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SHORT COMMUNICATION

Brazilian brown propolis elicits antileishmanial effect against promastigote and amastigote forms of *Leishmania amazonensis*

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Propolis is a complex matrix of chemical constituents extracted from plants and produced by bees which is used in folk medicine due to its several pharmacological properties. Its chemical composition varies according to the region where it is produced. This work has studied the antileishmanial activity and cytotoxicity of brown propolis (BP) originating from the semi-arid region of Piauí, Brazil. The BP showed significant inhibition of the *Leishmania amazonensis* promastigotes growth as well as being effective in reducing infection of murine macrophages and the number of internalised amastigotes in these cells. The dichloromethane fraction was the most active and showed the best selectivity index. The studied samples presented good activity and the fractioning improved the antileishmanial activity without an increase in the cytotoxicity against mammalian cells. Therefore, BP is a potential source for development of apitherapeutic products for the treatment of leishmaniasis.

Keywords: leishmaniasis; antiparasitic activity; cytotoxicity; propolis; brown propolis

1. Introduction

Leishmaniasis is a complex of diseases caused by protozoa of the genus *Leishmania* and constitutes a serious health problem around the world where 350 million people are considered at risk of contracting the diseases and two million new cases have been reported each year (WHO 2010). The treatment of infected individuals with pentavalent antimonial drugs is the main therapeutic strategy. However, their high toxicity and persistence of side effects, even after changing the dose and duration of treatment, have been serious drawbacks. Therefore, the discovery of new and safe natural-derived therapeutic agents against leishmaniasis is markedly important (Carvalho & Ferreira 2001; Croft & Coombs 2003; Kaiser et al. 2003).

Propolis is one of many natural products that have been used for centuries by mankind. It is prepared by *Apis mellifera* bees and is extracted from flower buds and plant exudates, together with salivary secretions, wax and pollen (Bankova 2005). Its chemical composition is not only rich and complex, but also closely related to the ecology and flora of each region visited by bees as well as its harvest period. Besides, the genetic variability of queen bees also influences in the propolis' chemical composition (Park et al. 2002).

A recent study has reported the potential of brown propolis (BP) against protozoa of *Plasmodium*, *Trypanosoma* and *Leishmania* genus (Monzote et al. 2012). Interestingly, a

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propolis ethanol extract may be useful for the stimulation of humoral immune responses in vaccine design for leishmaniasis (Rasouli et al. 2012).

Considering the importance of leishmaniasis and its endemic characteristic in the poor regions of Brazilian north-east, as well as the great variability of propolis' chemical composition, this study evaluates the antileishmanial and cytotoxic activities of BP and its fractions, as well as their chemical compositions by CG-MS analysis.

2. Results and discussion

Natural products have been systematically studied against *Leishmania amazonensis* and demonstrated a promising alternative due to their antileishmanial potential, not only obtained from plants, such as the geranylgeraniol obtained from *Bixa orellana* L. (Lopes et al. 2012), but also from marine organisms, such as the *Laurencia dendroidea*, a sesquiterpene-rich Brazilian red alga (Machado et al. 2011). In this work, the chemical composition and the antileishmanial effect of Brazilian BP was evaluated.

The chemical composition of the BP hydroalcoholic extract (BP-HAE) and its fractions was determined, and the major compounds were identified as follows: lupeol (25.77%) for hexanic fraction (BP-HEX); 2,3-dihydroxybenzofurane (20.04%) for dichlorometane fraction (BP-DCM) and *p*-coumaric acid (17.16%) for ethyl acetate fraction (BP-EtOAc) (See Supplementary Material, Table S1).

The antileishmanial activity was assessed by growth inhibition of *L. amazonensis* promastigotes for 24, 48 and 72 h of incubation. The results showed a marked time-dependent inhibitory activity, evidenced by the low IC_{50} values. After fractionation, an increase of 31% in BP-DCM activity was observed when compared with the BP-HAE. A less cytotoxicity was also observed for the BP-DCM according to the CC_{50} values and the selectivity index (SI), whereby the BP-HAE was six times more toxic against promastigote forms of the *L. amazonensis* than against macrophages (Table 1).

The high content of the triterpene lupeol in BP-HAE contributes for the BP-induced antileishmanial effect. In a previous study, lupeol obtained from *Pera benensis* Rusby was successfully evaluated against five *Leishmania* strains (Fournet et al. 1992). In addition, the diterpenic acids obtained from *Copaifera* spp. possess a great antileishmanial potential (Santos et al. 2013), confirming this property for the terpene-derived compounds encountered in BP-HAE.

