Effects of Brazilian propolis on *Leishmania amazonensis*

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Leishmaniasis, an endemic parasitosis that leads to chronic cutaneous, mucocutaneous or visceral lesions, is part of those diseases, which still requires improved control tools. Propolis has shown activities against different bacteria, fungi, and parasites. In this study we investigated the effect of four ethanolic extracts of typified propolis collected in different Brazilian states, on Leishmania amazonensis *performing assays with promastigote forms, extracellular amastigotes, and on infected peritoneal macrophages. Ethanolic extracts of all propolis samples (BRG, BRPG, BRP-1, and BRV) were capable to reduce parasite load as monitored by the percentage of infected macrophages and the number of intracellular parasites. BRV sample called red propolis, collected in the state of Alagoas, and containing high concentration of prenylated and benzophenones compounds, was the most active extract against* L. amazonensis. *The anti-*Leishmania *effect of BRV sample was increased in a concentration and time dependent manner. BRV treatment proved to be non-toxic to macrophage cultures. Since BRV extract at the concentration of 25 µg/ml reduced the parasite load of macrophages while presented no direct toxic to promastigotes and extracellular amastigotes, it was suggested that constituents of propolis intensify the mechanism of macrophage activation leading to killing of* L. amazonensis. *Our results demonstrate, for the first time, that ethanolic extracts of Brazilian propolis reduce* L. amazonensis *infection in macrophages, and encourage further studies of this natural compound in animal models of leishmaniasis.*

Key words: *Leishmania amazonensis* - leishmaniasis - natural products - propolis

Leishmaniasis is a parasitosis caused by several species of the protozoan *Leishmania* and it is currently endemic in 88 countries. Overall prevalence is 12 million people and the population at risk is 350 million (Desjeux 2004, Murray et al. 2005). Injected into mammalian hosts by phlebotomus sand flies as extracellular promastigotes, *Leishmania* bind to macrophage and are quickly phagocytosed. All *Leishmania* species are obligatorily intracellular parasites, which live within secondary phagolissosomes. In this way, the parasite is able to multiply, lyse host cell, and infect surrounding macrophages.

The severity of disease varies ranging from cutaneous or mucosal to visceral or diffuse cutaneous infection (Grimaldi & Tesh 1993, Murray et al. 2005). The former is generally caused by *L. amazonensis*, a species transmitted mainly in the Amazon region, which is associated with localized cutaneous lesions (Grimaldi & Tesh 1993). Chemotherapy remains the mainstay for the control of leishmaniasis, as effective vaccines have yet to be developed (Murray et al. 2005). The first line of therapy for all forms of the disease requires potentially toxic and painful multiple injections of pentavalent antimonials (Berman 2003). The problem is further aggravated by the appearance of resistance to these drugs in some endemic areas. Amphotericin B and pentami-

dine are second-line drugs and they present limited value because of their toxicity and difficulty in administration (Berman 2003). Many studies have been conducted to find an effective therapy for leishmaniasis that avoids exposure to potentially toxic drugs, including screening of plant extracts and plant-derived compounds (Abreu et al. 1999, Carvalho & Ferreira 2001, Rocha et al. 2005).

Propolis is a resinous substance that honey bees collect from different plant exsudates (Marcucci 1995). Propolis is claimed to posses versatile valuable pharmacological activities and has, to date, been taken in internal and external dosage forms for the treatments of various diseases (Burdock 1998, Marcucci & Bankova 1999). It is widely used in products like "healthy foods" and "biocosmetics" (Marcucci & Bankova 1999). Many authors have reported the in vitro activities of propolis against different microorganisms, among them some important human pathogens, such as *Staphylococcus aureus*, *Salmonella thyphimurium*, *Candida albicans*, *Trypanosoma cruzi*, and *Giardia duodenalis* (Higashi & de Castro 1994, Marcucci et al. 2001, Miorin et al. 2003, Uzel et al. 2005, Dantas et al. 2006, Freitas et al. 2006, Trusheva et al. 2006). Brazilian propolis is the subject of an intensive study of chemists, biologists, and physicians all over the world due to specific tropical flora and their different chemical components (Marcucci & Bankova 1999, Marcucci et al. 2001, Trusheva et al. 2006).

