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Antileishmanial assessment of leaf extracts from *Pluchea carolinensis*, *Pluchea odorata* and *Pluchea rosea*

Marley García[1], Wilmer H Perera[2], Ramón Scull[3], Lianet Monzote[1*]

[1]*Parasitology Department, Institute of Medicine Tropical "Pedro Kourí", Havana City, Cuba*
[2]*Institute of Ecology and Systematic, Havana City, Cuba*
[3]*Institute of Pharmacy and Food, Havana City, Cuba*

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ABSTRACT

Objective: To evaluate the antileishmanial activity of different extracts from three Cuban *Pluchea* species. **Methods:** In *in vitro* assays the IC_{50} was calculated in the promastigotes and amastigotes forms as cytotoxicity in murine macrophages. In leishmaniasis cutanea experiment, mortality, weight loss, lesion size and burden parasite were measured. **Results:** Extracts evaluated showed inhibitive effect on growing of promastigote form; however, active extracts caused a high toxicity. Ethanol and *n*-hexane extracts demonstrated specific antileishmanial activity. Ethanol and *n*-hexane extracts from *Pluchea carolinensis (P. carolinensis)* caused similar inhibition against amastigote form. The intraperitoneal administration of the ethanol extract of *P. carolinensis* at 100 mg/kg prevented lesion development compared with control groups. **Conclusions:** The antileishmanial experiment suggests that ethanol extracts from *P. carolinensis* is the most promising. Further studies are still needed to evaluate the potential of this plant as a source of new antileishmanial agents.

1. Introduction

Leishmaniasis is one of the most diverse and complex of all vector borne diseases. It is caused by an obligated intracellular protozoa parasite belonging to the genus *Leishmania*. The disease affects approximately 12 million people from 88 countries, with an increasing incidence of 1.5-2.0 million new cases diagnosed every year and 350 million people at risk. It is manifested mainly in three clinical forms: cutaneous, mucocutaneous and visceral leishmaniasis, from which the visceral disease constitutes the fatal form if untreated[1,2].

In the current scenario, the chemotherapy is one of the promising actions to control the disease. Conventional drugs have serious limitations such as high cost, toxicity, difficult route of administration and lack of efficacy in endemic areas. The development of safe, effective and affordable antileishmanial agents is a need[3]. In endemic areas, the population use plants to treat many infectious diseases, including leishmaniasis[4].

Cuba is well known for rich flora with a high percentage of endemic species. It is considered as an interesting source of plant species for searching bioactive metabolites. The genus *Pluchea* contains many species with important medicinal properties[5]. The genus includes 80 species from which 30-40 live in tropical regions (6) and only three of them are Cuban: *Pluchea carolinensis* (*P. carolinensis*)(Jacq.) G. Don., *Pluchea odorata* (*P. odorata*) (Sw.) DC. and *Pluchea rosea* (*P. rosea*) Godfrey. The Cuban species of *Pluchea* are well known for their ethnomedical use, including headaches, fever and slow digestions. Antimicrobial properties of different plant organs of some species of the genus *Pluchea* have been tested against bacterias, fungi and viruses[5,7]. On the other hand, some chemical studies have been carried out in the genus *Pluchea*. The most widely distributed metabolites are terpenoids followed by flavonoids[5] and it's well known that these kinds of phytochemicals have antileishmanial activity[8,9]. The present study is to evaluate

*Corresponding author: Lianet Monzote, Departamento de Parasitología, Instituto de Medicina Tropical "Pedro Kourí", Apartado Postal No. 601, Marianao 13. Ciudad de la Habana, Cuba.
Tel: +53-7-202-60-51
Fax: +53-7-204-6051
E-mail: beorimadegun@yahoo.com

the antileishmanial activity of different extracts from three Cuban *Pluchea* species.

