



Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: <http://www.elsevier.com/locate/jtcme>

Therapeutic effect of *Prosopis strombulifera* (LAM) BENTH aqueous extract on a murine model of cutaneous leishmaniasis



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ARTICLE INFO

Article history:

Received 4 May 2021

Received in revised form

10 August 2021

Accepted 11 August 2021

Available online 16 August 2021

Keywords:

Leishmania amazonensis

Neglected tropical disease

In vivo model

Natural extract treatment

Drug resistance

Infectious disease

Leishmaniasis

Herbal medicine

Treatment with natural extracts

ABSTRACT

Background and aim: *Prosopis strombulifera* (Lam.) Benth is a rhizomatous shrub native from different zones of Argentine Republic. *P. strombulifera* aqueous extract (PsAE) has different effects and several biological activities have been reported. The goal of this study was to analyze the activity of PsAE on a murine model of cutaneous leishmaniasis caused by *Leishmania amazonensis*.

Experimental procedure: PsAE was orally administered at 150 mg/animal/day on BALB/c mice infected in the right footpad (RFP) with 1×10^5 promastigotes of *L. amazonensis*. As a chemotherapeutic control of treatment, animals receive a commercial form of meglumine antimoniate (MA) (Glucantime®, Aventis, Paris, France).

Results and conclusion: We observe that the size of RFP lesions of infected mice without treatment showed a grade of inflammation, ulceration and necrosis at the site of infection much greater than that observed with PsAE or MA treatment. Moreover, PsAE was capable of decreasing parasite burden and splenic index. Furthermore, PsAE treated mice showed a significant decrease in O.D. of total anti-*Leishmania* IgG antibody responses against *L. amazonensis*. This decrease was similar to those observed when the reference drug, MA, was used. This would indicate that PsAE treatment inhibits or delays disease progression in mice. In conclusion, our findings suggest that PsAE could be a potential candidate to be used, as a new therapeutic strategy, to treat cutaneous leishmaniasis caused by *L. amazonensis*.

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1. Introduction

The leishmaniasis are a spectrum of diseases caused by infection

Abbreviations: PsAE, *Prosopis strombulifera* aqueous extract; MA, meglumine antimoniate; RFP, right footpad; TLA, total *L. amazonensis* antigen; BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; PBS, phosphate buffered saline; FBS, fetal bovine serum; MTT, thiazolyl blue tetrazolium bromide; EGF, endothelial growth factor.

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

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with protozoan pathogens of the *Leishmania* genus, with an estimated 2 million new cases *per annum*.¹ *Leishmania* parasites are transmitted to a mammalian host via the bite of an infected sand fly. The clinical forms of the disease (cutaneous, mucocutaneous and visceral leishmaniasis) depend on the species of *Leishmania* involved. Current treatments for leishmaniasis, like meglumine antimoniate (MA), are unsatisfactory due to high associated toxicity, pain, cost, complex administration and the emergence of resistant strains.^{2,3} Efforts have greatly increased over the last decade to identify novel compounds with antileishmanial properties. Thus, one strategy in the search for new compounds is the screening of molecules purified from plant sources. There are more than five hundred species of plants in Mendoza province, but a limited extent of that have been studied against the genus

<https://doi.org/10.1016/j.jtcme.2021.08.009>

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Leishmania.⁴

Prosopis strombulifera (Lam) Benth is a rhizomatous shrub from the Fabaceae family; it can be found in the northern and central zones of Argentine Republic. *P. strombulifera* aqueous extract (PsAE) has different effects and its several biological activities have been reported.⁵

The aim of this work was to evaluate the effect of PsAE as a possible treatment for cutaneous leishmaniasis on a murine model.

2. Materials and methods

2.1. Plant material and aqueous extract preparation

P. strombulifera was collected in Lavalley county, Mendoza, Argentina (33° 44'10" S, 68° 21' 30.5" W). PsAE was prepared according to Persia et al., 2020.⁶

2.2. Experimental animal

Male BALB/c mice of 8–10 weeks were used. All animals were kept in a controlled light-dark cycle and room temperature. Mice chow and drinking liquids were available *ad libitum*. All animals were cared in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. All procedures were approved by the Institutional Animal Care and Use Committee of School of Medical Science, UNCuyo, Mendoza, Argentina (Protocol approval N° 132/2018).