This report describes in vitro analyses of the effects of ethanolic extracts of typified Brazilian propolis samples on both promastigote and amastigote forms of *L. amazonensis* and on macrophages infected with the parasite.

MATERIALS AND METHODS

Parasite - L. amazonensis (MHOM/BR/73/M2269) promastigotes were cultured at 28°C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 25 μg/ml gentamicin, 2 mM L-glutamine, 100 mM HEPES, and 10% fetal calf serum (FCS) (Cultilab, Campinas, SP, Brazil), pH 7.2. Amastigotes were isolated from active skin lesions from BALB/c mice, and used immediately after isolation (Barbieri et al. 1993).

*Brazilian propolis sample*s - Two propolis samples collected in the Brazilian state of Paraná were green propolis, typified as BRG and BRPG. Propolis collected in the state of Minas Gerais was typified as BRP-1 (green propolis) (Miorin et al. 2003) and the sample collected in the state of Alagoas as BRV (red propolis) (Trusheva et al. 2006). The ethanolic extracts of propolis were prepared by using a modified technique described by Miorin et al. (2003). Propolis (30 g) was cut into small pieces and extracted with 100 ml absolute ethanol at room temperature for 24 h. The solution was filtered with Whatman paper number 3, and placed in amber flasks. Each solution was dried and the residue weighted to prepare stock solution in ethanol at concentration of 5%. The final concentration of the solvent in the experiments did not exceed 0.1% ethanol.

Macrophage infection with L. amazonensis - Primary mouse macrophages (5×10^5/ml) were obtained from normal BALB/c mice by peritoneal washing, cultured on 24-well plates containing 13 mm diameter glass coverslips and infected with amastigotes (3:1 parasite/host cell) for 1 h, as described previously (Colhone et al. 2004). After the interaction period, the cultures were washed to remove extracellular parasites and incubated in the presence or absence of different concentrations of propolis or diluent (0.1% ethanol), at 37°C in 5% CO_2 in air in a humidified incubator as established by Ayres et al. (2006). After the indicated periods of treatments, coverslips were fixed with methanol, stained with Giemsa, and examined under light microscope. Six hundred cells were counted per triplicate coverslip for the evaluation of the percentage of infected macrophages and the number of amastigotes per infected macrophage (Colhone et al. 2004). The infection levels were quantified using a light microscopy at 1000 magnification.

Assessment of propolis effects on L. amazonensis promastigotes, amastigotes, and macrophage cultures - Promastigotes growing in 25 cm² plastic flasks at 28°C were treated with different concentrations of propolis or diluent, and parasite number and morphology were determined using a Neubauer haemocytometer (Arrais-Silva et al. 2005). Amastigotes maintained under promastigote culture conditions, i.e. at 28°C in 25 cm² plastic flasks with RPMI 1640 medium supplemented with gentamicin, L-glutamine, HEPES, and 10% FCS, were treated with different concentrations of propolis or diluent, and left to transform into promastigote forms. After the indicated periods of incubation at 28°C, promastigote and amastigote numbers were recorded by microscopic observation (Lemesre et al 1997, Arrais-