2. Materials and methods

2.1. Extracts from P. carolinensis

Samples of *P. carolinensis* (HAC 41725) were collected in "Sierra del Rosario" (Pinar del Río) in March 2006; while *P. rosea* (LS 16648) and *P. odorata* (LS 17198) were collected in "Ciénaga de Zapata" (Matanzas) in May 2006. The blooming specimens were deposited in the HAC herbarium of the "Instituto de Ecología y Sistemática" (Havana), Cuba. Leaves of plants were extracted at room temperature during 3 days. The hydroalcoholic solution (70% ethanol) was pooled, evaporated and subjected to liquid-liquid extractions with solvents of increasing polarity[10]. One liter of the solutions was extracted eight times in a separating funnel, firstly with 400 mL of *n*-hexane and the other times with 200 mL each one. The *n*-hexane solution was pooled, filtered and dried at low pressure until *n*-hexane crude extracts was obtained. The same methodology was followed up by chloroform, ethyl acetate and *n*-butyl alcohol. All extractions were performed in duplicates. After evaporation, dried samples were dissolved in dimethyl sulfoxide (DMSO) at 20 mg/mL.

2.2. Parasite culture

Leishmania amazonensis (*L. amazonensis*) (MHOM/77BR/LTB0016) was kindly provided by the Department of Immunology, Oswaldo Cruz Foundation (FIOCRUZ), Brazil. Parasites were routinely isolated from mouse lesions and maintained as promastigotes at 26 ℃ in Schneider's medium (SIGMA, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (HFBS) (SIGMA, St. Louis, MO, USA), streptomycin 100 μg/mL, and penicillin 100 U/mL. All parasites were not used after the tenth passage.

2.3. Laboratory animals

Female BALB/c mice, with a body weight of approximately 20-22 g, were obtained from National Center of Laboratory Animals Production (CENPALAB). The maintenance and care of mice was followed according to guidelines from Ethics Committee for the Human Use of Laboratory Animals. The temperature and humidity were controlled, with 12 h light/dark cycle and given water and food *ad libitum* for all animals.

2.4. Reference drug

As a drug reference amphotericin B (AmB, IMEFA, Cuba) was used at a concentration of 2 mg/mL. The drug was diluted in sterile distilled water.

2.5. In vitro evaluations

2.5.1. Antipromastigote assay

Promastigotes of *L. amazonensis* (10^5 promastigotes/mL) were distributed in 96-well plates, treated with 1 μL of extracts or DMSO, and then incubated at 26 ℃. After 72 h, the parasites were incubated for 3 h with *P*-nitrophenol phosphate (20 mg/mL) dissolved in 1 mol/L sodium acetate buffer (BDH, Poole, England), pH 5.5, with 1% Triton × -100 (BDH, Poole, England) at 37 ℃. The absorbance was determined in an EMS Reader MF Version 2.4-0, at a wavelength of 405 nm[11].

2.5.2. Cytotoxicity assay

Peritoneal macrophages were collected from normal BALB/c mice in RPMI 1640 medium (SIGMA, St. Louis, Mo, USA) supplemented with antibiotics, and seeded at 30 000 cell/well. The cells were incubated for 2 h at 37 ℃ in 5% CO_2 and non-adherent cells were removed. Dilutions of the extracts or DMSO were added and incubated in the same conditions during 72 h. The viability was determined adding 15 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (SIGMA, St. Louis, MO, USA) at 5 mg/mL. After incubation for an additional 4 h the formazan crystals were dissolved by addition of 100 μL DMSO. The optical density was determined using an EMS Reader MF Version 2.4-0, at a test wavelength of 560 nm and a reference wavelength of 630 nm[12].

2.5.3. Antiamastigote assay

The peritoneal macrophages monolayer was obtained and infected with stationary-phase *L. amazonensis* promastigotes at 4:1 parasite/macrophage ratio and incubated for further 4 h. The monolayer's cell was washed and treated with 1 μL of extracts or DMSO for further 48 h. Parasites were then fixed, stained with Giemsa, and examined under light microscopy[13]. The number of intracellular amastigotes and infected macrophage was determined by counting and the percent of inhibition were calculated in comparison to cultures treated with DMSO[14].

The medium inhibitory concentration (IC_{50}) value and medium cytotoxicity concentration (IC_{50}) were determined from the lineal concentration-response curves. The selectivity index (SI) ratio was calculated trough division of IC_{50} for macrophage by IC_{50} for promastigotes[15].

2.6. In vivo studies

2.6.1. Infection and treatment

On day 0, mice were infected with 10^7 logarithmic promastigotes by subcutaneous route in the right footpads. On day 15 post-infection (pi.), mice were randomly divided into four groups: two control groups (one group treated with vehicle and other one untreated), one group treated with extract 100 mg/kg, and the last group treated with amphotericin B 1 mg/kg. Treatment was administered daily

by intraperitoneal route from day 15 pi. to the day 30 pi.