2.3. In vitro antileishmanial effect of PsAE

The antileishmanial effect of PsAE was determined on promastigotes of *L. amazonensis* MHOM/VE/84/MEL by MTT (thiazolyl blue tetrazolium bromide) assay.⁷ Promastigotes (10^6 /well) were incubated on 96-wells plates with or without different concentrations of the PsAE, Amphotericin B or Pentamycin® (as standard drugs) at 24 °C for 48 h on Grace's Insect Media (without phenol red) supplemented with fetal bovine serum (FBS) at 5 % and antibiotics (100 IU/mL penicillin, 100 mg/mL streptomycin). After incubation, MTT assay was made.⁸ Three independent experiments were performed in triplicate. Results were expressed as the mean concentration inhibiting 50 % of the parasite growth (IC50) + R².

2.4. Cytotoxic effect of PsAE

Human mammary epithelial line cells MCF10A were cultured at 1.3×10^4 /well on 96-well plates for 24 h at 37 °C 5 % CO₂ in humidified atmosphere on DMEM medium supplemented with insulin 0.006 UI/mL, glutamine 2.5 mM, EGF 20 ng/mL, FBS 5 %, penicillin and streptomycin. Cells were incubated with or without different concentrations of PsAE (25–0.39 mg/mL) for 24 and 48 h and their viability percentage were measured by MTT assay.⁸ Values were expressed as mean concentration of PsAE inhibiting 50 % of the viability (IC50) + R².

2.5. Animal infection and in vivo treatment

Male BALB/c mice were infected in the right footpad (RFP) with 1×10^5 promastigotes of *L. amazonensis* (MHOM/VE/84/MEL). PsAE was administered orally by diluting it in the drinking water at nontoxic dose of PsAE 150 mg/animal/day for 10 weeks.⁹ As a chemotherapeutic control treatment, animals receive a commercial form of MA (Glucantime®, Aventis, Paris, France). MA was administered by intraperitoneal route for 30 days, the dose being 400 mg/kg/day.¹⁰ Another control group was treated with phosphate buffered saline (PBS). Two independent protocols were conducted with

6 animals per experimental group.

2.6. Infection progression

The swelling of RFP was measured weekly using a digital caliper (SCHWYZ, ED-10P) until 10 weeks after infection. The value for uninfected footpads was subtracted from each infected footpad to estimate lesion size.

2.7. Splenic index

At week 10, mice were weighted and their spleens removed and weighted to calculate splenic index: Splenic index = (spleen weight/body weight)*100.

2.8. Parasite burden

At week 10, infected footpads were removed and homogenized in 1 mL of supplemented RPMI 1640 medium. The suspension was plated in 96-wells plate and 10-fold dilutions were made and incubated at 26 °C for 14 days in order to assess viable parasites under optic microscopy.

2.9. Humoral response

The humoral immune response induced by the experimental treatment was evaluated by measuring total specific IgG, IgG1 and IgG2a by enzyme linked immunosorbent assay (ELISA) in serum samples collected on week 3 and 10 of the protocol.¹¹

2.10. Statistical analysis

Data were analyzed employing the Tukey's-Kramer Multiple Comparisons Test using KyPlot 6.0 version software, KYENSLAB INC. (<https://www.kyenslab.com>). Shown data represent the mean values ± standard deviation (SD).

The percentages of reduction of antibodies, splenic index, parasite load and footpad swelling of treated groups with respect to control groups (PBS and MA) were determined:

$$\% \text{ reduction} = [(\text{control media value} - \text{treated media value}) / \text{control value}] * 100$$

These results were showed as supplementary data.

Half maximal inhibitory concentration (IC50) values of *in vitro* antileishmanial effect and cytotoxicity effect on MCF10A cells of PsAE and standard drugs were calculated by log(concentration) vs. normalized response of % response-variable slope and a post nonlinear regression test to analyze the dose-response inhibition relation, using Graphpad Prism version 5.00 software.

3. Results

3.1. Nontoxic dose of PsAE have not in vitro antileishmanial effect on promastigotes

In Table 1, it can be observed that PsAE did not inhibit the promastigotes growth at nontoxic concentration for mammalian line cells MCF10A.

3.2. PsAE reduces RFP swelling of infected mice

In Fig. 1, we observed that infected mice treated with PsAE showed a significant reduction in the size of the footpad lesion compared with infected mice treated with PBS. This decrease

Table 1
IC50 values of antileishmanial and cytotoxic effect of PsAE on promastigotes and mammalian line cell.