Silva et al. 2005). Macrophages cultured on 24-well plates were incubated for 72 h in the presence of different propolis samples or diluent (0.1% ethanol). Cell viability was analyzed by a dye-reduction assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) (Mosmann 1983).
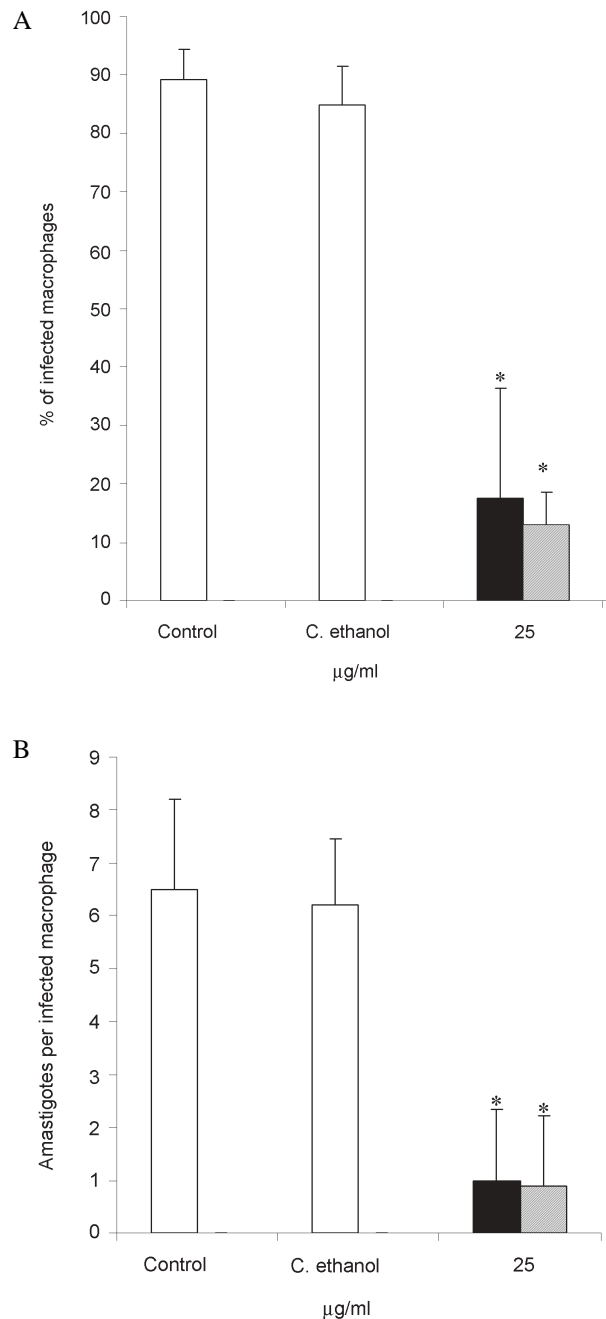

Fig. 1: effect of ethanolic extracts of Brazilian propolis (25 μg/ml) on *Leishmania amazonensis*- infected macrophages after 72 h of treatment: control, 0.1% ethanol, BRG (■) or BRPG (▨). The percentage of infected macrophages (A) and the number of parasites per infected cell (B) were determined as described in Materials and Methods. The results represent the mean ± SD of three independent experiments. Asterisks represent statistically differences (p < 0.01) between control and propolis-treated cultures.
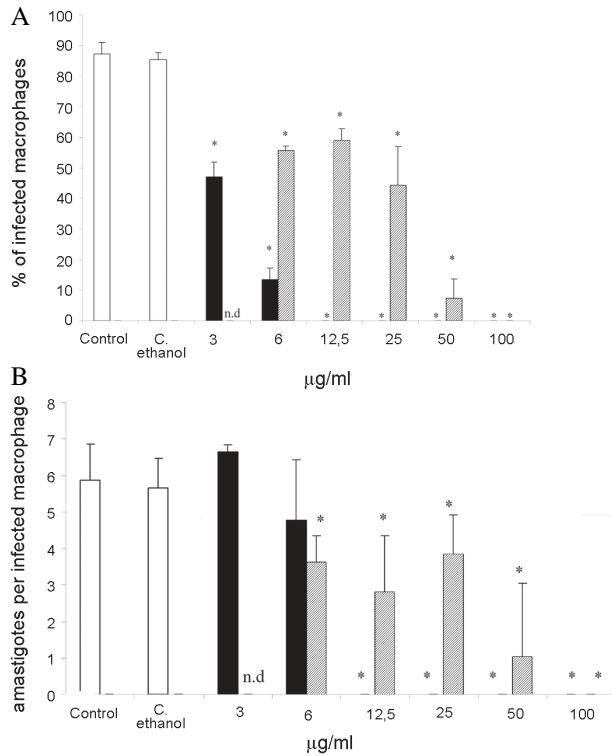
A

B

Fig. 2: effect of ethanolic extracts of Brazilian propolis (3-100 μg/ml) on *Leishmania amazonensis*-infected macrophages after 72 h of treatment: control, 0.1% ethanol, BRV (■) or BRP-1 (▨). The percentage of infected macrophages (A) and the number of parasites per infected cell (B) were determined as described in Materials and Methods. The results represent the mean ± SD of three independent experiments; n.d.: not determined. Asterisks represent statistically differences (p < 0.01) between control and propolis-treated cultures.

