	Punicaceae	Fruit bark	2006	NBG, Havana	HAJB 8300050
Tradescantia discolor Sw.	Commelinaceae	Leaves	2005	NBG, Havana	HAJB 9200504
Roystonea regia (Kunth) O. F. Cook	Arecaceae	Leaves	2010	NBG, Havana	HFC 87098
Simarouba glauca DC.	Simaroubaceae	Leaves	2006	NBG, Havana	HAJB 8300710
Stachytarpheta jamaicensis (L.) Vahl	Simaroubaceae	Leaves	2006	NBG, Havana	HAJB 9200475
Tabernaemontana citrifolia L.	Boraginaceae	Leaves	2006	NBG, Havana	HAJB 8500720
Tamarindus indica L.	Apocynaceae	Stem bark	2006	NBG, Havana	HAJB 8300068
Thevetia peruviana L.	Caesalpiniaceae	Leaves	2006	NBG, Havana	HAJB-HFC 87095
Trichilia havanensis Jacq.	Meliaceae	Leaves	2010	NBG, Havana	HAJB-HFC 87097
Turnera ulmifolia L.	Turneraceae	Leaves	2009	NBG, Havana	HAJB 8602024
Zerumbet speciosum J. C. Wendl.	Zingiberaceae	Leaves	2010	NBG, Havana	HAJB-HFC 87096

[a] NBG: the collection of plant was performed in the National Botanic Garden, Havana, Cuba.
[b] IPF: the collection of plant was performed in the Institute of Pharmacy and Food, Havana, Cuba.
[c] HFC: Herbarium of National Botanic Garden in the special series of Cuban Flora.
[d] HAC: Herbarium of Systematic and Ecology Institute.
[e] HAJB: Herbarium of National Botanic Garden.
[f] ROIG: Herbarium of Experimental Station of Medicinal Plants "Dr. Juan Tomás Roig."

at 26°C in Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (HFBS) (Sigma-Aldrich, St. Louis, MO, USA), 100 μg of streptomycin/mL, and 100 U of penicillin/mL, with passage each 3 or 4 days. The parasites were not used after 10 *in vitro* passages.

2.5. Antipromastigote Screening. Exponentially growing promastigotes (10^5 promastigotes/mL, 199 μL) were plated in 96-well plates. Two microliters of extracts or 2 μL of DMSO for control were added to the wells at a final concentration between 6.25 and 100 μg/mL. Plates were incubated at 26°C for 72 h. Then, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) solution (15 μL) at 5 mg/mL dissolved in saline solution was added to each well. After incubation for additional 4 h, the medium was removed and formazan crystals were dissolved by addition of 100 μL of DMSO. Absorbance was determined using an EMS Reader MF Version 2.4-0, at a wavelength of 560 nm and 630 nm as reference [12, 13].

2.6. Cytotoxicity Assay. We determined the IC_{50} of the extracts on peritoneal macrophage from BALB/c mice. Resident macrophages were collected from peritoneal cavity of healthy BALB/c mice in RPMI 1640 medium (Sigma, St. Louis, Mo, USA) supplemented with antibiotics (penicillin 200 UI, streptomycin 200 μg/mL), plated at 10^6/mL in 96-well Lab-Tek (Costar, USA) and left to adhere for 2 h at 37°C in 5% CO_2. Nonadherent cells were removed by washing with saline solution after 2 h of incubation at 37°C in 5% CO_2. Then, 198 μL of medium with 10% of HFBS and antibiotics (penicillin 200 UI, streptomycin 200 μg/mL) was added in each well, and later 2 μL of extracts dilutions, previously prepared in medium, was added. Macrophages were treated with the extracts from 1.5 to 200 μg/mL for 72 h. Cultures with DMSO were included as control treated. The cytotoxicity was determined using the colorimetric assay with MTT as described above in the promastigote assay [13].