Drug	Antileishmanial effect	Cytotoxicity on MCF10A	
	IC50 + R ²	IC50 + R ² (24 h)	IC50 + R ² (48 h)
Pentamycin (μM)	2.445 + 0.9537	–	–
Amphotericin B (μg/mL)	0.2035 + 0.8605	–	–
PsAE (μg/mL)	20810	4709 + 0.8505	3591 + 0.9014

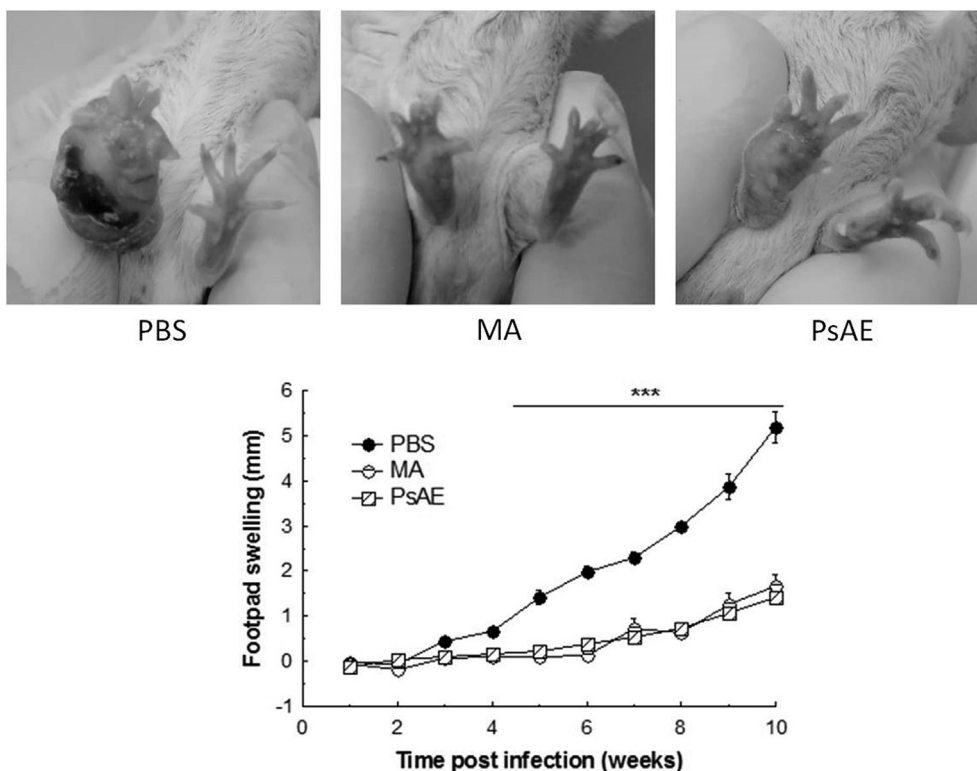


Fig. 1. Footpad swelling in male BALB/c mice caused by infective promastigotes of *L. amazonensis* after 10 weeks. Values represent the means ± SD from 6 animals under each condition. The asterisks indicate statistically significant differences between PBS group and the different treatments (MA or PsAE): ***p < 0.001.

becomes significant from week 5 until week 10. The animals treated with MA showed a similar footpad lesion to PsAE treated mice. This is due to the used concentration MA did not heal completely but significantly reduced the lesions compared to infected mice treated with PBS.¹⁰

3.3. PsAE treated mice showed a reduction in parasite burden and splenic index

To determine the effectiveness of the treatment with PsAE, different parameters were analyzed. Parasite burden and splenic index were determined 10 weeks after infection. Mice treated with PsAE and MA showed a significant reduction of parasite burden and splenic index compared to the PBS group where a strong splenomegaly was observed (Fig. 2).

3.4. PsAE treatment reduces the humoral immune response and induces a switch on the type of immune response generated by the infection

Regarding to humoral immunity, we could observe that at week 3 there were no significant differences in anti-*Leishmania* IgG levels among the groups. The levels of specific anti-*Leishmania* IgG

produced by mice treated with MA or PsAE showed a significant decrease compared to infected mice treated with PBS at week 10 after infection (Fig. 3A). Moreover, PsAE induced a switch in the type of humoral immune response at week 10 after infection, reducing statistically the IgG1 antibodies (Fig. 3B) compared to PBS group and maintaining high levels of IgG2a (Fig. 3C).

