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Pentalinon andrieuxii* root extract is effective in the topical treatment of cutaneous leishmaniasis caused by *Leishmania mexicana

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Abstract

Cutaneous leishmaniasis (CL) manifests as localized skin lesions, which lead to significant tissue destruction and disfigurement. In the Yucatan Peninsula, Mayan traditional healers use *Pentalinon andrieuxii* Muell.-Arg. (Apocynaceae) roots for the topical treatment of CL. Here, we studied the effect of *P. andrieuxii* root hexane extract (PARE) on the parasites and host cells *in vitro* and examined its efficacy in the topical treatment of CL caused by *L. mexicana*. PARE exhibited potent antiparasitic activity *in vitro* against promastigotes as well as amastigotes residing in macrophages. Electron microscopy of PARE-treated parasites revealed direct membrane damage. PARE also activated NF- κ B and enhanced IFN- γ R and MHC class II expression and TNF- α production in macrophages. In addition, PARE induced production of the Th1 promoting cytokine IL-12 in dendritic cells as well as enhanced expression of the co-stimulatory molecules CD40, CD80 and CD86. *In vivo* studies showed that *L. mexicana*-infected mice treated by topical application of PARE resulted in the significant reduction in lesion size and parasite burden

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compared to controls. These findings indicate that PARE could be used as an alternative therapy for the topical treatment of CL.

Keywords

Cutaneous leishmaniasis; *Leishmania mexicana*; *Pentalinon andreuxii* roots; phytomedicinal agent

Introduction

Over 12 million people currently suffer from leishmaniasis, and approximately 2 million are infected annually, making it a major global health problem and a neglected tropical disease. Cutaneous leishmaniasis (CL) manifests as localized skin lesions that may heal or lead to significant tissue destruction and disfigurement. This disease is the most common form of *Leishmania* infection worldwide. The pentavalent antimonials sodium stibogluconate (Pentostam™) and meglumine antimonite (Glucantime™) were introduced 60 years ago and are the recommended drugs for the treatment of CL (Croft et al., 2006; Croft and Yardley, 2002). However, these drugs are toxic, require daily administration via a parenteral route for 20-28 days, and are not approved currently by the FDA. Even in low doses these drugs show cardiac, kidney and liver toxicity (Laguna del Estal et al., 1994; Ribeiro et al., 1999; Sampaio et al., 1997; Veiga et al., 1985). Over the past decade, alternative drugs such as amphotericin B, paromomycin and pentamidine have been used to treat CL in different regions in the world and have been met with variable levels of success (Croft et al., 2006; Croft and Yardley, 2002). Therefore, there is a need for new antileishmanial drugs that are safe, effective, cheap and easy to administer. A topical treatment with minimal side effects, which can be applied after a sand fly bite or after the development of an ulcerating lesion, is ideal for CL. Such a strategy will likely prevent the development of disease pathology and the spread of infection, as well as have improved patient compliance.

Alternative therapies have long used natural products from plants to treat many infectious and non-infectious diseases. Plants belonging to the Apocynaceae, Araceae, Cycadaceae, Fabaceae, Piperaceae, Sapindaceae and Solanaceae families are used commonly in the topical treatment of CL in Latin America (Chan-Bacab et al., 2003; Chan-Bacab and Pena-Rodriguez, 2001; Davies et al., 2003; Estevez et al., 2007; Kvist et al., 2006; Mejía and Rengifo, 1995; Pinedo P et al., 1997; Soares et al., 2007; Villegas et al., 1997). Topical treatment usually involves the direct application of powder or juice (from the bark and roots) or ground dried leaves on to the affected area.

We have shown previously that a hexane-soluble extract of the roots of the plant *Pentalinon andreuxii* Muell.-Arg. (Apocynaceae) inhibits the growth of *L. mexicana* *in vitro* (Lezama-Davila et al., 2007b). Similarly, Chan-Bacab *et al* also found that methanol extracts from the roots of *Urechitis andreuxii* (syn. *P. andreuxii*) display a potent cytotoxic activity against *L. donovani*, *L. braziliensis* and *L. mexicana* promastigotes (Chan-Bacab et al., 2003). In the present study, we determined the effect of PARE on parasites and host macrophages, and evaluated its efficacy in topical treatment of CL using a mouse model.

