Journal of Antimicrobial Chemotherapy (2009) **64**, 84–87
doi:10.1093/jac/dkp170
Advance Access publication 19 May 2009

JAC

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

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Received 29 January 2009; returned 8 March 2009; revised 8 April 2009; accepted 16 April 2009

***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.[1,2] 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*.[4,5]

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/RI/–/WR-173) promastigotes were cultivated in liver-infusion

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tryptose (LIT) medium supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil), 5% sterile urine and 50 μg/mL gentamicin (Sigma, St Louis, MO, USA), pH 7.2 at 26°C until logarithmic phase. Physalins B, D and F were obtained from plant specimens collected in Belém do Pará, Brazil, as previously described.[5] Amphotericin (GIBCO BRL Life Technologies, Grand Island, NY, USA) was used as a positive control. Amastigotes were obtained *in vitro* as previously described.[6] 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 IC_{50} 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% CO_2/95% humidified air. Macrophages (5×10^5) 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.

In vivo infection

Stationary-phase promastigotes of *L. amazonensis* (5×10^6) 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 infected contralateral ear from the thickness of the uninfected ear. The number of parasites in the draining auricular lymph node was estimated by limiting dilution assay, as previously described.[7]

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.[7]

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 $P<0.05$.

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 IC_{50} values of 0.21 and 0.18 μM, respectively.

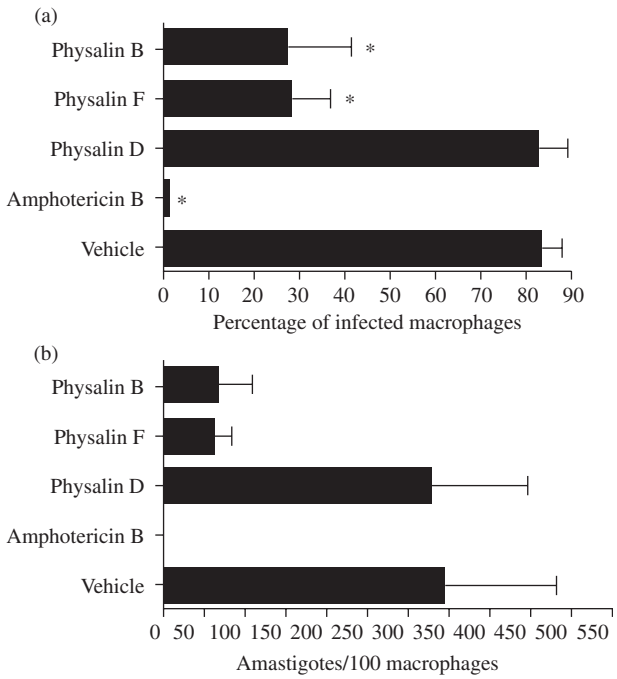

Figure 1. Effects of physalins B, F and D on the growth of intracellular amastigotes of *L. major*. J774-G8 macrophages were infected with *L. major* and treated with 2 μg/mL physalin B, F or D 4 h after infection. The percentage of infected macrophages (a) and the intracellular amastigote numbers (b) 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.

