Activity of Brazilian and Bulgarian propolis against different species of *Leishmania*

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Extracts of propolis samples collected in Brazil and Bulgaria were assayed against four Leishmania *species –* Leishmania amazonensis, L. braziliensis, L. chagasi *from the New World, and* L. major *from the Old World – associated to different clinical forms of leishmaniasis. The composition of the extracts has been previously characterized by high temperature high resolution gas chromatography coupled to mass spectrometry. Considering the chemical differences among the extracts and the behavior of the parasites, it was observed significant differences in the leishmanicidal activities with IC50/1 day values in the range of 2.8 to 229.3* μg/ml. *An overall analysis showed that for all the species evaluated, Bulgarian extracts were more active than the ethanol Brazilian extract. As the assayed propolis extracts have their chemical composition determined it merits further investigation the effect of individual components or their combinations on each* Leishmania *species.*

Key words: *Leishmania* - leishmaniasis - propolis - chemical composition - natural products

Leishmaniasis is endemic in 88 countries of Tropical and Sub-tropical regions, affecting more than 12 million people (WHO 2004). No vaccines are available for any form of the disease, and the chemotherapy of this disease is still inadequate and expensive (Croft & Yardley 2002). In this context, there is an intense search for potential new synthetic compounds and natural products for the treatment of leishmaniasis. In the last decades occurred a movement sometimes called "back to Nature", which in the area of drug development was accentuated by the success obtained, for example, by taxol in cancer chemotherapy and artemisinin for malaria (Newman et al. 2003). Such intensification in the search for drugs from natural sources was also observed in the area of leishmaniasis. In the literature there are several reports on the activity of a variety of crude natural extracts, especially from plants collected in tropical zones, against *Leishmania* species of the New World (Fournet et al. 1994, 1996, Akendengue et al. 1999, Weniger et al. 2001, Fournet & Munoz 2002). Most of these studies were performed with *L. amazonensis* and *L. braziliensis* that cause the cutaneous form of the disease (Lainson 1983, Hepburn 2000), and as second pathology, the first species led to the diffuse cutaneous form (Garnier & Croft 2002), and the second one to the mucocutaneous disease (Jones et al. 1987, Almeida et al. 1996); and also with *L. chagasi* the causal agent of visceral leishmaniasis (Lainson et al. 1987, Davidson 1999).

Our group has been engaged for several years in the investigation of propolis effect against pathogenic trypanosomatids, especially *Trypanosoma cruzi*, agent of Chagas disease (De Castro & Higashi 1995, Marcucci et al. 2001, Cunha et al. 2004, Dantas et al. 2005, 2006). Propolis is a resinous substance that honeybees use to fill gaps and to seal parts of the hive and is collected from different plant exudates resulting in a complex mixture containing known bioactive constituents (Bankova et al. 2000). This product possesses microbicidal, anti-inflammatory, and anti-tumoral activities, which have been associated with the presence of phenolic compounds (reviewed in De Castro 2001). We have previously determined the chemical composition as well as the analgesic and anti-inflammatory activities of a standard ethanol extract of a Bulgarian sample (Et-Blg) (Prytzyk et al. 2003, Paulino et al. 2003) and compared its activity to that of a corresponding Brazilian extract (Et-Bra) against *T. cruzi* and several species of fungi and bacteria of medical importance (Salomão et al. 2004). In the present work, we analyzed the effect of these extracts against different species of *Leishmania.*

MATERIALS AND METHODS

Propolis samples - The Bulgarian propolis (Blg) sample was collected at Burgas (Southeast Bulgaria) (Prytzyk et al. 2003) and the Brazilian one (Bra) at Mar de Espanha (state of Minas Gerais, Brazil) (Salomão et al. 2004). The exudates collected by bees in Bulgaria were mainly from buds of *Populus nigra*, and those in Brazil from a mixture of tropical plants. Each sample was cut in small pieces, after cooling bellow –10°C and extracted with 70% ethanol (1:10 w/v), under agitation at room temperature. After 24 h, the extracts were filtered and evaporated to dryness under vacuum at 40°C and stored in a desiccator at 4°C. The yield of the extracts was 62 and 58% for Et-Blg and Et-Bra, respectively. Also, an acetone extract from the Bulgarian sample was obtained after extraction of the resin with hexane (1:25, w/v) followed by extraction of the residue with acetone at room temperature leading to Ket-Blg, obtained in a yield of 7.5%. The composition of the extracts has been

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previously characterized by high temperature high resolution gas chromatography coupled to mass spectrometry (HT-HRGC-MS) (Table I) (Prytzyk et al. 2003, Salomão et al. 2004).