Materials and methods

Animals

Eight- to twelve-week old sex-matched 129Sv/Ev mice purchased from Taconic Farms and C57BL/6 mice purchased from Harlan Labs were used in the study. 129Sv/Ev mice were used to maintain the stock of *L. mexicana* (MNYC/BZ/62/M379) and C57BL/6 mice were used for infection studies. All mice were maintained in a pathogen-free animal facility at The Ohio State University and experiments were performed in accordance with NIH and Institutional guidelines.

Parasites

L. mexicana (MNYC/BZ/62/M379) was maintained by inoculation of amastigotes subcutaneously into the shaven rumps of 129Sv/Ev or BALB/c mice. Amastigotes obtained from the infected lesions were grown *in vitro* in complete M199 medium supplemented with 10% fetal bovine serum (FBS), 100 IU penicillin and 100 µg streptomycin (Lezama-Davila et al 2007) to generate stationary phase promastigotes (SPP) for *in vitro* and *in vivo* studies.

Plant material and preparation of *P. andrieuxii* root hexane extract (PARE)

The plant roots were collected from Campeche, Mexico in a region of subdeciduous forest (19° 46'N 90° 29' W). This plant was identified in the Herbarium at the Autonomous University of Campeche (Universidad Autonoma de Campeche), Mexico, under voucher No. 6921 (Zamora-Crescencio & Lezama-Davila) and in the Herbarium of the Autonomous University of Yucatan (Universidad Autonoma de Yucatan), Mexico, under voucher no. 1 (Viscencio de la Rosa & Lezama-Davila). PARE was prepared from air-dried *P. andrieuxii* roots as described recently by us (Pan et al., 2012). Briefly, air-dried roots were milled and subsequently extracted with methanol overnight at room temperature (3 × 4 L). The methanol macerate was concentrated *in vacuo* and partitioned to produce a hexane-soluble extract.

Assay for screening leishmanicidal activity against promastigotes

A flow cytometry-based propidium iodide uptake assay developed in our laboratory was used to screen the leishmanicidal activity of PARE against *L. mexicana* promastigotes. One million stationary phase *L. mexicana* promastigotes were treated with different concentrations (0 to 100 µg/mL) of PARE dissolved in DMSO or DMSO alone at 28 °C for 48 h. Following the treatment, parasites were centrifuged at 3000 RPM for 5 min and the pellet was re-suspended in 100 µL of PBS containing 20 µg of propidium iodide (PI) for 1 h in the dark at 4 °C. Dead parasites stained with PI were quantified by flow cytometry using a FACS Calibur flow cytometer. Three controls were included, parasites treated with 1) saponin (100 µg/ml) as a positive control, 2) sodium stibogluconate (reference drug) and 3) 2% DMSO. Data analysis was performed with CellQuestPro software (Beckton Dickinson) or FlowJo software (Tree Star, Inc.).