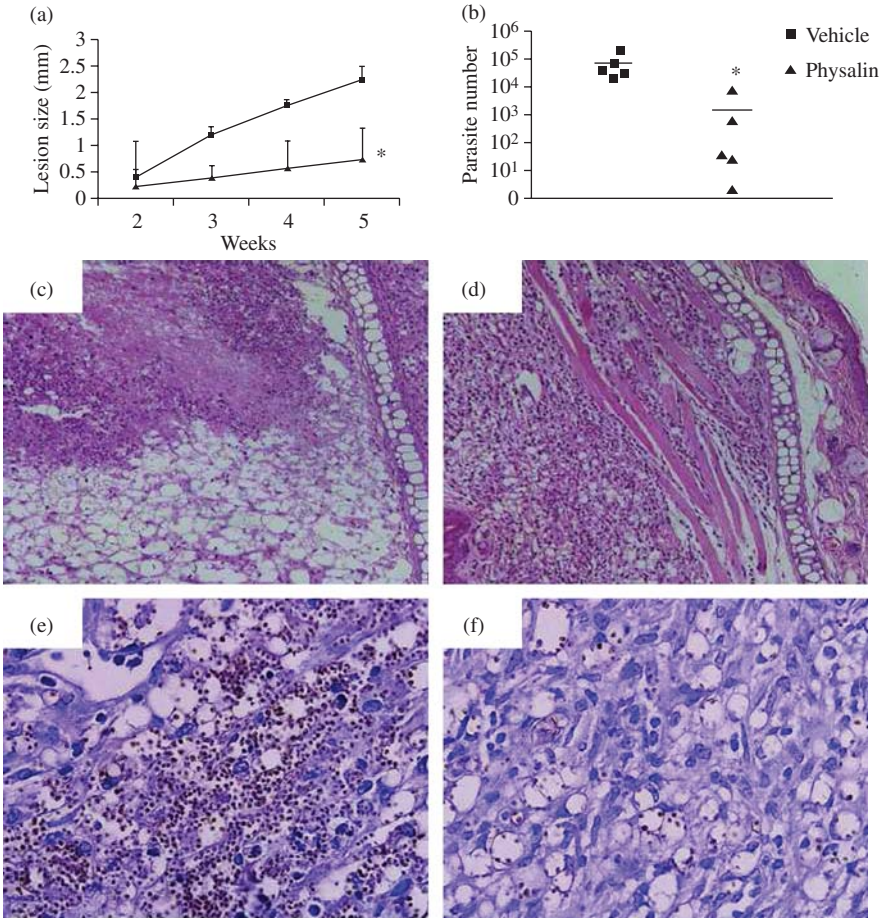

Figure 2. Topical treatment with physalin F decreases the lesion growth and the parasite number in mice infected with *L. amazonensis*. BALB/c mice infected subcutaneously with *L. amazonensis* in the right ear dermis were treated with physalin F daily, after 2 weeks of infection. (a) Lesion development was monitored weekly using a digital caliper. Values represent the means ± SEM of five mice per group, in one representative experiment of three performed. $*P < 0.01$, physalin F versus vehicle. (b) Decreased parasite burden in auricular draining lymph nodes of mice treated with physalin F. The number of viable parasites was estimated by limiting dilution assay. The results are expressed as −log of the highest cell suspension dilution with wells positive for leishmania. Data represent the individual mice ($n = 5$ per group) in one experiment of three performed. $*P < 0.001$, vehicle versus physalin F. (c–f) Morphological and histological aspects of ear lesions in BALB/c mice infected with *L. amazonensis*. (c and e) Vehicle-treated mice. (d and f) Mice treated with physalin F. Monomorphic inflammatory infiltrate with extensive necrotic areas (c) and vacuolated parasite-containing macrophages (e). (d and f) Pleomorphic inflammatory infiltrate with non-vacuolated and vacuolated, parasite-containing macrophages. (d) Necrotic areas were small or absent. (c and d) H&E, ×20. (e and f) Immunohistochemistry using a polyclonal anti-*Leishmania* antibody, ×40. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

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 monomorphic 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 monomorphic 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] Jacobo-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.[4] IL-12 stimulates interferon-γ (IFN-γ) secretion by T and natural killer (NK) cells that, in turn, activates macrophages to destroy *Leishmania* parasites.[9] TNF-α also activates macrophages to eliminate intracellular forms of the parasite through a mechanism involving the production of NO.[10] 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.

Funding

This work was supported by FIOCRUZ/PDTIS, CNPq, Institutos do Milênio, IMSEAR, MCT, FINEP, RENORBIO and BIOINOVA.

Transparency declarations

None to declare.

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