2.7. Antiamastigote Activity. Peritoneal macrophages from BALB/c mice were collected, plated at 10^6/mL in 24-well Lab-Tek (Costar, USA), and incubated 2 h at 37°C in 5% CO_2. Nonadherent cells were removed, and stationary-phase *L. amazonensis* promastigotes were added at a 4 : 1 parasite/macrophage ratio. Cultures were added for further 4 h, and cell monolayers were washed to remove free parasites. Then, 1990 μL of the RPMI complete medium and 10 μL of the different extracts were added, following serial dilutions 1 : 2, to obtain final concentrations between 12.5 and 100 μg/mL. Plates were incubated for a further 48 h [14]. Cultures as control were included, which were treated with DMSO. Cultures were then fixed with absolute methanol, stained with Giemsa, and examined under light microscopy. The number of intracellular amastigotes was determined by counting the amastigotes resident in 100 macrophages per each sample. Results were expressed as percent of reduction of the infection rate (% IR) in comparison with those obtained with positive controls. The infection rates were obtained by multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophages [15].

2.8. Statistic Analysis. All tests were performed in triplicate, and the median inhibitory concentration (IC_{50}) to parasite and median cytotoxic concentration (CC_{50}) to peritoneal macrophage from BALB/c mice were obtained directly from linear equation of dose-response curves. The results were expressed as their average and standard deviation.

The active extracts that showed an $IC_{50} < 100$ μg/mL were selected as active against promastigote form, and cytotoxicity was determined. Then, the selectivity index (SI), calculated as ratio of CC_{50} for macrophage/IC_{50} for promastigotes, was used to compare the toxicity and activity of the extracts. The extracts with an SI more than 5 were tested against the amastigote form. The extracts with an $IC_{50} \leq 50$ μg/mL were considered as active against intracellular amastigotes of *Leishmania*.

3. Results

The activity of extracts against *L. amazonensis* promastigotes, the cytoxicity against peritoneal macrophage from BALB/c mice, and the selectivity are shown in Table 2. Between them, 20 extracts showed activity, with an $IC_{50} < 100$ μg/mL, although only 4 extracts (*Bambusa vulgaris, Hura crepitans, Mangifera indica,* and *Simarouba glauca*) demonstrated selective activity against the parasite and were tested against intracellular amastigotes.

Among the four extracts evaluated against *L. amazonensis* amastigotes, three showed inhibition of growth with $IC_{50} \leq 50$ μg/mL. The highest antileishmanial activity was exhibited by *H. crepitans,* with the lower IC_{50} value (27.7 ± 0.6 μg/mL). The IC_{50} of *B. vulgaris* and *S. glauca* was 41.5 ± 0.6 μg/mL and 45.5 ± 0.3 μg/mL, respectively. *M. indica* extract showed an IC_{50} value of 60.1 ± 3.3 μg/mL.

4. Discussion

Natural products are potential sources of new and selective agents for the treatment of important tropical diseases caused by protozoan and other parasites [16]. Only few laboratories are involved in drug evaluation and development against these devastating diseases, particularly against leishmaniasis, which has been considered as a "neglected disease" [17]. In this sense, the potential of plant products as a source of antileishmanial drugs has been demonstrated and considered as a promising approach. Several studies about screening of plants extracts against *Leishmania* have been reported [18–23].

Different models to evaluate drugs have been used, including promastigote, intracellular amastigote, or axenic amastigote forms of the parasite. The most important method is the counting of intracellular amastigotes, which are the clinical relevant stage of *Leishmania* in the mammalian host. Conventional procedures involve staining with Giemsa after treatment and manual counting. However,

Table 2: Antileishmanial activity and cytotoxicity of Cuban plants extracts.