2.6.2.Biological evaluation

Animals were checked daily to record the death and the individual corporal weigh were determined each 7 days. Disease progression was monitored by measuring footpads swelling, using an automatic calliper. Average lesion size was calculated as the differences obtained between infected and uninfected footpads. On day 45 pi., six animals of each group were killed by cervical dislocation and the parasite burden was determined using the culture microtitration method described by Buffet and collaborators[16].

2.7. Statistical analyses

Data on lesion progression were analyzed for statistical significance by the analysis of variance test, following of a post hoc test (LDS test or planned comparison) using the Statistical for Windows Program (Release 4.5, StatSoft, Inc. 1993).

3. Results

The three extracts evaluated inhibited promastigotes form growing. *P. carolinensis* extracts showed the best activity, with IC_{50} values below of 100 μg/mL from all extracts, except for *n*-butanol extract. The active extracts caused a high toxicity; although the ethanol and *n*-hexane extracts demonstrated the more specific antileishmanial activity (Table 1). The ethanol and *n*-hexane extracts from *P. carolinensis* caused similar inhibition against amastigote form, which IC_{50} values of 30.9 μg/mL and 29.6 μg/mL, respectively. Amphotericin B showed an IC_{50} of 0.034 μg/mL.

The intraperitoneal administration of the ethanol extract of *P. carolinensis* at 100 mg/kg prevented lesion development compared with the animals treated with the vehicle, untreated mice, as well as mice treated with amphotericin B ($P<0.05$). However, the treatment with *n*-hexane extract did not decrease the lesion size ($P>0.05$) respecting untreated animals. These results were corroborated when the parasite burden was determined (Figure 1).

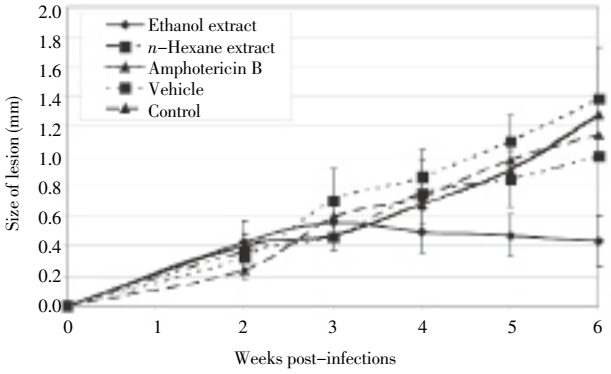

Figure 1. Effect of the ethanol and *n*-hexane extracts from *Pluchea carolinensis* extracts (100 mg/kg), Vehicle (0.1 mL) and Amphotericin B (1 mg/kg) on lesion growth. BALB/c mice were infected in the footpad by subcutaneous infection with 10^7 promastigotes of *L. amazonensis* and treated by intraperitoneal route during 15 days. Lesion sizes were measured at the indicated times post-infection and the results are expressed as mean standard deviation.

Table 1
Activity of *Pluchea* spp. extracts and amphotericin B on promastigotes of *L. amazonensis* and macrophage from BALB/c mice.

Plant Specie	Extract	IC_{50}[a] ±SD[b] (μg/mL)		SI[c]
		Promastigote	Macrophage	
P. carolinensis	EtOH	30.4±1.2	172.2±1.0	6
	$CHCl_3$	61.2±2.4	64.8±1.1	1
	n-hexane	54.5±4.8	241.5±1.3	4
	EtOAc	90.3±9.1	57.4±2.7	1
	n-BuOH	> 100	ND[d]	–[e]
P. odorata	$CHCl_3$	> 100	ND	–
	n-hexane	93.5±7.6	59.7±2.7	0
	EtOAc	> 100	ND	–
	n-BuOH	> 100	ND	–
P. rosea	$CHCl_3$	67.9±8.6	80.1±7.4	1
	n-hexane	> 100	ND	–
	EtOAc	> 100	ND	–
	n-BuOH	> 100	ND	–
Amphotericin B		0.026±0.003	5.9±0.5	172

a: IC_{50}: Concentration of drug that caused 50 % of inhibition. b: SD: Standard deviation. c: SI: Selectivity index. d: ND: activity not detected. e: –: Not done.