Electron microscopy

L. mexicana parasites were treated with 100 µg/mL of hexane extract of *P. andrieuxii* (PARE) for 48 h and after that period, the culture medium was eliminated by centrifugation and the pellets resuspended in glutaraldehyde. Parasites fixed in 3% buffered glutaraldehyde were washed twice with sodium cacodylate buffer (pH=7.4, 10 minutes each and centrifuged at 1500 RPM/5 min.) Cell samples were then post-fixed in 1% osmium tetroxide in sym-collidine buffer (pH=7.6) for 1 hour at room temperature. Samples were washed twice with s-collidine buffer (10 minutes each), and the cell pellet was stained with a saturated aqueous uranyl acetate solution (pH=3.3) for 1 hour. After the last centrifugation, samples were dehydrated in a graded ethanol series up to absolute (10 minutes each). Acetone was used as the transitional solvent, two changes for 10 minutes each. Parasite suspensions were infiltrated overnight with a 1:1 mixture of acetone and Spurr's epoxy resin (Electron Microscopy Sciences, Fort Washington, PA). Finally, cell pellets were placed into BEEM™ embedding capsules containing 100% Spurr's resin. Polymerization of epoxy blocks was carried out at 70°C overnight. Polymerized blocks were sectioned with a Leica Ultratut UCT ultramicrotome (Leica Microsystem GmbH, Vienna, Austria) and semi-thin sections (750 nm) were stained with Methylene Blue–Basic Fuschin and two representative *areas* were thin sectioned for ultrastructural examination. Ultrathin (80 nm) sections were collected on 200 mesh copper grids (Electron Microscopy Sciences) and post stained with lead citrate (3 minutes). Electron micrographs were generated on a JEOL JEM-1400 TEM (JEOL Ltd. Tokyo, Japan), operating at 80K equipped with a Veleta digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Macrophage and dendritic cells infection assays

Bone marrow-derived macrophages (BMDMs) or dendritic cells (BMDDC) from femurs and tibias of C57BL/6 mice were prepared by differentiating them from bone marrow stem cells using complete media supplemented with 20% supernatant from L-929 cells to produce macrophages or low concentrations of recombinant granulocyte macrophage colony stimulating factor (GM-CSF) to produce dendritic cells (Inaba et al., 2009; Lezama-Davila et al., 2007a). BMDMs or BMDDC (0.5×10^6) were infected overnight with 2.5×10^6 stationary phase promastigotes of *L. mexicana* (ratio 5:1) in 24-well tissue culture plates containing glass rounded coverslips. Cells were washed 10 hours later to remove non-phagocytosed parasites, and were treated with 1, 10 or 100 µg of PARE. Sham controls were treated with equal volumes of vehicle. Leishmanicidal activity was determined 72 hours later by recording the number of parasites per 100 BMDM or BMDDC. In each experiment, infection of cells was determined by counting 100 cells on each slide in triplicate in a blinded fashion.

Cytokine ELISA

Levels of IL-12, TNF-α and IL-10 in above culture supernatants were determined by sandwich ELISA as described previously (Lezama-Davila et al., 2007a). Briefly, 96 well flat-bottom plates (Nunc, Thermo Scientific, Pittsburgh PA) were coated with primary capture antibody at 4°C overnight. Plates were blocked by incubating with PBS supplemented with 10% FBS for 2 hours at room temperature, then incubated overnight with

50 μ L of culture supernatants or serial diluted recombinant cytokine in duplicates. Plates were washed four times with PBS-Tween (0.05% Tween 20 in 1 $\times$ PBS, pH 7.4), and incubated with biotinylated detection antibody against specific cytokine (BD Biosciences, San Diego CA) for 1 hour at room temperature, then washed four times and incubated with streptavidin conjugated Alkaline Phosphatase for 30 minutes at room temperature in dark. Finally, plates were washed five times before the addition of PNPP (Thermo, Rockford IL). Absorbance was read at 405 nm using Molecular Devices SpectraMax M3 microplate reader (Sunnyvale, CA), and cytokine levels were quantified against a standard curve by SoftMax Pro software. All reagents for ELISA were purchased from Biolegend Inc.

Flow cytometry analysis

PARE-treated cells were incubated with FITC or PE- conjugated antibodies against IFN- γ R, MHC class II and co-stimulatory molecules CD80, CD86 and CD40 (Biolegend, San Diego CA). Cells were acquired on a FACS Calibur flow cytometer and analysis was performed with CellQuestPro software (Beckton Dickinson) or FlowJo software (Tree Star, Inc.).