4. Discussion

Cutaneous leishmaniasis is one of the clinical forms of leishmaniasis diseases depending on the species of *Leishmania* involved. One of the species of *Leishmania* that has been identified in human cases in the Argentine Republic is *L. amazonensis*, which is associated with different clinical manifestations, including cutaneous leishmaniasis.¹

There are many natural products with antiparasitic activity, including different species of *Leishmania*.^{12,13} They were obtained by different extraction methods^{14,15} using different parts of plant materials.^{16,17} Most of them have been tested *in vitro* and *in vivo* against visceral leishmaniasis,¹⁸ but fewer of them have been tested *in vivo* against cutaneous leishmaniasis with different ways of administration.^{14,19}

There are only 2 previous reports about *Prosopis* gender

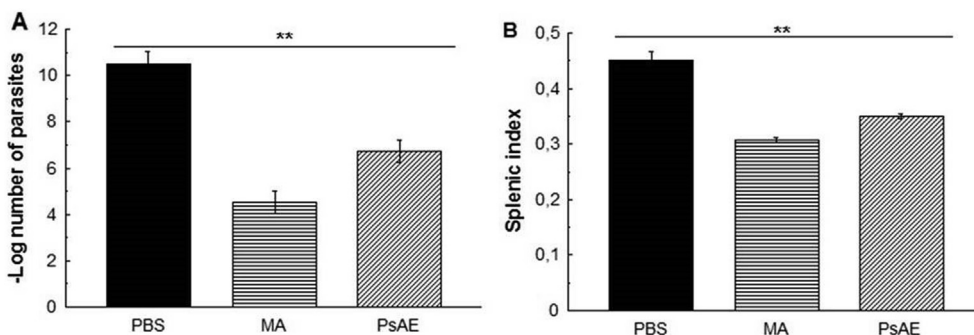


Fig. 2. Parasite burden in infected footpads, the numbers of viable parasites were determined after 10 weeks of infection by a limit dilution assay (A). Splenic index was evaluated 10 weeks after infection (B). Values represent the means \pm SD from 6 animals under each condition. The asterisks indicate statistically significant differences between PBS group and the different treatments (MA or PsAE): ** $p < 0.01$.