4. Discussion

Plant extracts or plants-derived compounds are likely to provide a valuable source of new medicinal agents[17,18] and the urgent need for alternative treatments has led to a program to screen natural products for potential use in therapy of leishmaniasis. The leishmanicidal activity of several extracts has been evaluated on different plant family[19]. Approximately, 20 different species of Asteraceae family[4,20,21] have demonstrated to have antileishmanial potentialities. Among them, the genus *Pluchea* has been the less explored, but reports shows that some species of the genus have been identified as sources of antimicrobial crude extracts[5]. In addition, etnobotanical reports have described the use of *P. carolinensis* to treat migraine in Puerto Rico population[22] and *P. odorata* as anticancer in Guatemala[23]. Here, we report for the first time the antileishmanial evaluation of different extracts from three species of *Pluchea*.

Different *in vitro* activities against promastigote form were recorded of the extracts, which were prepared using different solvents, as well as cytotoxicity effect on macrophage from BALB/c mice. The best selectivity was demonstrated by ethanol and *n*-hexane extracts from *P. carolinensis*, which inhibited the growth of intracellular amastigote with an IC_{50} value of 30 μg/mL, approximately. These results led us to evaluate both extracts *in vivo* with the aim to validate the antileishmanial potentialities of these plants.

The leaf ethanol extract from *P. carolinensis* showed the greatest activity in BALB/c mice experimentally infected. In addition, no death or weight losses higher than 10% were observed in the treated animals with the extract. Toxicity of natural products is important because of the interest in alternative therapies and the therapeutic use of medicinal plants in endemic populations, which should be safe to use. One limitation of conventional antileishmanial treatments result their side effects. Many people worldwide have no access to conventional pharmacological treatments but depend on folk remedies. The widespread use of traditional medicine suggests that natural products are harmless, but their safety require scientific demonstrations[24].

The amphotericin B showed better activity *in vitro* compared with the extracts evaluated, which are complex mixture of substances. The purification of active compounds might result in a considerable increase of their antileishmanial activity. In addition, amphotericin B is a pure compound and constitutes the most active antileishmanial drug. Nevertheless, due to their toxicity the amphotericin B is considered as a second option in areas refractory to antimony[25,26]. In contrast, experiment the ethanol extract from *P. carolinensis* cause higher efficacy against cutaneous leishmaniasis *in vivo*. Previous studies have suggested the low effect of amphotericin B against leishmaniasis caused by *L. amazonensis* in BALB/c mice[27,28].

Previously has been demonstrated that among the phenolic compounds, flavonoids are constituents from *P. carolinensis* extracts. In deep, aglycone flavonols with kaempferol, myricetin and quercetin skeletons were detected from crude ethylacetate extract, in (374 ± 1), (62 ± 3) and (2 622 ± 2) μg per gram of leaf dry weight/g, respectively[29]. These flavonols have hydroxyl group in the positions 3′ and 4′ of the flavan skeleton. In this sense, the 3′,4′,5,6,7-pentahydroxy-3-methoxyflavone was also identified from leaves of *P. carolinensis*[30]. So, it is possible that these flavonols would be responsible in part for the antileishmanial activity found for this extract. In addition, some terpenoids with skeleton type cuauthemone and eudesmane[31] have been isolated from crude leaf extract from the species *Pluchea carolinensis*. This kind of phytochemicals could be also the responsible of the antileishmanial activity found in the non-polar extract.

The mechanism of action related with antileishmanial activity of *P. carolinensis* has not been studied, although in this study we demonstrated their direct effect on *Leishmania* parasites growing. In addition, Rosales *et al* reported the anti-inflamatory effect of *P. carolinensis* extracts[32], which could influence the decreased of lesion growing. During the infection by *Leishmania* parasite occurs an inflammatory process in the site of infection due to the influx of cytokines and others effectors cells of the immune system, which have direct relation with lesion size[11].

The assessment of different extracts from three Cuban species of the genus *Pluchea* could suggest that *P. carolinensis* is the most promising species among the studied and further studies can be performe to evaluate the flavonols compounds isolated from this plant as a source of new antileishmanial agent.

Conflict of interest statement

We declare that we have no conflict of interest.

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