Treatment of *L. mexicana*-infected mice with PARE

Age and sex matched *L. mexicana* susceptible C57BL/6 mice were infected by intradermal inoculation of 1000 *L. mexicana* SPPs into the left ear dermis as described previously (Cummings et al., 2012). Following development of a palpable lesion, mice were treated with topical application of 10 μ g PARE once a day for six weeks. Two control groups included: 1) sham-treated mice that received topical treatment with vehicle or 2) once a week intraperitoneal injection of antileishmanial drug sodium stibogluconate (20 mg/kg). Disease progression was monitored in each mouse by measuring the difference in the thickness of infected ear and contralateral uninfected ear. Lesion parasite loads were quantified at the end of treatment by limiting dilution analysis, as described previously (Cummings et al., 2012).

Statistical analysis

Student's *t* test was used for statistical analysis. IC₅₀ values for promastigotes were calculated using a LdP Line® and Prism 5® software.

Results

PARE exhibits direct cytotoxicity against *L. mexicana* promastigotes *in vitro*

To determine whether PARE is cytotoxic to *Leishmania* parasites, 2×10^6 stationary phase promastigotes of *L. mexicana* were treated with different concentrations of PARE, sodium stibogluconate (SSG), saponin (positive control) or DMSO (sham control) for 48 hours, and IC₅₀ values were determined by quantifying dead parasites using a flow cytometry based propidium iodide uptake assay. Treatment with 100 μ g/mL of PARE for 48 hours killed almost all promastigotes (Figs. 1C and 1H). The IC₅₀ values for promastigotes treated with PARE and SSG were 7 and 243 μ g/mL (Table 1), respectively, indicating that PARE was more potent in killing *L. mexicana* promastigotes *in vitro* compared to SSG. Electron microscopy of parasites treated with PARE showed complete destruction of parasite membranes associated with disintegration or loss of intracellular components (Fig. 1F)

compared to sham- treated parasites, which appeared normal with intact cell membrane, nucleus, mitochondria and lipid droplets (Fig. 1D).

PARE displays leishmanicidal activity against intracellular amastigotes residing in mouse bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDDC)

Since PARE displayed cytotoxic activity against extracellular promastigotes, we next determined whether PARE is also active against intracellular amastigotes, BMDMs were infected with *L. mexicana* overnight and then treated with different concentrations (1, 10, 100 µg/ml) of PARE, SSG or DMSO. Intracellular parasites residing in BMDM or BMDDC were enumerated 48 hours post-treatment and IC₅₀ values were calculated. Infected BMDM and BMDDC treated with PARE as well as SSG showed a significant reduction in intracellular parasites compared to sham controls. Moreover, the IC₅₀ data for intracellular amastigotes treated with PARE were 4.1 µg/mL for BMDM or 11.06 µg/mL for BMDDC, which were significantly lower than SSG treated BMDM (14.66 µg/mL) or BMDDC (20.04 µg/mL), indicating that PARE is more potent than SSG against amastigotes (Table 1). Furthermore, PARE exhibited no considerable toxicity against BMDMs at these concentrations (Table 1). These findings indicate that PARE is active against intracellular amastigotes and that it is not toxic to mammalian cells since its therapeutic index is considerable higher than that of SSG (Table 1).

PARE up-regulates expression of IFN-γR, MHC class II and CD80 on macrophages

To determine whether PARE also exerts immunomodulatory effects on host cells, we analyzed the expression of immune system-associated molecules that are involved in regulating resistance to *Leishmania*: MHC class II, co-stimulatory molecules, and receptors for macrophages (e.g., IFN-γR) on PARE-treated BMDMs by flow cytometry. We observed that PARE enhanced the expression MHC class II and CD80 as well as induced a dose-dependent increase in IFN-γR in BMDMs (Figs. 2A – 2C). These results suggest that PARE enhances antigen presentation, co-stimulatory activity, and the responsiveness of macrophages to IFN-γ activation.

