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Leishmanicidal activity of brazilian propolis hydroalcoholic extract in Leishmania amazonensis

Atividade leishmanicida de extrato hidroalcoólico de própolis brasileira em Leishmania amazonensis

Suelen Santos da Silva[1]; Milena Menegazzo Miranda[1]; Idessania Nazareth Costa[2]; Maria Angélica Ehara Watanabe[3]; Wander Rogério Pavanelli[4]; Ionice Felipe[5]; José Mauricio Sforcin[6]; Ivete Conchon-Costa[7]

Abstract

As leishmanioses são consideradas doenças negligenciadas devido às altas incidências, ampla distribuição geográfica e dificuldade no tratamento sendo incluídas na relação de doenças prioritárias pela Organização Mundial da Saúde. Os tratamentos disponíveis para estas doenças apresentam elevada toxicidade, justificando a busca por fármacos alternativos. Estudos prévios com própolis, resina produzida por abelhas, demonstraram sua atividade antiparasitária e imunomoduladora em diversos modelos experimentais. O objetivo deste trabalho foi avaliar o efeito *in vitro* do extrato hidroalcoólico de própolis brasileira, coletado na cidade de Botucatu no estado de São Paulo, sobre formas promastigotas de *Leishmania amazonensis,* bem como analisar seu efeito *in vivo* sobre a carga parasitária em baço de camundongos susceptíveis à infecção. Assim, formas promastigotas tratadas com extrato hidroalcoólico de própolis brasileira nas concentrações 5, 10, 25, 50 ou 100 µg/mL apresentaram efeito inibitório sobre a proliferação desses parasitos nos tempos de 24, 96 e 168 h. No entanto, as concentrações de 50 e 100 µg/mL mostraram-se mais eficazes quando comparadas ao controle e às demais concentrações em todos os tempos avaliados. Em relação à carga parasitária, após 30 dias de infecção com *L. amazonensis,* camundongos BALB/c foram tratados diariamente com a própolis (5mg/kg), via oral ou intraperitoneal, durante 60 dias. Posteriormente, o baço destes animais foi coletado para análise da carga parasitária. O tratamento por via oral reduziu 40% da carga parasitária. Desta forma, a amostra de própolis brasileira testada apresentou ação leishmanicida sobre *L. amazonensis* em cultura e em camundongos infectados com este protozoário.

Keywods: Leishmaniose cutânea. Própolis. *Leishmania.*

[1] Doutoranda do Programa de Patologia Experimental, Universidade Estadual de Londrina, Paraná, Brasil. E-mails: suelenbiomedica@gmail.com; milenamenegazzo@yahoo.com.br

[2] Doutora em Imunologia e Parasitologia Aplicadas pela Universidade Federal de Uberlândia. Docente adjunta do Departamento de Ciências Patológicas, área de Parasitologia, Centro de Ciências Biológicas (CCB), Universidade Estadual de Londrina, Paraná, Brasil. E-mail: idessania@hotmail.com

[3] Doutora em Ciências Biológicas-Bioquímica. Docente adjunta do Departamento de Ciências Patológicas, área de Imunologia Básica, Centro de Ciências Biológicas (CCB), Universidade Estadual de Londrina, Paraná, Brasil. E-mail: maewatuel@gmail.com

[4] Doutor em Imunologia pela Universidade de São Paulo-USP. Docente adjunto do Departamento de Ciências Patológicas, área de Parasitologia, Centro de Ciências Biológicas (CCB), Universidade Estadual de Londrina, Paraná, Brasil. E-mail: wanderpavanelli@yahoo.com.br

[5] Doutora em Ciências Biológicas (Biofísica) pela Universidade Federal do Rio de Janeiro. Docente adjunta do Departamento de Ciências Patológicas, área de Imunologia Básica, Centro de Ciências Biológicas (CCB), Universidade Estadual de Londrina, Paraná, Brasil. E-mail: ionice@uel.br

[6] Doutor em Nutrição e Produção Animal, FMVZ, UNESP, Campus de Botucatu. Docente adjunto do Departamento de Microbiologia e Imunologia, Universidade Estadual Paulista, UNESP, Botucatu, São Paulo, Brasil. E-mail: sforcin@ibb.unesp.br