Fig. 3: effect of BRV propolis extract (25 μg/ml) on *Leishmania amazonensis*-infected macrophages after 24, 48, and 72 h of treatment: control (☐), 0.1% ethanol (▨), and BRV (■). The percentage of infected macrophages (A) and the number of parasites per infected cell (B) were determined as described in Materials and Methods. The results represent the mean ± SD of three independent experiments. Asterisks represent statistically differences (p < 0.01) between control and propolis-treated cultures.

Statistical analyses - All experiments were repeated at least three times in triplicate wells. The results were expressed as mean ± SD. Data obtained with different propolis extracts were analyzed by 1-way ANOVA and Student's *t*-test (P < 0.01).

RESULTS

Experiments were undertaken to study a possible effect of four typified Brazilian propolis samples on *L. amazonensis*-infected macrophage cultures. As shown in Fig. 1 murine macrophages were efficiently infected with *L. amazonensis* amastigotes (around 90% of infected cells and 6 intracellular parasites per infected cell). Macrophages infected with the parasite and treated with 25 μg/ml of BRG and BRPG extracts for 72 h have showed significant reduction of both the percentage of infection (Fig. 1A) and of the number of intracellular parasites (Fig. 1B). At concentrations higher than 25 μg/ml both extracts were toxic to the cells, because light microscopy showed cellular debris and few intact macrophages present on the surface of glass cover slips and in the culture supernatants. It must notice that at the percentage of 0.1% ethanol, amount present in experiments performed, this solvent had no effect on the cultures. The same protocol was employed to test BRP-1 and BRV samples (Fig. 2). The extract from BRP-1 sample was
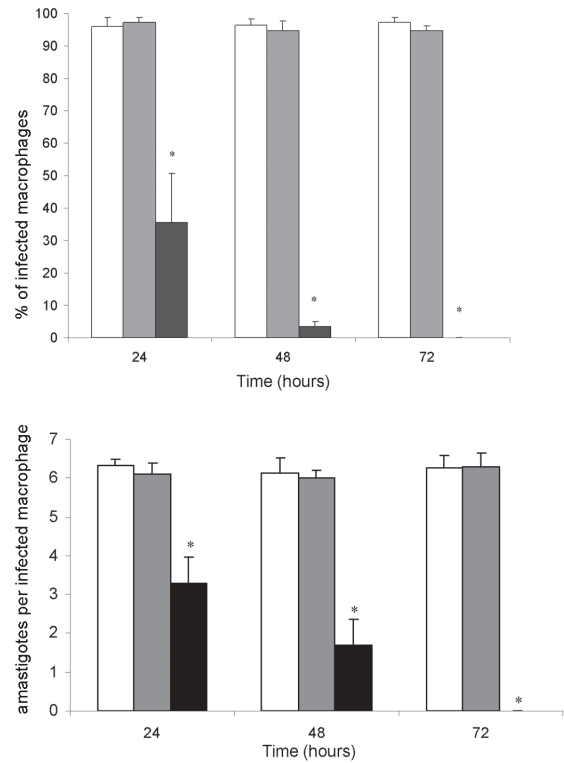
capable to reduce significantly the macrophage infection at concentrations ranging from 6 to 100 μg/ml. The treatment with 6 μg/ml of BRV for 72 h led to a reduction of 84.5% of the infection level and at higher concentrations of the extract no infected macrophage was observed. Since BRV was the most active, further experiments were performed, treating the infected cultures for 1, 2, and 3 days with 25 μg/ml of this extract, and being observed a time-dependent decrease of both the percent of infection and of the intracellular proliferation of the parasites (Fig. 3). The viability of macrophages treated for 72 h with 0.1% ethanol, 25, 50 or 100 μg/ml BRV was further analyzed by MTT assay. Formazan production was similar between control, macrophages treated with 0.1% ethanol or with 100 μg/ml BRV (Fig. 4). Interestingly, treatment with 25 or 50 μg/ml of the extract induced an increase in the MTT-reducing activity (Fig. 4). Since peritoneal macrophages are non-dividing differentiated cells (Handel-Fernandez & Lopez 2000), we can exclude the possibility that BRV stimulates the proliferation of macrophages.