PARE activates NF-κB and/or AP1 transcription factors, induces TNF-α and suppresses IL-10 production in RAW macrophages

The transcription factors NF-κB and AP1 play a critical role in macrophage activation and induction of pro-inflammatory responses. We therefore treated RAW-Blue™ NF-κB/AP1 reporter cells with various concentrations of PARE and measured NF-κB/AP1 activation as per the manufacturer's instructions. In addition, we measured cytokine levels in the culture supernatants. PARE induced significant activation of NF-κB/AP1 in RAW cells (Fig. 3A). Furthermore, PARE-treated cells also produced significantly more TNF-α (Fig. 3B) and less IL-10 (Fig. 3C) compared to sham-treated controls. No IL-6 was detectable in culture supernatants from either group (data not shown). These findings show that PARE mediates macrophage immunomodulation by activating the transcription factors NF-κB and/or AP1 and cytokine production, which favor an antileishmanial microenvironment.

Effect of PARE on dendritic cells (DCs)

Dendritic cells play a critical role in the development of protective immunity in cutaneous leishmaniasis by promoting Th1 development. We therefore examined the effect of PARE on bone marrow-derived mouse dendritic cells (BMDDC) *in vitro*. Following treatment with 100 µg/ml of PARE, DCs showed a significant increase in the production of the Th1 inducing cytokine IL-12, but not the Th2 associated IL-10 (Figs. 4A and 4B). Furthermore, DCs treated with PARE showed significant up-regulation of the co-stimulatory molecules CD40 (Figs. 4C and 4F), CD80 (Figs. 4D and 4G) and CD86 (Figs. 4E and 4H). These data indicate that PARE also activates DCs and induces preferential production of Th1 associated IL-12.

Topical treatment with PARE reduces parasite growth *in vivo* in *L. mexicana*-infected mice

To evaluate the *in vivo* efficacy of PARE in the topical treatment of CL, sex and age matched C57BL/6 mice were infected intradermally by inoculating 10⁴ *L. mexicana* promastigotes into the ear dermis and then treated with PARE, SSG or vehicle as described previously (Cummings et al., 2012). PARE treatment for 6 weeks resulted in a significant reduction in lesion size as compared to sham controls or animals treated with sodium stibogluconate (Fig. 5A). SSG treatment of mice using doses normally applied to humans (20 mg/Kg) did not alter lesion size as compared to controls and parasite loads were also similar to controls (Figs 5A,D). Furthermore, lesions of mice treated with PARE contained significantly fewer parasites compared to those from vehicle-treated controls and SSG-treated mice (Fig. 5B). Histological analysis of infected ears from PARE treated mice showed inflammation comprised of lymphocytes and macrophages containing few or no parasites (Fig. 5E). In contrast, infected ears from sham-treated control animals contained macrophages full of parasites (Fig. 5C see arrows). These data proved that topical treatment with PARE is effective in limiting lesion growth following *L. mexicana* infection.

Discussion

The findings in this study show that a hexane extract of the roots of the plant *P. andrieuxii* (PARE) displays both antileishmanial and immunomodulatory activities *in vitro*. Our results demonstrate that topical treatment using PARE is effective in controlling cutaneous leishmaniasis (CL) caused by *L. mexicana*, suggesting that PARE could become a novel phytomedicine for CL. Further, since PARE displays minimal cytotoxicity to mammalian cells and is effective when used topically as demonstrated by our *in vivo* studies, it presents an advantage over antimonial drugs currently used for the treatment of CL, which are toxic, require parenteral administration leading to poor patient compliance, and could be expensive and are not always readily accessible (Pinedo P et al., 1997; Ribeiro et al., 1999; Sampaio et al., 1997).