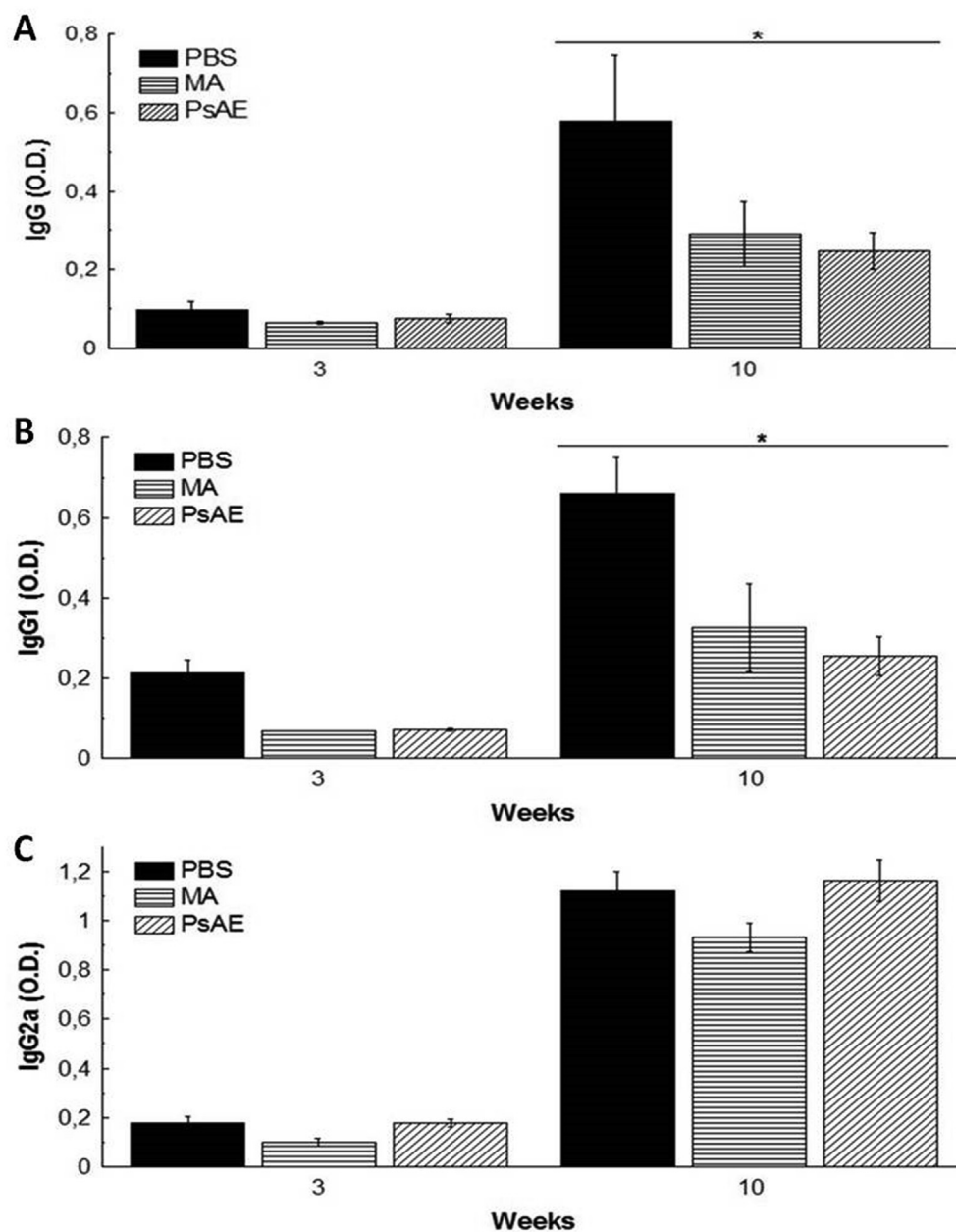


Fig. 3. Humoral immune response in mice of different experimental groups. Anti-*Leishmania* IgG (A), IgG1 (B), IgG2a (C) antibodies responses to *L. amazonensis* by the mice. Values represent the means \pm SD from 6 animals under each condition. The asterisks indicate statistically significant differences between PBS group and the different treatments (MA or PsAE): * $p < 0.05$.

leishmanicidal activity against different strains of *Leishmania* and its multiple development forms.^{20,21}

PsAE was administered orally in drinking water on animals at concentrations up to 150 mg/animal/day. This dose was chosen due to it has been demonstrated that it does not produce toxic effects in BALB/c mice, without alteration on anatomical, hematological and biochemical parameters.⁹ PsAE reduced the lesion sizes in mice infected with *L. amazonensis* in a similar way to mice treated with MA. The size of RFP lesions of infected mice treated with PBS shows a grade of inflammation, ulceration and necrosis at the site of infection much greater than that observed with PsAE or MA treatment.

It has been demonstrated that *L. amazonensis* can produce visceral leishmaniasis, reach inner organs like spleen, increasing its size.²² PsAE and MA were capable of decreasing parasite burden, which was related to a reduction in splenic index. This reduction in splenic index might indicate a decrease in parasite visceralization due the treatment.²³

Regarding total anti-*Leishmania* IgG antibody responses, treated mice with PsAE or MA showed a significant decrease in O.D. values of IgG compared to PBS group at 10 weeks after infection. This would indicate that the treatment with PsAE and MA inhibit or delay disease progression in mice. Mwololo et al., 2015 indicated that there is a correlation of high antibody levels during active disease and a reduction in antibody levels following successful cure.²⁴ Furthermore, it has been demonstrated that IgG1 antibodies are increased in mice infected by *Leishmania*.²⁵ For that, a reduction of IgG1 observed in the PsAE treated group compared to the control PBS group, indicates PsAE induces a switch in the immune response with bias to Th1 profile.

Our results show that in spite of PsAE has not *in vitro* antileishmanial activity on promastigotes, its effect is clear when it is administered on animals, as it is observed with MA.²⁶ Chemical modifications on natural extracts during its metabolism could explain the observed effect.²⁷ However, more experiments are necessary to understand the action mechanism of PsAE.

Previously, PsAE chemical composition was identified by UHPLC–Q/Orbitrap/MS/MS, including simple organic acids, phenolic acid, procyanidins, flavonoids and oxylipins.⁶ Many studies demonstrate the antileishmanial effect of phenolic compounds by inhibiting arginase, inducing morphological changes and cell cycle arrest, altering mitochondrial activity and DNA arrangement of *Leishmania*.^{28,29} Due to the PsAE is constituted by different chemical compounds, many different action mechanisms could be acting at the same time; therefore more experimental analysis has to be made.

Our results suggest that, the non-toxic natural PsAE could be used as a potential new therapeutic strategy to treat cutaneous leishmaniasis.

Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Funding

Universidad Nacional de Cuyo (SIIP J081), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 11220150100210Co 2015-2017), Agencia Nacional de Promoción Científica y Tecnológica (PICT N° 2018-00739).

Declaration of competing interest

The authors declare that there is no conflict of interests

regarding the publication of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtcme.2021.08.009>.

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