We also addressed the question concerning whether BRV extracts presented a direct effect on promastigotes and extracellular amastigotes of *L. amazonensis*. Up to 96 h, at 25 μg/ml, the extract did not affect promastigotes proliferation (Fig. 5A). In experiments with lesion-derived
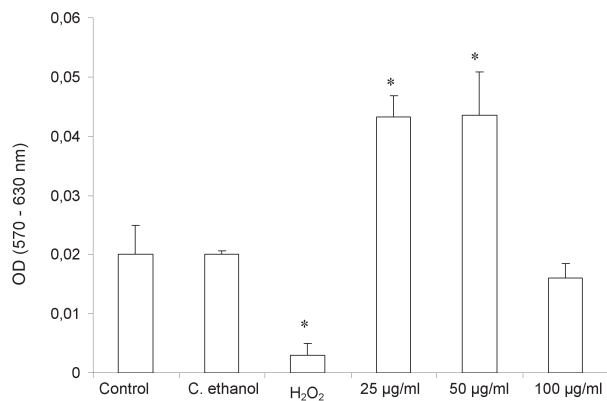
Fig. 4: MTT production by non-infected macrophages: control, 0.1% ethanol, 88 mM H_2O_2, or BRV extract at 25, 50, 100 µg/ml. The results represent the mean ± SD of three independent experiments. Asterisks represent statistically differences (p < 0.01) between control, H_2O_2 and BRV-treated cultures.
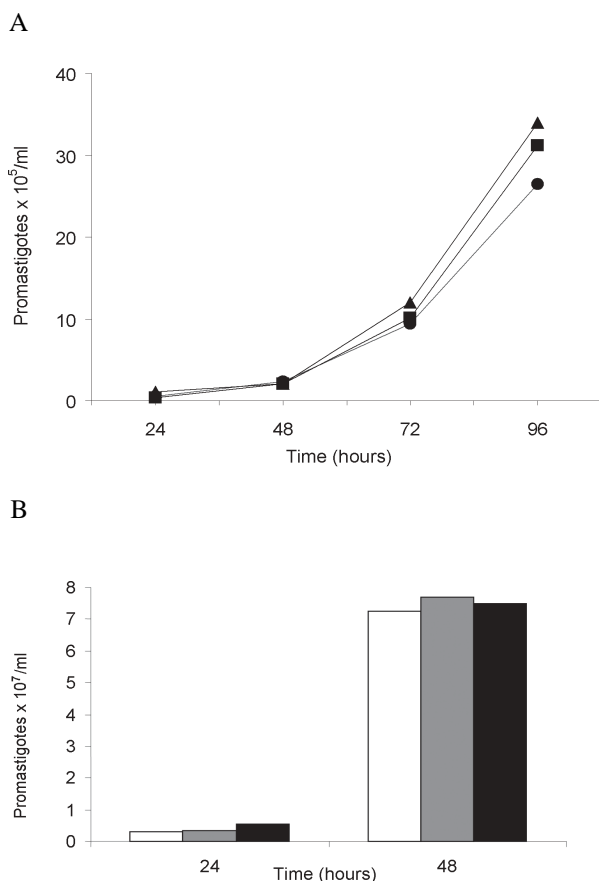
A

B

Fig. 5: effect of BRV propolis extract on *Leishmania amazonensis* promastigotes and extracellular amastigotes. A: promastigotes were left untreated (●), treated at 28ºC with 0.1% ethanol (■), 25 µg/ml BRV extract (▲) up to 96 h. Parasite viability was assessed by microscopic examination; B: extracellular amastigotes were left untreated (□), treated at 28ºC with 0.1% ethanol (▤) or 25 µg/ml BRV extract (■). The transformation of amastigotes into promastigotes was monitored after 24 h and 48 h. These are the results of a typical experiment, representative of a total of 3.

amastigotes, 25 µg/ml BRV did not interfere with their viability (around 98% of control viability after 24 h of treatment) or with their morphology (data not shown). Previous studies indicated that even if amastigotes remain viable, only molecularly undamaged amastigotes are expected to be able to transform into promastigotes (Lemesre et al. 1997, Arrais-Silva et al. 2005). Addition of 25 µg/ml BRV in medium did not affect amastigotes differentiation to promastigotes (48 h) (Fig. 5B).