Parasites - Promastigote forms of *L. amazonensis* (strain MHOM/BR/77/LTB0016) were maintained in Schneider's *Drosophila* medium supplemented with 10% fetal calf serum (FCS), pH 7.2, at 26°C. Promastigotes

TABLE I

Chemical characterization of the Bulgarian and Brazilian propolis extracts by high temperature high resolution gas chromatography coupled to mass spectrometry

	% area		
Compound	Et-Blg[a]	Ket-Blg[a]	Et-Bra[b]
3-Acetobutyric acid	-	-	0.53
α-Amyrin	-	-	0.53
β-Amyrin	-	-	0.93
β-Amyrin acetate	-	-	0.56
Amyrin 3-methoxy	-	-	0.52
Benzoic acid	0.14	tr	-
Benzyl caffeate	1.64	0.72	-
Caffeic acid	2.61	6.87	0.57
Chrysin	2.22	3.85	-
Cinnamic acid	-	-	4.81
Diethyl 2-methylsuccinate	-	-	7.00
3,4-Dimethoxy-cinnamic acid	0.37	-	-
2-Ethylhexanoic acid	-	-	1.44
Ethyl hydrocinnamate	-	-	0.27
Ethyl indolacetate	-	-	1.11
Ferulic acid	0.40	-	0.40
Fructose	0.36	10.03	-
Geranyl acetal	-	-	2.99
Glicyrrizic acid	-	-	1.03
Glucose	0.62	1.22	-
Glycerol	0.92	5.00	-
Hexadecanoic acid	-	-	1.14
Hydrocinnamic acid	-	-	4.48
Hydroxymethyl indolacetate	-	-	2.58
2-Indolcarboxylic acid	-	-	0.35
Inositol	-	-	0.39
Isobutylquinoline	-	-	4.30
Isoferulic acid	0.30	-	-
3-Ketoadipic acid	-	-	0.46
Linoleic acid	-	-	1.30
Mannose	-	-	0.66
Menthol	-	-	4.00
Myristic acid	0.65	0.95	-
Oleic acid	2.33	0.24	2.63
Palmitic acid	2.46	0.61	-
Palmitoleic acid	0.87	0.24	-
Patchouli alcohol	-	-	5.60
p-Coumaric acid	0.60	0.41	-
Pentanedioc acid	-	-	4.84
Pentenoic acid	-	-	1.60
Pentenyl caffeate (isomer)	0.74	tr	-
Pentenyl caffeate (isomer)	1.41	tr	-
Pentenyl ferrulate (isomer)	0.97	tr	-
Pentenyl ferrulate (isomer)	0.79	tr	-
Phenethyl caffeate	2.10	0.43	-
Pinobanksin 3-butanoate	9.85	1.36	-
Pinobanksin 3-etanoate	11.23	3.85	-
Pinobanksin 3-pentanoate	2.75	0.58	-
Pinocembrin	9.44	3.60	-
Pinostrobin	-	-	1.06
Pinostrobin chalcone	0.40	-	-
Squalene	4.41	-	-
Stearic acid	0.66	tr	-
Undecanoic acid	-	-	2.89

a: Prytzyk et al. 2003; *b*: Salomão et al. 2004.

of *L. braziliensis* (MCAN/BR/98/R619), *L. chagasi* (MCAN/BR/97/P142), and *L. major* (Cão4) were grown in the same medium supplemented with 20% FCS plus 2% human urine, pH 7.2, at 26°C (Howard et al. 1991, Shamsuzzaman et al. 1999). The parasites were maintained only up to five passages, in order to guarantee to perform the experiments with infective forms (Cysne-Filkelstein et al. 1998). The parasites were harvested in the late log phase of growth, which varies for each species, by centrifugation at 4000 rpm for 10 min and the sediment was resuspended in the supernatant of the corresponding culture, counted in a Neubauer chamber and the concentration adjusted to 2×10^6 promatigotes/ml.