The results obtained for the BP-HAE and BP-DCM against infection of macrophages by *L. amazonensis* showed a significant concentration-dependent decrease in infection rates by the comparison between the number of amastigotes in treated and untreated macrophages. These results are supported by Monzote et al. (2011). Macrophages treated with BP-DCM presented

Table 1. *In vitro* effect of BP on the growth inhibition of promastigotes forms, cytotoxicity to murine macrophages and selectivity for *L. amazonensis*.

BP samples	IC_{50} value (μg/ml)[a] 24 h	48 h	72 h	CC_{50} (μg/ml)[b] 48 h	SI[c] 48 h
BP-HAE	11.87 (6.65–20.17)[d]	12.08 (6.69–20.71)	4.64 (2.27–8.31)	21.69 (16.65–28.66)	1.80
BP-HEX	17.34 (6.58–40.28)	10.11 (3.46–23.58)	4.79 (1.31–12.00)	35.10 (27.04–45.10)	3.47
BP-DCM	4.96 (2.75–8.00)	4.95 (2.75–7.97)	3.22 (1.66–5.43)	30.50 (10.94–49.81)	6.16
BP-EtOAc	36.95 (10.45–132.95)	28.17 (7.70–93.41)	8.83 (1.60–28.05)	> 400	ND

[a] Concentration that caused 50% inhibitory growth of promastigotes.
[b] 50% cytotoxicity concentration to macrophages.
[c] SI (CC_{50}/CI_{50}).
[d] 95% confidence interval; ND, not determined.

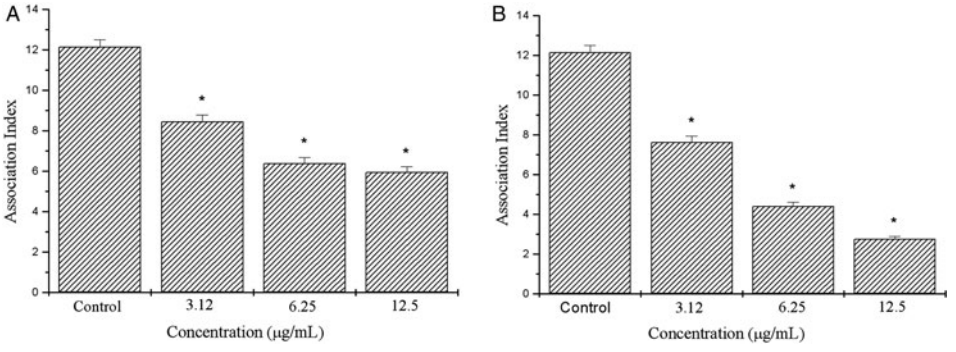

Figure 1. *In vitro* effect of BP on internalised amastigotes of *L. amazonensis* in murine macrophages after 48 h of incubation. (a) BP-HAE; (b) BP-DCM. The statistical differences were analysed using the Mann–Whitney's test and considered significant at $p < 0.05$. Each column represents the mean of experiments carried out in triplicate with standard error. The asterisk denotes the significance level when compared with the control group: $*p < 0.001$.

5.9 amastigotes per macrophage and those treated with BP-HAE presented 7.89 amastigotes per macrophage, while the control group had a mean of 12.51 amastigotes per macrophage. Hence, the Association Index obtained for the BP-HAE was also analysed and the differences were statistically significant from the control until the concentration of 6.25 μg/ml ($p < 0.001$). Between 6.25 and 12.5 μg/ml, no significant difference ($p = 0.69$) was found. For the BP-DCM, the differences were statistically significant ($p < 0.001$), and a concentration-dependent effect was also observed (Figure 1).

These results lead us to propose the main compound from BP which contributes to a marked antileishmanial activity is the 2,3-dihydrobenzofuran, the major constituent present in the BP-DCM (20.04%), also found in the BP-HEX (1.39%) and BP-EtOAc (0.80%). Accordingly, several synthetic dihydrobenzofurans were reported as promising antiprotozoals, including a marked activity against *L. donovani* (Van Miert et al. 2005).

3. Conclusions

The Brazilian BP presented a marked antileishmanial activity and low cytotoxicity against macrophages. Especially, the dichloromethane fraction showed the best activity and safety due to its highest SI. Accordingly, the chemical composition by CG-MS indicates the dihydroxybenzofurane-derivative compounds as possible antileishmanial biomarkers which leads this work to further studies concerning the development of apitherapeutic products directed to the treatment of leishmaniasis.

Supplementary material

Supplementary material relating to this article is available online, alongside Table S1.

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