[7] Doutora em Microbiologia pela Universidade Estadual de Londrina. Docente adjunta do Departamento de Ciências Patológicas, área de Imunologia Básica, Centro de Ciências Biológicas (CCB), Universidade Estadual de Londrina, Paraná, Brasil. E-mail: icconchon@gmail.com

Resumo

Leishmaniosis are considered neglected diseases due to its high incidence, widespread and difficulty in treatment being included in the list of priority diseases by the World Health Organization. Available treatments for these diseases have high toxicity, which explains the search for more effective drugs. Previous studies with propolis - a resinous substance produced by bees - demonstrated immunomodulatory and anti-parasitic activity in several experimental models. The objective of this study was to evaluate the effect *in vitro* of Brazilian propolis hydroalcoholic extract, collected in the city of Botucatu in São Paulo State, on promastigotes forms of *Leishmania amazonensis* as well as its effect on the parasite load in the spleen of infected mice. Thus, promastigote forms treated with 5, 10, 25, 50 or 100 µg/mL of Brazilian propolis hydroalcoholic extract at 24, 96 and 168 hours showed inhibitory effect on the spread of these pararasite at all indicated times. However, the concentrations of 50 and 100 µg/mL were more effective, reducing the parasite spread when compared to the control and other concentrations at all times. Regarding parasitic load, after 30 days of infection with *L. amazonensis*, BALB/c mice were treated on a daily basis with propolis (5mg/kg) orally or intraperitoneally for 60 days. Further, the spleen was collected for parasite load analysis. Oral treatment reduced 40% of the parasitic load. Thus, the tested Brazilian propolis sample showed antileishmanial activity on *L. amazonensis* in culture and in parasite- infected mice.

Palavras-chave: Cutaneous leishmaniasis. Propolis, *Leishmania*.

Introduction

Leishmaniosis are infectious parasitic diseases showing worldwide distribution in temperate, tropical and subtropical regions. They constitute a serious public health problem and are present in most Brazilian states (HEPBURN, 2000).

The etiological agents of leishmaniosis are protozoans of Trypanosomatidae family and *Leishmania* genus that are transmitted by females of phlebotomine sandflies. The life cycle of these parasites involves two distinct developmental forms: promastigote- extracellular and flagellated found in the gut of the insect vector and the amastigote form- which lacks mobility and is found inside the cells of the immune system of the vertebrate host (NYLÉN; GAUTAM, 2010).

The disease can present a wide spectrum of clinical manifestations in different clinical forms as the appearing of self-healing skin lesions to severe visceral involvement of multiple organs (MICHALICK; RIBEIRO, 2011).

Despite the existence of an association between *Leishmania* species and clinical forms presented by the host, it has already been reported that the same species can produce different clinical manifestations related to the interaction of the host immune response and the characteristics of the etiological agent, as tropism and invasive capacity (BARRAL et al., 1991; NYLÉN; GAUTAM, 2010).

Therefore, it is important to report that *Leishmania amazonensis* has high pathogenic potential, since this species has been reported in cases of cutaneous, diffuse and visceral leishmaniosis (BARRAL et al., 1986; BARRAL et al., 1991).

The available drugs for the treatment of patients affected by this disease are pentavalent antimonials as Meglumine antimonate (commercialized in Brazil as Glucantime) and sodium stibogluconate (commercialized in Europe as Pentostam). Besides antimonials, amphotericin B, pentamidine and miltefosine can be used as therapeutic alternatives (DE VRIES; REEDIJK; SCHALLIG, 2015). However, the treatment of leishmaniasis patients is still a challenge, since these drugs have toxic side effects. Furthermore,

there are cases of post-treatment recurrence with antimonials, showing that clinical cure is not accompanied by parasitological cure (MEDEIROS; NASCIMENTO; HINRICHSEN, 2005).

In this sense, various natural or synthetic substances with antileishmanial, immunomodulatory and anti-inflammatory capacity have been investigated as an alternative to conventional treatment of this disease (ROCHA et al., 2005).