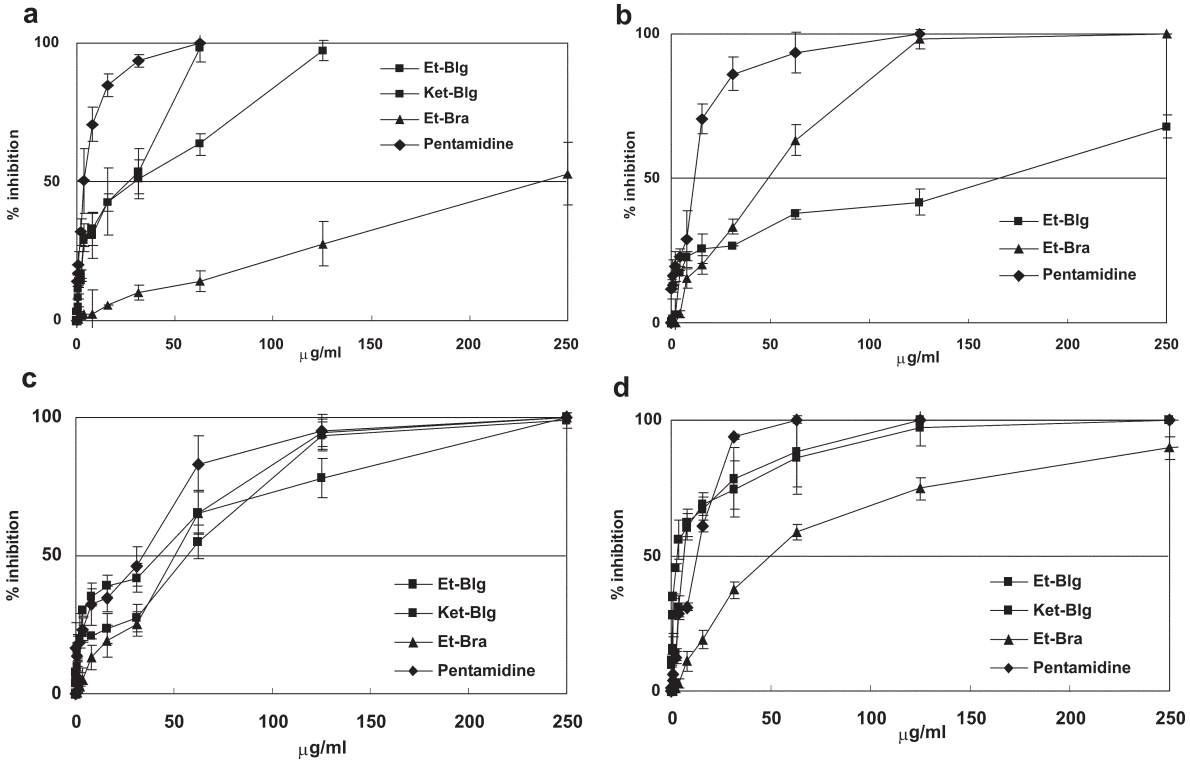
Leishmanicidal activity - Stock solutions of Et-Blg, Et-Bra and Ket-Blg were prepared in dimethylsulfoxide at 100 mg/ml. A volume of 100 µl of each parasite suspension (2×10^5 promastigotes) was added in 96-well microplates to the same volume of each propolis extract, previously prepared at twice the desired concentrations in the medium employed for each species. Untreated and pentamidine isethionate-treated parasites were used as controls. The extracts were assayed in the range of 0.5 to 500 µg/ml, with solvent concentration never exceeding 0.5%, which has no deleterious effect on the parasites. The incubation was performed at 26°C and after 24 h the parasite concentration was counted and the activity of the extracts was expressed as IC_{50}, corresponding to the concentration that leads to 50% of inhibition of promastigote proliferation.

Statistical analysis - Statistical significance ($p < 0.05$) was evaluated by the ANOVA test followed by Student-Newman-Keuls, by Kruskall-Wallis or Mann-Whitney tests software SPSS for Windows.

RESULTS

The effect of the propolis extracts against different species of *Leishmania* is displayed in Table II. Et-Blg showed the following decreasing order of activity against the parasites: *L. major* > *L. amazonensis* > *L. chagasi* > *L. braziliensis*, always with statistical differences among the IC_{50} values for the four species. A similar order of activity was observed for Ket-Blg. For Et-Bra, *L. major, L. chagasi*, and *L. braziliensis* showed a similar sus-ceptibility to the extract, while *L amazonensis* was the less susceptible species.