Multiple mechanisms seem to be involved in the protective activity of PARE against *L. mexicana*. Our results indicate that in addition to direct leishmanicidal activity against extracellular and intracellular *L. mexicana* parasites, PARE displays immuno-modulatory activities, enhancing the ability of macrophages and dendritic cells to kill intracellular parasites. PARE induced the expression of IFN-γR on macrophages, suggesting that it could

potentially enhance macrophage responsiveness to IFN- γ activation. IFN- γ activated macrophages are critical to the resolution of CL caused by *L. mexicana* since they contribute to increased antigen presentation, amplification of Th1 immune responses, and subsequent parasite killing (Pinheiro and Rossi-Bergmann, 2007). Not surprisingly, we observed an increase in MHCII, co-stimulatory molecule CD80 as well as TNF α and IL-12 production in PARE-treated macrophages and DCs compared to untreated controls. IL-12 production by antigen presenting cells is critical for directing Th1 differentiation and effector mechanisms required for the control of CL (Scharton-Kersten et al., 1995). We also observed increased NF- κ B activation in PARE-treated RAW macrophages, which either could be a direct result of PARE stimulation or mediated by TNF α (Schütze et al., 1992), and is also induced by PARE. PARE also reduced the expression of IL-10 by macrophages, which could further contribute to increased antileishmanial immune responses (Jones et al., 2002). Our present study underscores the importance of lipid-modulatory effects of PARE in both host macrophages and *L. mexicana* parasite that could lead to the observed immunomodulation and anti-leishmanial actions caused by PARE. Lipid signaling pathway mediated by phospholipase A₂ has been established as a crucial step in the upstream regulation of cytokine release by the lung epithelial cells and macrophages (Kotha et al., 2011). Therefore, it is conceivable that PARE containing the lipophilic molecules could have caused immunomodulation and anti-leishmanial actions through the (i) alterations of the lipid composition of the membranes of macrophages and the parasite thus leading to the changes in the membrane phase-transition and activation of receptors towards modulation of cytokine synthesis and secretion in macrophages and parasite killing and (ii) activation of lipid signaling cascades involving phospholipases that could cause formation of bioactive lipids in the host macrophage and parasite leading to immunomodulation and anti-leishmanial actions, respectively. It has also been proposed antileishmanial drugs, in general, present immunomodulation activity (Ghosh et al., 2013). Paromomycin is a recently developed drug to treat cutaneous leishmaniasis and it fails to enhance production of IL-12 or TNF- α by macrophages stimulated *in vitro* (Ghosh et al., 2013). PARE on the other hand induces TNF production in dendritic cells, indicating that paromomycin and PARE follows different immunomodulatory pathways than paromomycin. Taken together, these direct antiparasitic effects coupled with its immunomodulatory activities make PARE a potent novel candidate phytomedicine for the management of CL in humans.

In the Yucatan peninsula of Mexico, Mayan traditional healers frequently use the root of the plant *P. andrieuxii* to treat CL. Previous reports have demonstrated leishmanicidal activity in the leaves of this plant (Chan-Bacab et al., 2003) and studies performed by our group (Lezama-Davila et al., 2007b) showed that root extracts are most effective in killing promastigotes of *L. mexicana*. More recently, we have identified and characterized six sterols in the hexane root extract, which displayed considerable leishmanicidal activity against promastigotes as well as amastigotes within murine macrophages (Pan et al., 2012). Although additional studies will be required to fully identify and characterize the various immunomodulatory components as well as host immune pathways mediated by the various components of PARE, this study validates this plant product as effective in killing extracellular *Leishmania* promastigotes as well as intracellular amastigotes. Most significantly, we demonstrate the efficacy of PARE in the treatment of mice infected with *L.*

mexicana. The results from this study provide a strong case for the development of PARE as a novel phytomedicine in the management of CL.

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Figure 1. PARE exhibits direct cytotoxicity against *L. mexicana* promastigotes *in vitro*
 (A – C) Flow cytometric analysis of *L. mexicana* promastigotes treated with (A) DMSO (sham control), (B) 100 µg/mL of Sodium Stibogluconate, or (C) 100 µg/mL of PARE. Parasites were stained with propidium iodide after treatment and dead parasites were measured by flow cytometry. Numbers represent percentages of dead parasites after treatment. (D – F) Transmission electron microscopy (TEM) of *L. mexicana* promastigotes treated with (D) DMSO (sham control), (E) 100 µg/mL of Sodium Stibogluconate, or (F) 100 µg/mL of PARE. (H) Bar graph showing the number of live promastigotes after treatment with vehicle control (DMSO) or 100 µg/mL PARE. Intracellular parasites were enumerated after 48 h of treatment by microscopy. TEM images show parasite nuclei (N), kinetoplast (K), lipid droplet (L) and flagella (F).