Plants species	IC_{50}[a] ± SD[b] (μg/mL)	CC_{50}[c] ± SD (μg/mL)	SI[d]
A. sativum	153.2 ± 2.1	—	—
A. barbadensis	**77.5 ± 0.9**	150.3 ± 3.4	2
A. sessilis	>200	—	—
A. glabra	37.8 ± 0.1	—	—
A. mexicana	>200	—	—
A. absinthium	129.0 ± 1.5	—	—
A. vulgaris	**55.0 ± 3.2**	107.7 ± 5.1	2
A. indica	>200	—	—
***B. vulgaris* (leaves)**	**60.5 ± 7.3**	**276.5 ± 1.2**	**5**
B. vulgaris (root)	191.6 ± 1.1	—	—
B. pilosa	**73.7 ± 0.1**	222.8 ± 1.1	3
B. simaruba	163.3 ± 1.8		
C. cajan	**51.7 ± 0.7**	132.5 ± 5.7	3
C. grandis	>200	—	—
C. peltata	>200	—	—
C. ambrosioides	>200	—	—
C. sicyoides	>200	—	—
C. limetta	**73.6 ± 1.3**	210.6 ± 3.9	3
C. maxima	**62.2 ± 0.1**	161.7 ± 0.9	3
C. sempervirens	>200	—	—
C. longa	>200	—	—
C. citrate	>200	—	—
H. crepitans	**16.4 ± 2.1**	**390.5 ± 8.6**	**24**
I. suffruticosa	**75.8 ± 4.5**	158.5 ± 6.2	2
K. villosum	>200	—	—
L. virginicum	109.7 ± 1.8	—	—
L. cylindrica	127.9 ± 1.0	—	—
M. indica	**51.2 ± 0.1**	**442.4 ± 2.9**	**9**
M. leucadendron	**57.2 ± 2.9**	199.2 ± 4.7	3
M. azedarach	168.6 ± 1.9	—	—
M. charantia	**59.8 ± 0.4**	26.7 ± 0.3	0
O. sanctum	**96.9 ± 0.1**	216.9 ± 4.8	2
P. hysterophorus (leaves)	**54.7 ± 1.2**	89.2 ± 1.5	0
P. hysterophorus (root)	**46.9 ± 1.7**	140.2 ± 0.2	3
P. alliaceae	151.5 ± 4.1	—	—
P. pentandra	140.8 ± 1.4	—	—
P. oleracea	**63.9 ± 2.6**	125.9 ± 1.9	2
P. granatum	**39.4 ± 8.8**	129.0 ± 4.6	3
R. spathacea	**71.4 ± 1.4**	244.6 ± 1.6	3
R. regia	>200	—	—
S. glauca	**47.5 ± 0**	**228.7 ± 8.0**	**5**
S. jamaicensis	111.8 ± 1.2	—	—
T. citrifolia	>200	—	—
T. indica	>200	—	—
T. peruviana	>200	—	—
T. havanensis	>200	—	—
T. ulmifolia	>200	—	—
Z. speciosum	133.1 ± 0.8	—	—
Pentamidine	0.37 ± 0.01	11.7 ± 1.7	32

[a] IC_{50}: concentration of drug that caused 50% of growth inhibition of promastigotes of *L. amazonensis*.
[b] SD: standard deviation.
[c] CC_{50}: concentration of drug that caused 50% of mortality of peritoneal macrophage from BALB/c.
[d] SI: selectivity index. Bold data indicate the extract selected.

this conventional procedure has limitations such as being time consuming, the use of animals for extraction of macrophages, and the possible error in the counting. Axenic amastigotes forms have been developed to obtain a more simple and reproducible method. These are technically easier and less expensive and require a very shorter time for execution. However, this model lacks information about the behavior of macrophages during the treatment, their possible influence on drug activity, or possible damage received due to toxicity [24]. Alternatively, the promastigote form has been used in screening investigations. Although it is not a clinical relevant stage, it was reported that this parasitic form gives information on specific antileishmanial activity respect to toxicity showed (selectivity of the product). In addition, the tests are easy and highly reproducible [22]. The use of the promastigote form has been widely demonstrated as the preliminary test in screening of plant extract [8, 25, 26].

As part of a screening project of natural plants against protozoan parasites, we tested 48 extracts against *Leishmania*, which were selected according to the previous literature that mentioned these plants with antiparasitic properties [27]. In addition, the evaluated plants present an easy cultivation and can be obtained in high quantities of samples.