It is known that propolis is a natural product produced by bees which presents antibacterial (SFORCIN et al., 2000), antiviral (VYNOGRAD; VYNOGRAD; SOSNOWSKI, 2000), anti-inflammatory (KHAYYAL et al., 2003) and immunomodulatory activities (SFORCIN; KANENO; FUNARI, 2002).

In relation to the parasites of *Leishmania* genus, the results showed the leishmanicidal effect of propolis collected in different regions of Brazil and other countries against promastigote and amastigote forms of different species of this protozoan (AYRES; MARCUCCI; GIORGIO, 2007; DA SILVA et al., 2013; DURAN et al., 2008; MACHADO; LEON; CASTRO, 2007; PONTIN et al., 2008; OZBILGE et al., 2010).

Despite the evidences already presented in the literature, it is known that the chemical composition of propolis is dependent on the biodiversity of each area visited by bees. Thus, various propolis extracts may present variations in color, consistence and amount of biologically active substances, depending on the plant visited, bee species, age of collection and the substance used for extracting (WATSON et al., 2006).

In this context, this paper presents results concerning the leishmanicidal action, both *in vitro* and *in vivo*, of the hydroalcoholic extract of Brazilian propolis collected in Botucatu/São Paulo (SP) on promastigote forms of *L. amazonensis*.

Materials and Methods

Leishmania amazonensis

L. amazonensis promastigote forms (MHOM/BR/1989/166MJO) maintained in culture medium 199 (GIBCO Invitrogen®, Grand Island, USA) supplemented with 10% fetal bovine serum-FBS (GIBCO Invitrogen®, Grand Island, USA), 1M of HEPES biological buffer (AMRESCO®, Solon, USA), 1% human urine, 1% L-glutamine (Synth®, Diadema, Brazil), penicillin and streptomycin (10 U/mL-10 µg/mL, GIBCO Invitrogen®, Grand Island, USA) and 10% sodium bicarbonate (Synth®, Diadema, Brazil). The cell cultures were grown in a BOD type incubator at 25°C in 25 cm^2 culture flask.

Hydroalcoholic extract of Brazilian propolis

The propolis sample used in this study was collected at *Fazenda Lageado* apiculture section, *Universidade Estadual Paulista (UNESP),* Campus Botucatu, SP, from colonies of *Apis mellifera* bees. The extraction method, as well as the chemical composition of this sample have been documented in previous studies in which chemical analysis showed that major constituents of this sample are phenolic compounds such as flavonoids, aromatic acids and benzopyrans as well, di and tri terpenes and essential oils (SFORCIN, 2007). The final concentration of ethanol solvent in the experiments did not exceed 0.1%.

Proliferation Kinetics

L. amazonensis promastigote forms (10^6/mL) were treated with different concentrations of the hydroalcoholic extract of Brazilian propolis (5, 10, 25, 50, or 100 µg/mL) diluted in culture medium 199 supplemented and maintained at 25°C. Treatments were maintained for seven days, on which the promastigote forms were

counted in a Neubauer chamber after 24, 96, and 168 hours for the establishment of kinetics spread. As a means of control, promastigotes were used without treatment.

Animals

BALB/c mice weighing 25-30 g, 6-8 weeks old, from the central biotery at the State University of Maringá (UEM), Maringá, Paraná (PR) were used. The animals were kept in a biotery of parasitology at the State University of Londrina (UEL), Londrina, PR, with water and *ad libitum* fed, controlled luminosity (light-dark cycle, 12/12 h) and temperature (23° C ± 1° C). The project was approved by the Ethics Committee on Animal Experiments of UEL, under the registration number 09/2011 and Circular Letter N° 24/2011.

Experimental infection and treatment

The animals were divided into 4 groups (n= 6/ group): control without infection (animals that did not receive infection or treatment), control (infected but not treated animals), Propolis p.o. (infected animals orally treated with propolis), Propolis i.p. (infected animals treated with propolis intraperitoneally).