Comparing the two New World species associated to cutaneous leishmaniasis (Figs 1a,b), *L. amazonensis* was 7.8 times more susceptible to Et-Blg than Et-Bra, while an inverse order was observed in the case of *L. braziliensis*. Furthermore, *L. chagasi,* related to visceral leishmaniasis, showed a similar sensitivity to the three extracts assayed (Fig. 1c). The IC_{50}/1 day values for pentamidine were 4.7 ± 1.6, 13.4 ± 1.4, 34.2 ± 2.5, and 11.5 ± 0.1 µg/ml for, respectively, *L. amazonensis*, *L. braziliensis*, *L. chagasi,* and *L. major*. For this latter species, from the Old World, the susceptibility to both Bulgarian extracts was even higher in comparison with pentamidine.



Effect of the propolis extracts Et-Blg, Ket-Blg, and Et-Bra on promastigote forms of *Leishmania* after 1 day at 26°C - a: *Leishmania amazonensis*; b: *L. braziliensis*; c: *L. chagasi*; d: *Leishmania major*. The percent of inhibition corresponds to the mean value ± standard deviation of at least four experiments.

TABLE II

Values of IC_{50}/1 day, expressed in mg/ml, for the activity of the propolis extracts on promastigote forms of different species of *Leishmania*

Extract	*L. amazonensis*	*L. braziliensis*	*L. chagasi*	*L. major*
Et-Blg	29.3 ± 7.6 [a]	142.2 ± 14.5	53.9 ± 7.3	7.2 ± 1.4
Ket-Blg	26.9 ± 6.4	nd	41.3 ± 0.9	2.8 ± 0.3
Et-Bra	229.3 ± 24.2	48.6 ± 3.7	49.9 ± 4.4	48.2 ± 3.6

a: mean ± standard deviation of at least four independent experiments; nd: not determined.

DISCUSSION

Propolis samples from Bulgaria and Brazil present striking differences in their chemical composition (Marcucci & Bankova 1999). In temperate zones, the main sources of propolis are bud exudates from different poplar trees (*Populus* spp.) and the microbicidal activity is associated with the presence of flavonoids and derivatives of caffeic acid (Kujungiev et al. 1999, Hegazi et al. 2000, Sawaya et al. 2002, Bankova 2005). On the other hand, in Tropical regions, like Brazil, other plant sources are presented to the bees, such as *Araucaria*, *Baccharis*, and *Eucalyptus*, leading to samples with a totally distinct composition, in which flavonoids are usually present in very small quantities and, the main bioactive compounds are phenolic acids, specific terpenoids, and prenylated derivatives (Kumazawa et al. 2003, Trusheva et al. 2004, Mishima et al. 2005, Teixeira et al. 2005, Pisco et al. 2006).The chemical composition of the three propolis extracts was established by HT-HRGC-MS (Prytzyk et al. 2003, Salomão et al. 2004). This technique, for separation of complex mixtures and identification of high molecular weight compounds, is an excellent alternative to classical analytical phytochemistry and a potent tool for the rapid evaluation of the composition of crude natural products, such as propolis (Pereira et al. 2000).

Et-Blg presents a predominance of flavonoids (pinostrobin, pinocembrin, chrysin, and a series of pinobanksins) corresponding to 35.9% of the total identified area, besides aromatic acids and esters and fatty acids. Ket-Blg shares several constituents with Et-Blg, possessing, however, higher levels of monossacharides, and no major difference was observed between the activities of both Bulgarian extracts against the species of *Leishmania* assayed. The composition of Et-Bra was totally distinct from that of the other two extracts, with a very low content of flavonoids (1.1%), corresponding to pinostrobin, besides the presence of amyrins, diethyl methyl succinate, isobutylquinoline, and geranyl acetal. The higher activity of Et-Blg against the bacteria *Staphylococcus aureus*, *Neisseria meningitides*, and *Streptococcus pneumoniae*, when compared to that of Et-Bra, was previously associated with the high content of flavonoids in the Bulgarian extract (Salomão et al. 2004). In the present work, a higher effect of Et-Blg was also observed for *L. amazonensis* (7.8X) and *L. major* (6.7X). However, against *L. chagasi* both extracts presented similar, while for *L. braziliensis* Et-Bra was about three times more active, indicating that other phenolic compounds, besides flavonoids, and also amyrins are probably the compounds involved in the leishmanicidal activity. As the composition of the extracts have been already determined, the next step will be the analyses of individual components and their combinations on each *Leishmania* species, such as the flavonoids found mainly in the Bulgarian extracts and amyrins found only in Et-Bra.

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