Cuba presents a rich plant population that has been unexploited in the field of antiprotozoals. Nevertheless, previous studies about antileishmanial potentialities of Cuban plants were reported, including *Chenopodium ambrosioides* [28], *Piper auritum* [29], and *Bidens pilosa* [23]. We found that 42% (20 extracts) of the tested products showed leishmanicidal activity with an $IC_{50} \leq 100\,\mu g/mL$ against promastigotes, of which only 20% (4 extracts) exhibited selectivity (SI > 5) and of them 75% (3 extracts) caused growth inhibition in the antiamastigote assay.

Among the plant species evaluated here, *H. crepitans* caused the higher inhibition of promastigotes growth (IC_{50} = 16.4 μg/mL) and lower toxicity against host cell (IC_{50} = 390.5 μg/mL), with an SI = 24. This plant showed the highest activity with an IC_{50} = 27.7 μg/mL against the amastigote form. The results demonstrated the presence of compounds with reasonable potency. *H. crepitans* is a native plant from tropical America that has been used for amoebiasis treatment [30] and against other protozoa such as *Plasmodium falciparum* [31]. In Nicaragua, this plant is used to treat helmintic diseases as ascariasis [30] and in Loreto, Peru, is used by the population to treat the leishmaniasis [8] with acceptable efficacy. In addition, this extract has shown promising activity against *P. falciparum* [32], with a low toxicity and a SI > 10. In the literature uses of its seeds are reported as emmenagogue although they can cause toxic effects, including the death of patients [33]. The toxicity of this plant is caused by two toxic albumins (toxalbumins), hurina and crepitina, which are distributed among all plant organs [34, 35].

On the other hand, the leaves extract from *B. vulgaris* showed an IC_{50} = 60.49 μg/mL against the promastigote form and SI = 5, while the activity was better against the amastigote form, with an IC_{50} = 41.5 μg/mL. However the root extract of this species did not show antileishmanial activity, which would be due to the differential distribution of metabolites in the plant. This finding should indicate that the component(s) responsible for leishmanicidal activity is (are) found in a major percent in the leaves, resulting in the parasite inhibition presented. *B. vulgaris* is an original tropical plant from Old World that has been cultivated in America [27]. Previously, it has been reported that *B. vulgaris* leave extract was inactive in a different screening approach because the extract was dissolved in ethanol [23]. This demonstrates the importance of the dissolvent in the screening approaches. DMSO is a membrane permeabiliser and is known for its ability to serve as carried to transport the drugs into cells [36]. Additionally, this extract has shown activity against other protozoa, such as *P. falciparum* [32]. Other uses include antiparasitic preventive control for dogs [37], cuts, injuries, and swellings [38] and as diuretic in the east population of Cuba [27]. Chemical analysis of *B. vulgaris* leave extract revealed the presence of different bioactive components, including: alkaloids, tannins, phenolics, glycosides, saponins, flavonoids, and anthraquinones [39] *S. glauca* also showed a promising antileishmanial activity. The IC_{50} against promastigotes was similar to that against amastigotes (IC_{50} of 47.5 and 45.5 μg/mL, resp.) and an SI of 5 was obtained. This plant is present in Latin America and has been used for different purposes, for example, in Cuba as emmenagogue, febrifuge, antidysenteric, antihelmintic, and antiherpetic [27], in Haití for the cutaneous lesions [40], and in Guatemala against malaria and amoebiasis [41]. The glaucarrubin is a terpenoids, present in this plant, has been described as responsible for the activity against Gram-positive bacteria and protozoa parasite, specially *Entoameba histolytica* and *P. falciparum* [40].

Several reports in the literature have shown the antileishmanial activity of some plant extracts, including the hidroalcoholic extract from *C. ambrosioides* [42] and *Ocimum sanctum* [43]. However, we did not observe antileishmanial activity of these plants in this study. This apparent controversial result can be due to the variation of chemical components between plants from different geographic areas, which have been documented [44], including plants from the same country [9].

5. Conclusion

In sum, in this study, 48 Cuban plants extracts were evaluated against *L. amazonensis*. The extracts of *H. crepitans*, *B. vulgaris*, and *S. glauca* showed promising antileishmanial activity against life cycle stages of the parasite and high selectivity compared with the activity against mouse peritoneal macrophages. For this reason the next step should be the purification and identification of the active principles of these plants.

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