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Anti-leishmanial and immunomodulatory activities of extracts from *Portulaca hirsutissima* and *Portulaca werdermannii*

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Abstract

Ethyl acetate and chloroform extracts from aerial parts of *Portulaca werdermannii* and *P. hirsutissima* were tested in lymphoproliferation assays and axenic cultures of *Leishmania amazonensis* and *Trypanosoma cruzi*. Both extracts of *P. werdermannii* and *P. hirsutissima* had a potent inhibitory activity on lymphocyte proliferation. On the contrary, only the chloroformic extract of both plants inhibited *L. amazonensis* growth, without effect on *T. cruzi* cultures. These results indicate these *Portulaca* species as potential sources of new active molecules for the treatment of leishmaniasis and immune-mediated pathologies.

Keywords: *Portulaca werdermannii*; *Portulaca hirsutissima*; Anti-protozoal activity; Immunomodulatory activity

1. Introduction

Portulacaceae comprises 25–30 genera and between 450–500 species. Most species are succulent herbs or subshrubs from tropical to subtropical arid areas around the World [1]. Two genera are found in Brazil: *Portulaca* and *Talinum*. *Portulaca* is the largest genus in the family, but specific delimitations are difficult and the estimates of the number of species range from about 40 to near 150 [2]. A recent survey of *Portulaca* in Brazil found 21 species, seven of which occurring in the semi-arid area [3].

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Some species of *Portulaca* are cultivated with ornamental purposes and the weedy *P. oleracea* is sometimes cooked as a vegetable or eaten raw in salad. Species of this genus have been used in folk medicine for centuries. *P. oleracea* has been used in Chinese medicine since 500 a.C. as gastroprotector, for treatment of infectious diseases and inflammatory processes. Indian people from Guyanas used this plant to treat diabetes [4]. In Brazil, *P. oleracea* is used as diuretic, against fever and intestinal parasites and kidney complications in the Amazonic region, whereas in the Atlantic forest region this plant is used against gastric ulcer [5].

Leishmaniasis, found in 88 countries, are a group of diseases affecting more than 12 million of persons in the whole World [6]. The treatment of leishmaniasis is based on antimonial compounds, pentamidine and liposomal amphoterycin B [7], and is made under ambulatorial or hospital level, sponsored by the State, mainly in developing countries. The drugs employed can cause diverse side-effects, decreasing the treatment adherence by the patients. In addition, the rate of co-infection leishmaniasis-HIV has increased, leading to the development of resistance to the available drugs [8]. Chagas disease, a zoonosis caused by the *Trypanosoma cruzi*, is found in 18 Latin American countries and also belongs to the neglected diseases group. Benznidazole and nifurtimox are the drugs currently available for the treatment, which are effective only in the acute phase of the pathology and have high toxicity [9].

Thus, the finding of new active drugs for both groups of pathologies, with less toxicity, is of great importance [10].

This work aimed to study the anti-parasitic and immunomodulatory activities of extracts of two species of *Portulaca* native from the semi-arid area of Northeastern Brazil, a little explored region regarding the pharmacological potential which possesses typical vegetation, the Caatinga.

2. Experimental

2.1. Plants

P. werdermannii Poelln. and *P. hirsutissima* Cambess. (Portulacaceae), collected in the municipalities of Delfino and Sento Sé, in the State of Bahia, Brazil were identified in the Herbarium of the State University of Feira de Santana (HUEFS) where vouchers are deposited.

2.2. Preparation of extracts

Dried aerial parts of *P. werdermannii* (PW) and *P. hirsutissima* (PH) macerated in MeOH:H_2O (6:4) and extracted were partitioned with $CHCl_3$ and AcOEt, successively to give PH1, PH2, PW1, PW2 (yields: 0.051%, 0.017%, 0.093 and 0.076%, respectively).

For biological assays PH1, PH2, PW1, PW2 were dissolved in DMSO (10 mg/ml). Samples were sterilized with gamma radiation (dose of 60,000 rad in a ^{137}Cs source irradiator purchased from CisBio International, France).

2.3. Animals

Male and female 4–6 weeks old BALB/c mice were used as spleen cell donors. They were housed in standard environmental conditions and maintained at the animal facilities at Gonçalo Moniz Research Center, FIOCRUZ (Salvador, Brazil). Mice were provided rodent diet and water ad libitum. All mice were killed according to the Oswaldo Cruz Foundation guidelines for laboratory animals.