Animals from control groups, Propolis i.p. and Propolis p.o. were subcutaneously infected in the right hind paw with promastigote forms of *L. amazonensis* (10^7/20μL). After 30 days of infection, daily treatment with propolis (5 mg/ kg) was initiated orally (p.o.) or intraperitoneally (i.p.) for 60 days. Infected mice used as controls received only phosphate buffer vehicle (PBS) (i.p. and p.o.). At the end of the experiment (30 days of infection and 60 days of treatment) the animals were euthanized and their spleens collected for weighing and parasite load analysis. Every effort was made to minimize the number of animals used and their suffering.

Parasite load

L. amazonensis parasite load in the spleen of BALB/c mice was performed according to microtiter methodology described by Buffet et al. (1995), with modifications. The spleen of each animal was removed aseptically, weighed and homogenized in culture medium 199 supplemented. The suspensions were serially diluted in 96-wells that were subsequently incubated at 25°C. Every week, during thirty days, plaques were observed in inverted light microscope. Each well was examined with 200x magnification, looking for *L. amazonensis* promastigotes. The highest dilution in which at least one parasite was found was defined as titer. The following formula was carried out in order to determine the parasite load:

Parasitic Load = (geometric average of the titer of each triplicate / body mass (mg) x 400, considering 400 as the fraction of homogenized organ and placed in the first well before dilution. The results were expressed in $\log_{10}$.

Statistical analysis

Data were analyzed using GraphPad PRISM statistical software (Graph-Pad Software Inc., USA, 5.00). Significant differences between treatments were determined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was accepted at $p < 0.05$.

Results

Extract of Brazilian propolis presents leishmanicidal effect on promastigote forms of L. amazonensis

The promastigote forms of *L. amazonensis* were treated with different concentrations of propolis (5, 10, 25, 50 or 100 μg/mL) for 24 h (Figure 1A), 96 h (Figure 1B) and 168 h (Figure

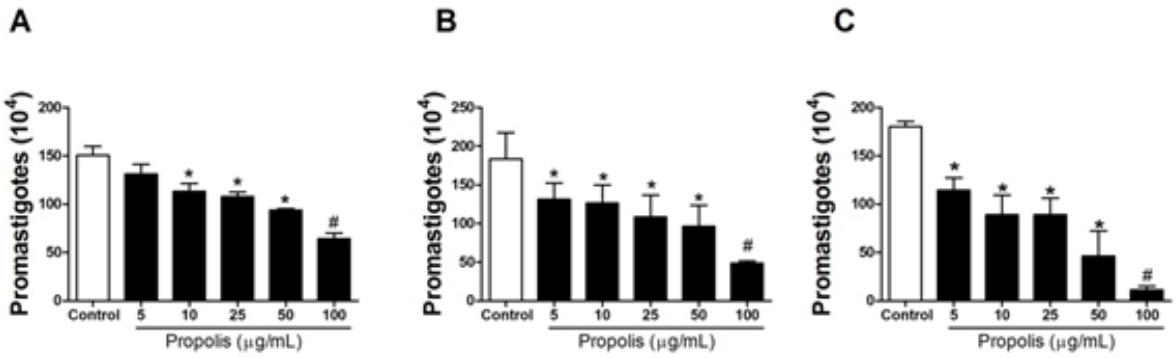
1C). Through counting in a Neubauer chamber, it was observed that treatment with Brazilian propolis reduced the spread of promastigotes at all evaluated times. The concentrations of 10, 25, 50 and 100 µg/mL reduced spread by 25, 29, 38 and 57% respectively after 24 h of treatment. On the other hand, the propolis concentration of 5 µg/mL inhibited the spread of *L. amazonensis* in 13% with no significant difference compared to the control.

After 96h treatment, the percentages of reduction reached 29, 31, 41, 48 and 74% for Brazilian propolis treatments with 5, 10, 25, 50 and 100 µg/mL, respectively (Figure 1B). After 168 h of incubation with the treatment, the reduction of parasites spread was 37, 51, 51, 74 and 94% for propolis treatments with 5, 10, 25, 50 and 100 µg/mL, respectively (Figure 1C).

