Activity of physalins purified from Physalis angulata in *in vitro* and *in vivo* models of cutaneous leishmaniasis

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**Objectives:** We have previously demonstrated the immunomodulatory effects of physalins, secosteroids purified from *Physalis angulata*. Here we investigate the antileishmanial activity of physalins *in vitro* and *in vivo* in a model of cutaneous leishmaniasis.

**Methods:** The antileishmanial activity of physalins B, D and F was tested in *Leishmania*-infected macrophage cultures. For the *in vivo* studies, BALB/c mice were infected with *Leishmania amazonensis* subcutaneously in the ear pinna and treated with physalin F by topical administration.

**Results:** Physalins B and F were able to reduce the percentage of *Leishmania*-infected macrophages and the intracellular parasite number *in vitro* at concentrations non-cytotoxic to macrophages. More importantly, topical treatment with physalin F significantly reduced the lesion size, the parasite load and histopathological alterations in BALB/c mice infected with *L. amazonensis*.

**Conclusions:** Our results demonstrate the potent antileishmanial activity of physalins, especially physalin F, and suggest these molecules as the basis for the development of new therapeutic options for cutaneous leishmaniasis.

Keywords: infections, macrophages, mice, secosteroids, therapy

**Introduction**

The use of natural products in the treatment of a variety of diseases has increased due to the considerable number of medicinal plants with proven biological activity applicable to the treatment of some diseases. *Physalis angulata* L. is an annual herb distributed in many countries located in tropical and subtropical regions of the world. This plant is widely used in popular medicine as a treatment for a variety of diseases and antitumour activity has been demonstrated.¹² In addition, this plant has compounds with anti-*Trypanosoma cruzi* activity.³ We have demonstrated the immunomodulatory activity of physalins, a group of secosteroids isolated from *P. angulata*, *in vitro* and *in vivo*.⁴⁵

In this work we investigate the antileishmanial effects of physalins B, D and F *in vitro* against intracellular amastigotes of *Leishmania amazonensis* and *Leishmania major*, and *in vivo* in a model of cutaneous leishmaniasis in BALB/c mice infected with *L. amazonensis*. Our results demonstrate the therapeutic potential of these substances for cutaneous leishmaniasis.

**Materials and methods**

**Mice**

Specific-pathogen-free, 8-week-old female BALB/c mice were maintained at the animal facilities at the Gonçalo Moniz Research Center–FIOCRUZ, and provided with rodent diet and water *ad libitum*. Animals were handled according to the NIH guidelines for animal experimentation. All procedures described here had prior approval from the local animal ethics committee.

**Parasites and drugs**

*L. amazonensis* (MHOM/BR88/BA-125 Leila strain) and *L. major* (MHOM/RJ/–/W-173) promastigotes were cultivated in liver-infusion

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Corresponding IC50 values were calculated by curve fitting. For inhibition was evaluated for up to 5–7 days of treatment and the observed and counted using a Neubauer chamber. Parasite growth in an optical microscope (Olympus, Tokyo, Japan). The percentage of infected macrophages was determined by counting the slides after Giemsa staining of infected macrophages and the mean numbers of amastigotes/infected macrophages were determined by counting the slides after Giemsa staining in an optical microscope (Olympus, Tokyo, Japan). The percentage of infected macrophages and the number of amastigotes per macrophage were determined by counting 100 cells per slides.

**In vitro infection**

Stationary-phase promastigotes of *L. amazonensis* (5 x 10^6) were used to infect BALB/c mice subcutaneously in 10 μL of saline, into the right ear dermis. After 2 weeks of infection, groups of five mice were topically treated daily with physalin F or the cream base (vehicle). Lesion development was monitored weekly using a digital caliper (Mitutoyo, Yokohama, Japan). The lesion size was determined by subtracting the thickness of the uninfected contralateral ear from the thickness of the infected ear. The number of parasites in the draining auricular lymph node was estimated by limiting dilution assay, as previously described. The parasites were observed and counted using a Neubauer chamber. Parasite growth inhibition was evaluated for up to 5–7 days of treatment and the corresponding IC50 values were calculated by curve fitting. For in vivo treatment, physalin F was used as a 1% formulation in a cream base, composed of 0.025% propylparaben, 0.042% methylparaben, 26% mineral oil, 16% glycerol, 23% emulsifying wax and 100 mL of distilled water (all purchased from Galena, Campinas, SP, Brazil).

**In vitro macrophage infection and treatment with physalins**

The murine macrophage cell line J774-G8 was maintained by culture in RPMI medium supplemented with 10% FBS, 7.5% sodium bicarbonate (Sigma), 1 M HEPES (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1 mM sodium pyruvate (Sigma), 2 mM l-glutamine (Sigma), 0.05 μM 2-mercaptoethanol and 50 μg/mL gentamicin (Sigma) at 37°C in 5% CO2/95% humidified air. Macrophages (5 x 10^5) cultured in polypropylene tubes were infected with *L. amazonensis* or *L. major* stationary-phase promastigotes at a ratio of 10 parasites per macrophage. After 4 h of infection, the free promastigotes were eliminated by successive washes using Hanks balanced salt solution (HBSS; GIBCO BRL, Gaithersburg, MD, USA). Physalin B, F or D (2 μg/mL) was added to the macrophage cultures. Amphotericin B was used as a positive control at the same concentration. At the concentration used, the viability of J774 cells was 100% after incubation with all the drugs tested, as determined by Trypan Blue exclusion. After 24 or 48 h, the cells were fixed in methanol and the percentage of infected macrophages and the mean numbers of amastigotes/infected macrophage were determined by counting the slides after Giemsa staining in an optical microscope (Olympus, Tokyo, Japan). The percentage of infected macrophages and the number of amastigotes per macrophage were determined by counting 100 cells per slides.