DISCUSSION

This report provided evidences that ethanolic extracts of Brazilian propolis reduced *L. amazonensis* infection in macrophage cultures. Based on high performance liquid chromatography and nuclear magnetic resonance analysis ethanolic extract of Brazilian propolis samples have been typified in four groups (Marcucci 2000). BRG contains high concentration of coniferaldehyde compounds, BRPG contains high concentration of prenylated and coniferaldehyde compounds, BRP-1 contains high concentration of prenylated compounds and BRV contains high concentration of prenylated and benzophenones compounds (Marcucci & Bankova 1999, Marcucci et al. 2001, Miorin et al. 2003, Sawaya et al. 2004, Trusheva et al. 2006). The four propolis samples (BRG, BRPG, BRP-1, and BRV) evaluated in this study were able to reduce parasite load, as monitored by the percentage of infected cells and the number of intracellular parasites. Additional experiments with BRG and BRPG samples were abandoned because at concentrations higher than 25 µg/ml both extracts were toxic to macrophages. In fact some studies have demonstrated propolis toxicity for different cell types (Higashi & de Castro 1994, Chen et al. 2001, Ferguson 2001, Dantas et al. 2006, Tavares et al. 2006). For example, damage to murine macrophages was observed after treatment with ethanolic extract of propolis at concentrations above 30 µg/ml (Higashi & de Castro 1994), and genotoxic effect of ethanolic extract of Brazilian propolis (100 µg/ml) was detected in Chinese hamster ovary cells (Tavares et al. 2006). The mechanism of propolis cytotoxicity is still unknown (Tavares et al. 2006). On the other hand, BRP-1 and BRV extracts were not toxic to macrophages and inhibited the intracellular proliferation of *L. amazonensis*. The BRV extract was the most active and treatment of macrophages led to no morphological alterations as judged by light microscopy. Interestingly, 25 or 50 µg/ml BRV extract induced in macrophages an increase of the MTT-reducing activity. These results are apparently paradoxical, since macrophages are non-dividing cells (Handel-Fernandez & Lopes 2000), but the present results match those obtained with interferons and plant-derived polyphenols, which induce cell cycle arrest in cancer lineages but increase MTT-reducing activity (Jabber et al. 1989, Pagliacci et al. 1993, Bernhard et al. 2003). Such increase may be attributed to an increase in cell volume and mithocondrial number and/or activity of cells treated with the BRV extract (Bernhard et al. 2003). Although BRV is active against intracellular parasites it presented no direct effect on promastigotes or extracellular amastigotes. Our data corroborate results

obtained by Higashi and de Castro (1994). The authors observed that concentrations of ethanolic extract of propolis that inhibited the levels of *T. cruzi* infection in macrophages did not affect proliferation of axenic amastigotes. These results and our findings suggest that factors associated with host cell metabolism may contribute to intensify the effects of propolis (Higashi & de Castro 1994). Another possibility is that constituents of propolis intensify the mechanism of macrophage activation, leading to production of cytokines and reactive nitrogen intermediates engaged in the killing of intracellular parasites (Solbach & Laskay 2000). It was demonstrated that Korean propolis induces macrophages by producing interleukin-1, tumor necrosis factor-α, and nitric oxide (Han et al. 2002); these results suggest that propolis may function through macrophage activation. The precise mechanism by which BRV propolis treated macrophages are able to control *L. amazonensis* infection needs further investigations. This sample was collected in Alagoas, Brazil and it is a new propolis type called red Brazilian propolis containing high concentration of prenylated and benzophenones compounds (Marcucci 2000, Trusheva et al. 2006). The recent study of Trusheva et al. (2006) identified 14 chemical constituents of red Brazilian propolis, three of them with antibacterial and antimycotic activities, and encourages further investigations of the chemical constituents which are responsible for the leishmanicidal activities of red Brazilian propolis showed in this report. The investigation in animal models of *Leishmania* infection is currently under investigation in our laboratory.

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