Propolis extract can reduce the parasitic load on the secondary lymphoid organ (spleen) of BALB/c mice infected with L. amazonensis

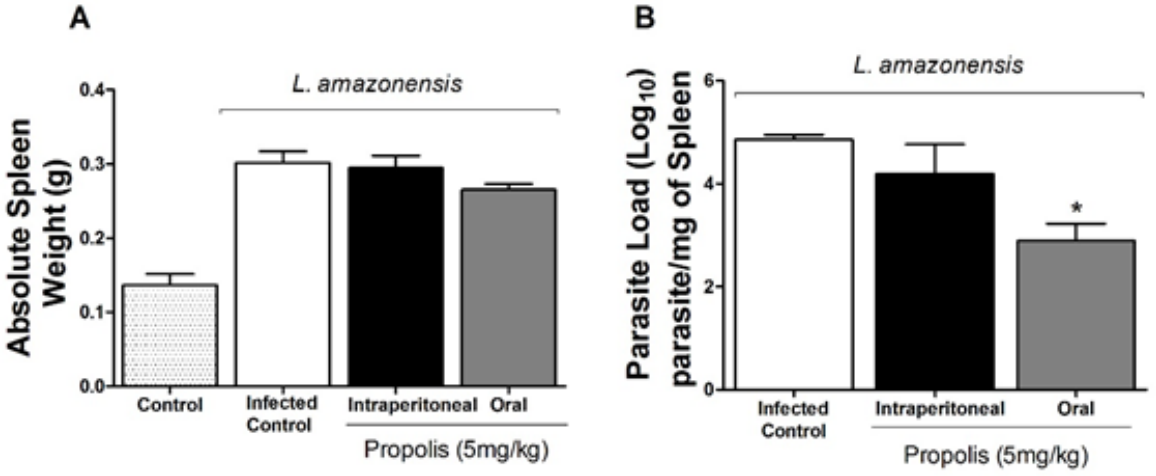
The obtained results considering the spleen weight and parasite load are shown in Figures 2A and 2B, respectively. Data showed that *L. amazonensis* was able to migrate to secondary organs, such as the spleen. Treatment with a dose of 5 mg/kg, p.o., of Brazilian propolis extract was able to reduce 40% the parasitic load in the spleen when compared to infected control. Yet, the treatment via i.p. promoted 13% of reduction in parasite load, showing no differences with the infected control (Figure 2B). Moreover, both treatments did not reduce organ weight (spleen) as compared to organ weight of the animals in the infected control group (Figure 2A).

Figure 1- Antileishmanial activity of Brazilian propolis extract in the kinetics of spread of *Leishmania amazonensis* promastigote forms treated with different concentrations of Brazilian propolis extract (5, 10, 25, 50, or 100 µg/mL) for 24 hours (A), 96 hours (B) and 168 hours (C). As a control, we used *L. amazonensis* promastigotes maintained in culture medium 199 without treatment. One-way ANOVA followed by Tukey test. Results represent the mean ± SEM of five independent experiments. * Significantly different from control ($p < 0.05$). # Significantly different of treatments with concentrations 5, 10 and 25 µg/mL of propolis ($p < 0.05$).



Fonte: authors

Figure 2 - Leishmanicidal activity of Brazilian propolis extract in secondary lymphoid organ of BALB/c mice. BALB/c mice infected with *Leishmania amazonensis* promastigote form (10^7) subcutaneously in the right hind paw. After 30 days of infection it was begun daily treatment with propolis (5 mg/ kg) by oral or intraperitoneal route for 60 days. The spleens of animals were collected and evaluated as the mass, expressed in g (A) and the parasitic load expressed in $\log_{10}$/mg spleen (B) by means of serial dilutions of spleen homogenate culture. One-way ANOVA followed by Tukey test. Results represent the mean ± SEM of quadruplicate 6 animals per experimental group. * Significantly different from infected control ($p < 0.05$).



Fonte: authors

Discussion

This study evaluated the leishmanicidal activity of the hydroalcoholic extract of propolis collected in SP region on promastigote forms of *L. amazonensis*. We used models *in vitro* and *in vivo*, in which the apitherapic sample used demonstrated direct activity decreasing the proliferation of promastigote forms and indirect activity reducing the parasite load in the spleen of mice after infection.