2.4. Cytotoxicity assay

The cytotoxicity (LC_{50}) of PH1, PH2, PW1, PW2 was determined using BALB/c mice splenocytes (5×10^6 cells/well) cultured in 96 plate-well in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% of fetal calf serum (FBS; Cultilab, Campinas, SP, Brazil) and gentamycin (50 μg/ml) (Novafarma, Anápolis, GO, Brazil). Each extract was evaluated at 1, 10 and 100 μg/ml, in triplicates. A positive control was made with cells treated with a 1% saponin solution. Cultures were incubated in the presence of ^3H-thymidine (1 μCi/well) during 24 h at 37 °C and 5% CO_2. After this period, the content of the plate was harvested to determine the ^3H-thymidine incorporation using a Beta Radiation Counter (β-matrix 9600, Packard, Meriden, CT). The viability of the cells was determined by counting the incorporation of thymidine and the cytotoxicity was calculated in relation to untreated cultures.

2.5. Anti-Leishmania amazonensis assay

L. amazonensis (MHOM/BR88/BA-125 Leila strain) promastigotes were cultured in liver infusion tryptose (LIT) medium supplemented with 10% FBS, 5% sterile human urine and 50 μg/ml gentamycin, pH 7.2, at 26 °C until logarithmic phase. Parasites were cultured in 96-well plate at 5×10^6 cells/well in 200 μl, in triplicate wells, alone or in the presence of the samples analyzed in five different concentrations (100, 33, 11, 3 and 1 μg/ml). After 24, 72 and 120 h a direct counting of viable parasites was carried out in a Neubauer chamber using a phase contrast microscope. Glucantime and Amphoterycin B were used as a reference.

2.6. Anti-T. cruzi assay

T. cruzi (Y strain) epimastigotes were cultured in liver infusion tryptose (LIT) medium supplemented with 10% FBS, 5% sterile human urine and 50 μg/ml gentamycin, pH 7.2, at 26°C until logarithmic phase. Parasites were cultured in 96-well plates at 10^7 cells/well in 200 μl, in triplicate, alone or in the presence of the samples analyzed in one concentration (100, 10 or 1 μg/ml) or alone. After 24 h plates were pulsed with 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl-tetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO), 50 ng in saline/well. Plates were incubated again at 37 °C during 3 h and read in spectrophotometer (Spectra Max 190, Molecular Devices, Sunnyvale, CA) at 570 nm.

2.7. Lymphoproliferation assay

BALB/c splenocytes suspensions were prepared in RPMI medium (Life Technologies, GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2 mM of L-glutamine, 0.1% RPMI 1640 vitamins solution (Sigma), 1 mM of sodium pyruvate, 10 mM of Hepes, 50 μM of 2-mercaptoethanol, and 50 μg/ml of gentamycin (Sigma). Splenocytes were cultured in 96-well plates at 4×10^5 cells/well, in 200 μl, in triplicate wells, in the presence of concanavalin A (Con A) at 2 μg/ml alone or in the presence of 100–10-1 μg/ml of PH1, PH2, PW1, PW2. After 48 h, plates were pulsed with 1 μCi of methyl-^{3}H thymidine (Amersham, Little Chalfont, England) for 12 h, and proliferation was assessed by measurement of ^{3}H-thymidine uptake in a β-plate counter (Packard). The percentage of inhibition of proliferation by the extracts was determined in relation to controls stimulated by Con A in absence of drugs.

2.8. IC_{50} calculation and statistical analyses

The inhibitory concentrations for 50% of inhibition (IC_{50}) of lymphoproliferation and Leishmania growth, at third day of incubation, were calculated based in a nonlinear regression (curve fit) and the statistical analyses were made by one-way ANOVA and Newman–Keuls multiple comparison test using Prism version 4.00 for Windows, GraphPad Software (San Diego, CA). All data were reported as the mean ± standard error. The level of statistical significance for all tests was $P < 0.05$.

3. Results

Immunomodulatory and anti-leishmanial activity of *P. hirsutissima* and *P. werdermannii*, was evaluated. The species were collected in the countryside at the state of Bahia, Brazil, and the aerial parts of the plants were used to prepare extracts with two organic solvents, ethyl acetate and chloroform, generating samples PH1, PH2, PW1 and PW2 (Table 1).