**Histopathological evaluation and immunohistochemistry**

Infected ears of BALB/c mice were removed after 5 weeks of infection and fixed in 10% formaldehyde. After 12–24 h of fixation, tissue slices were embedded in paraffin. Sections 3–5 μm thick were stained with conventional haematoxylin and eosin (H&E) and analysed by light microscopy. Immunostaining for *Leishmania* detection was performed as previously described.

**Results**

**Physalins B and F inhibit the infection of macrophages with *L. amazonensis* or *L. major***

J774-G8 macrophages were infected with *L. amazonensis* or *L. major* and treated with physalin B, D or F after 4 h of infection. A reduction in the percentage of macrophages infected with *L. major* after 24 or 48 h of infection was observed in cultures treated with physalins B and F, but not with physalin D (Figure 1a and b), at concentrations non-toxic to macrophages. Treatment with amphotericin B reduced the number of infected cells as well as the number of intracellular parasites. Similar results were obtained in macrophage cultures infected with *L. amazonensis* (data not shown). Physalins B and F against *L. amazonensis* amastigotes obtained in axenic cultures showed IC50 values of 0.21 and 0.18 μM, respectively.

**Statistical analyses**

Differences among the means of the different groups were analysed using one-way analysis of variance (ANOVA) followed by Newman–Keuls test for parametric data. For non-parametric data a paired multiple group Friedman test followed by Dunn’s test was used. The critical level of significance was established as *P* < 0.05.

**Figure 1.** Effects of physalins B, F and D on the growth of intracellular amastigotes of *L. major*. J774-G8 macrophages were infected with *L. major* and treated with 2 μg/mL physalin B, D or F 4 h after infection. The percentage of infected macrophages (a) and the intracellular amastigote numbers (b) were determined 48 h after infection by *L. major*. Amphotericin B (2 μg/mL) was used as a reference drug. Results shown are from one representative of three independent experiments performed. *One way ANOVA, *P* < 0.05, physalin B (95% CI = 20.58–90.76), physalin F (95% CI = 19.58–89.76) and amphotericin B (95% CI = 19.01–89.54) compared with vehicle-treated group.

[85]
Effects of physalin F treatment on experimental cutaneous leishmaniasis

To evaluate the in vivo effects of physalin F treatment in cutaneous leishmaniasis, BALB/c mice were infected with L. amazonensis in the ear dermis and treated daily with physalin F (topical administration) or vehicle starting 2 weeks after infection. Physalin F treatment significantly reduced the lesion size when compared with mice treated with vehicle (Figure 2a). Physalin F treatment also caused a significant reduction in the number of parasites compared with vehicle-treated mice (Figure 2b). Histopathological and histochemical changes also correlated with lesion progression. The animals treated with the vehicle alone had an extensive and monomorphlic collection of vacuolated and heavily parasitized macrophages, extensive or focal necrotic and ulcerative areas and microabscess (Figure 2c and e). In contrast, mice treated with physalin F were normal or presented either small collections of monomorphonic macrophagic inflammatory infiltrate or a mixed inflammatory infiltrate, containing macrophages, lymphocytes and neutrophils. Necrotic areas, microabscess and parasitism were less frequent in physalin-treated animals (Figure 2d and f).

Discussion

In the present study we showed the antileishmanial activity of steroids purified from P. angulata. Whereas physalins B and F had potent antileishmanial activity against intracellular amastigotes of L. amazonensis and L. major, this was not found with physalin D. In a similar way, physalin D also lacks immunomodulatory/anti-inflammatory activity, in contrast with physalins B and F.4,5 Jaco-bo-Herrera et al.8 also found that physalins B and F, but not D, inhibited the activation of NF-κB, an important transcription factor in inflammatory responses, and suggested that the presence
of a double bond and an epoxy ring between carbons 5 and 6 in physalins B and F, respectively, which are not present in physalin D, are critical for the anti-inflammatory activity.

The control of *Leishmania* infection is highly dependent on macrophage activation and nitric oxide (NO) production. Physalins B and F possess immunomodulatory activities, strongly inhibiting the production of NO and pro-inflammatory cytokines, such as interleukin-12 (IL-12) and tumour necrosis factor-α (TNF-α), by activated macrophages. IL-12 stimulates interferon-γ (IFN-γ) secretion by T and natural killer (NK) cells that, in turn, activates macrophages to destroy *Leishmania* parasites. TNF-α also activates macrophages to eliminate intracellular forms of the parasite through a mechanism involving the production of NO. Thus, although the beneficial effect of physalin treatment *in vivo* may seem contradictory due to its immunosuppressive activity, the inhibition of growth of *Leishmania in vivo*, as observed in this work, may be due to a direct action of these secosteroids in the parasite. The inflammatory response during cutaneous leishmaniasis also has an important role in tissue damage and necrosis in the lesions. Thus, in addition to the antileishmanial activity, the anti-inflammatory properties of physalins may also be relevant for the healing process in cutaneous leishmaniasis lesions. These results suggest that the two mechanisms of action of the physalins, immunomodulatory and antileishmanial activities, play a role in the treatment of cutaneous leishmaniasis.

In conclusion, this study demonstrated an antileishmanial action of physalins B and F with potential action *in vitro* and *in vivo* for cutaneous leishmaniasis. The identification of the mechanisms by which these secosteroids exert antileishmanial and anti-inflammatory activities may contribute to the development of an alternative therapy in the treatment of leishmaniasis.

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