Previous studies in experimental models *in vitro* and *in vivo* have demonstrated that propolis extracts collected in different regions of Brazil have leishmanicidal activity in promastigote and amastigote forms of *L. amazonensis, L. braziliensis, L. major* and *L. chagasi* (AYRES et al., 2007; DA SILVA et al., 2013; MACHADO; LEON; CASTRO, 2007; MIRANDA et al., 2015; PONTIN et al., 2008).

It is known that the propolis samples may exhibit different chemical composition or different concentrations of these components (WATSON et al., 2006). The sample used in this study presents a greater amount of phenolic compounds, di and tri terpenes and essential oils (SFORCIN, 2007). According to Cunha et al. (2011), these constituents have been previously reported to be associated with antileishmanial activity against promastigote and amastigote forms of various species of this protozoan.

Although the mentioned studies have demonstrated the leishmanicidal activity of various propolis extracts, it is known that the susceptibility of different species and parasite strains of the genus *Leishmania* when subjected to the action of various compounds is not homogenous and varies considerably between species/strains tested (MORAIS-TEIXEIRA et al., 2011; VILA-NOVA et al., 2013).

The propolis sample used in this study was previously conducted *in vitro* against *L. braziliensis* presenting leishmanicidal and immunomodulatory action, and in *in vivo* model against *L. amazonensis* where therapeutic potential associated with a nitric oxide donor was evaluated(DA SILVA et al, 2013;. MIRANDA et al., 2015). In the present study, we demonstrate that this Brazilian propolis extract was also effective in inhibiting *in vitro* spread of *L. amazonensis* promastigote forms in dependent dose manner, consolidating the leishmanicidal effect of this compound.

Among the tested concentrations, 5, 10 and 25 µg/mL have inhibitory effect on the proliferation of these parasites. However, the concentrations of 50 and 100 µg/mL were more effective over time when compared to the control and other concentrations.

When the action of Brazilian propolis extract on *in vivo* model was evaluated, the parasites were quantified in the spleen of BALB/c mice infected with *L. amazonensis* by microtiter technique in culture, after 60 days of treatment, a total of 90 days of infection. The presence of *L. amazonensis* promastigote forms was detected in the spleen of all infected animals. Thus, we can affirm that this protozoan species associated with the cutaneous form of the disease are able to visceralize in animal infection model. In addition, we confirm the susceptibility of BALB/c mice to infection by *L. amazonensis.*

These data corroborate the reported findings of Barral and colleagues (1986 and 1991) that *L. amazonensis* as the etiologic agent of visceral leishmaniasis in humans. Furthermore, in animal models, some authors reported that infection with *L. amazonensis* and other species associated with cutaneous disease, are able to visceralize in an animal infection model (ABREU-SILVA et al., 2004; MAGILL et al., 1993; ROBERTS; ALEXANDER; BLACKWELL, 1989; SOLIMAN, 2006; WALTON; INTERMILL; HAJDUK, 1977).

As for the different routes of propolis extract administration, most studies only show their chemical composition and pharmacological activities, not showing the peculiarities of pharmacokinetics (LUSTOSA et al., 2008). The few studies on the pharmacokinetics are made only with specific chemical compounds isolated from the extracts (MESBAH; SAMIA, 2011; METZNER et al., 1979; PAULINO et al., 2009).

We found that only p.o. treatment decreased the parasite load, reducing at 40% the number of parasites in the assessed body. This parasite burden reduction in secondary organs is extremely important in resolving the infection in the host.

Propolis extract therapeutic potential has been shown in several studies, including models using this apitherapic in combination with Glucantime, a drug already used for the treatment of leishmaniases (AYRES et al., 2011; FERREIRA et al., 2014). Thus, the presented data justify further study of the use of this natural compound in leishmaniosis treatment support.

Conclusion

This study demonstrated that Brazilian propolis hydroalcoholic extract collected in SP region presents leishmanicidal activity in *L. amazonensis in vitro* and *in vivo* models. These results represent an encouragement for research and mechanisms description of action and general pharmacology in using this apitherapic as a support in the therapy of leishmaniosis.

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