Table 1
The extracts from *P. hirsutissima* and *P. werdermannii* aerial parts collected in semi-arid areas of the Bahia State, Brazil

Material	Plants	Local of collection	Solvents	Voucher specimens
PH1	*P. hirsutissima*	Sento Sé	Chloroform	*K.R.B.Leite 168* (HUEFS n°58995)
PH2	*P. hirsutissima*	Sento Sé	Ethyl acetate	*K.R.B.Leite 168* (HUEFS n°58995)
PW1	*P. werdermannii*	Delfino	Ethyl acetate	*A.Oliveira 111* (HUEFS n°59056)
PW2	*P. werdermannii*	Delfino	Chloroform	*A.Oliveira 111* (HUEFS n°59056)

Table 2
The inhibitory concentration (IC_{50}) of lymphoproliferation and of *L. amazonensis* anti-leishmanial activity of *P. hirsutissima* and *P. werdermannii* aerial parts extracts

Material	IC_{50} (μg/ml)Lymphoproliferation inhibition	IC_{50} (μg/ml) [a]Anti-leishmanial activity
PH1	13.1	7.6
PH2	41.0	–
PW1	15.4	–
PW2	39.4	11.1
Glucantime	–	11,000
Amphoterycin B [7]	–	0.24–0.71

[a] Calculated after 72 h of incubation.

The immunomodulatory potential of these species was evaluated in a lymphoproliferation assay. The four samples tested had inhibitory activity in the proliferation of Con A-activated lymphocytes. The effective concentrations for 50% inhibition of lymphoproliferation are shown in Table 2. PH1 and PW1 had the lowest IC_{50} values (13.1 and 15.4 μg/ml, respectively). The inhibitory activity on lymphocytes was not due to a cytotoxic effect, since samples were tested in concentrations with toxicity equal or below 30% (Fig. 1).

The anti-protozoal activity of *P. hirsutissima* and *P. werdermannii* was investigated using two trypanosomatid parasites, *T. cruzi* and *L. amazonensis*. The extracts tested did not have activity against *T. cruzi* (data not shown). Of the four extracts tested, two (PH1 and PW2) had inhibitory activity in *L. amazonensis* cultures. In the third day of culture with the higher dose of extract tested (100 μg/ml), the number of parasites was significantly reduced compared to control cultures grown in the absence of extracts. The two samples active against *L. amazonensis* had their effective concentration for 50% calculated (Table 2).

4. Discussion

Immunomodulatory and anti-leishmanial activities were detected in samples extracted from *P. hirsutissima* and *P. werdermannii*. According to the literature, there are many descriptions confirming pharmacological activities for species of *Portulaca*, such as gastroprotection [11], bronchodilatory effect, analgesic and anti-inflammatory activity [12,13], action on central and peripheral nervous system [14] and acceleration of wound healing processes [15].

In the present work we demonstrated a potent immunomodulatory activity of *P. hirsutissima* and *P. werdermannii* extracts. Chloroform extract from aerial parts of *P. hirsutissima* (PH1) and ethyl acetate from aerial parts of *P. werdermannii* (PW1) had the lower values of EC_{50} values. At a concentration of 100 μg/ml, PH1 and PW2 had the highest immunomodulatory activity with values superior to 95%. PH1 and PW2 were extracted with chloroform from different species. The immunomodulatory potential for the species studied is being tested on in vivo models of

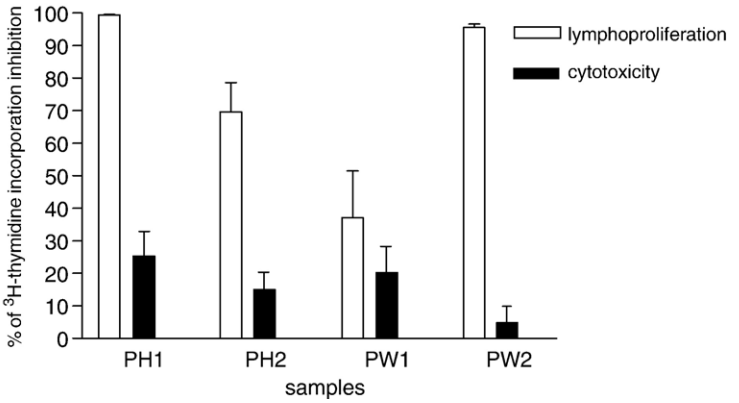

Fig. 1. Cytotoxicity and lymphoproliferation of the *P. hirsutissima* and *P. werdermannii* whole plant extracts on mice splenocytes. Values represent the means + S.D. of values obtained in five individual experiments.

immune-mediated diseases. It is important to note that inhibition was not due to the cytotoxicity of samples, since the cytotoxicity values of extract concentrations assayed were lower than 30% with the highest dose used and thus the lethal concentration for 50% (LC_{50}) could not be calculated.

In conclusion the results of this study indicated a promising immunomodulatory and anti-leishmanial activities for the *P. hirsutissima* and *P. werdermannii* extracts. Investigations should be continued aiming to identify the active compounds present in these extracts in order to obtain potential new compounds for the treatment of leishmaniasis and immuno-mediated the pathologies.

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