Figure 2. PARE up-regulates expression of IFN- γ R, MHC class II and CD80 on macrophages (A) Histogram plot showing IFN- γ R expression on bone marrow derived macrophages treated with vehicle control or various concentrations of PARE. (B and C) Dot plot showing expression of (B) MHCII and (C) CD80 on the surface of bone marrow derived macrophages treated with vehicle control or various concentrations of PARE. Numbers correspond to percentages of positive populations.

Figure 3. PARE activates NF-κB and/or AP1 transcription factors, induces TNF-α and suppresses IL-10 production in RAW macrophages

(A) NF-κB induction in treated RAW blue macrophages as determined by the presence of alkaline phosphatase in culture supernatants measured using the Quanti blue assay. Cells were stimulated with vehicle control, LPS/IFN-γ, SSG or PARE at indicated concentrations for 72 h. Colorimetric detection was performed at 656 nm. (B) TNFα and (C) IL-10 cytokine production in culture supernatants of vehicle control or PARE treated RAW blue macrophages as determined by ELISA. Statistical analysis was performed using Student T test (*p<0.05).

Figure 4. Effect of PARE on Dendritic cells

(A) IL-12 and (B) IL-10 cytokine production in culture supernatants of BMDCs from C57BL/6 mice stimulated with vehicle control (DMSO) or 50ug/ml of PARE for 24 hrs as determined by ELISA. (C – E) Histogram plots showing (C) CD40, (D) CD80 and (E) CD86 expression in BMDCs of C57BL/6 mice stimulated with vehicle control (DMSO) or 50ug/ml of PARE for 24hrs and analyzed by flow cytometry. (F – H) Bar graphs showing mean fluorescence intensities (MFIs) of (F) CD40, (G) CD80 and (H) CD86 expression in BMDCs of C57BL/6 mice stimulated with vehicle control (DMSO) or 50ug/ml of PARE for 24 hrs and analyzed by flow cytometry.

Figure 5. Topical treatment with PARE reduces parasite growth *in vivo* in *L. mexicana*-infected mice

(A) *L. mexicana* infected C57BL/6 mice were treated with topical application of PARE for 6 weeks and lesion development was measured and compared to untreated or sodium antimony treated controls. (B) Parasite numbers in the ears of *L. mexicana* infected C56BL/6 mice treated with PARE, SSG or untreated controls. (C – E) Histological analysis of infected ear tissue in *L. mexicana* infected C56BL/6 mice treated with (E) PARE, and compared to (C) untreated or (D) SSG treated controls.

Table 1
***P. andrieuxii* root extract kills *L. mexicana* promastigotes and amastigotes inside of macrophages or dendritic cells**

Compound	IC ₅₀ (µg/mL) Promastigotes [¶]	IC ₅₀ (µg/mL) Amastigotes inside of BMDM [§]	IC ₅₀ (µg/mL) Amastigotes inside of BMDDC ^{§§}	Therapeutic index ^{¶¶}
SSG	257.22 ± 23.3	14.66 ± 4.57	20.04 ± 12.12	7.8
PARE	43.04 ± 7.73 [‡]	4.1 ± 1.5 [‡]	11.06 ± 2.31 [‡]	137.2

[¶]IC₅₀ is the concentration of drug (µg/mL) to achieve 50% killing of parasites per 10⁶ axenic promastigotes, 2.5×10⁶ amastigotes inside of 0.5×10⁶ bone marrow derived macrophages (BMDM)

[§] or bone marrow derived dendritic cells (BMDDC)

^{§§} after 48 h of culture.

^{¶¶} Therapeutic index = CC₅₀ of BMDM / IC₅₀ of BMDM.

[‡] Significantly different (*p* < 0.05) as compared to SSG.