Antitrypanosomal Activity of Brazilian Propolis from *Apis mellifera*

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Extracts from different samples of Brazilian propolis were obtained by Soxhlet extraction or maceration at room temperature using ethanol, water, and accombination of both solvents. Analysis of their composition using HPLC revealed that no major differences were seen when a propolis sample was subject to different extraction methods. The activity of the 15 extracts was assayed against bloodstream trypomastigotes of *Trypanosoma cruzi*, the etiologic agent of Chagas' disease. Multivariate analysis was applied to evaluate the efficiency of the different extracts and the trypanocidal activity. The extracts could be divided into two groups. In the first, in which, extracts were obtained by reflux in Soxhlet using 100% ethanol, there was a lower content of bioactive compounds and consequently lower trypanocidal activity. Extract 136-Et100 stands out in this group, since it had the highest levels of bioactive compounds together with highest activity against the parasite when compared with all other extracts. The second group comprises extracts with intermediate levels of bioactive compounds and higher activity against *T. cruzi*.

Key words Brazilian propolis; extraction procedure; chemical composition; HPLC; *Trypanosoma cruzi*

Propolis, a bee product, has various biological and therapeutic activities, which have been associated with the presence of flavonoids and aromatic acids and esters.[1)] In a recent review,[2)] we discussed the remarkable differences between propolis from tropical and temperate zones. In Brazilian samples other classes of bioactive components, instead of flavonoids, have been described, such as prenylated phenolic acids and specific terpenoids. It is also known that the biological activities of a sample depend on the extraction method employed.

In the present study we analyzed the chemical composition of different extracts from Brazilian propolis using HPLC and their activity against *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, which is endemic in Latin America with about 16 million of people infected.[3)] There is a current effort to investigate alternative synthetic and natural drugs for both the clinical treatment of the disease and the chemoprophylaxis of blood.[4)] In this context, propolis has been investigated by our group.[5—8)]

Results and Discussion

Samples of propolis from *Apis mellifera* from different regions of Brazil were collected and submitted to different procedures (Table 1). The percentage of humidity of the samples was in the range of 5% and 10%, in accordance with Brazilian law,[9)] and the yield of the Et100 extracts between 40% and 70%.

Comparison of the composition of the extracts 136-Et100,

Table 1. Propolis Samples and Extraction Procedures

No.	Sample		Extract	
	Locale[a)]	Date	Conditions	Code
110	Pedra Bela, SP	05/96	Soxhlet,[b)] absolute ethanol	110-Et100
112	Maringá, PR	05/09	Soxhlet, absolute ethanol	112-Et100
118	Salto de Pirapora, SP	12/96	Soxhlet, absolute ethanol	118-Et100
124	Amparo, SP	10/96	Soxhlet, absolute ethanol	124-Et100
125	Amparo, SP	10/96	Soxhlet, absolute ethanol	125-Et100
126	Itu, SP	02/97	Soxhlet, absolute ethanol	126-Et100
133	Brag. Paulista, SP	08/97	Soxhlet, 70% ethanol in water	133-Et70
			Soxhlet, 50% ethanol in water	133-Et50
			Soxhlet, absolute ethanol	133-Wt100
134	Jarinu, SP	08/94	Soxhlet, absolute ethanol	134-Et100[b)]
			Soxhlet, absolute ethanol	134-Et100[c)]
136	Jarinu, SP	03/98	Soxhlet, absolute ethanol	136-Et100
			Soxhlet, absolute ethanol	136-Wt100
			Maceration, 96° GL,[d)] in presence of light	136-Mac[L+]
			Maceration, 96° GL, in absence of light	136-Mac[L−]

a) States of Brazil: SP (São Paulo), MG (Minas Gerais), RJ (Rio de Janeiro), PR (Paraná). *b*) Resin extracted after trituration. *c*) Resin extracted as small pieces. *d*) GL, grain alcohol. Soxhlet extractions were performed for 1 d and maceration for 20 d.

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Table 2. Determination Using HPLC of the Composition of Extracts of Different Propolis Samples (in mg/g of Dried Extract) from Different Regions of Brazil Obtained by Extraction in Soxhlet for 1 d Using Absolute Ethanol

	110-Et100	112-Et100	118-Et100	124-Et100	125-Et100	126-Et100	134-Et100[b)]	134-Et100[c)]
2,2-Dimethyl-6-carboxyethenyl-2*H*-1-benzopyran (DCBEN)[a)]	7.6	9.0	1.2	0.9	1.8	0.9	—	—
2,2-Dimethyl-8-prenyl-2*H*-1-benzopyran-6-propenoic acid (DPB)	8.3	13.8	20.5	12.1	14.0	9.0	18.3	17.5
3,5-Diprenyl-4-hydroxycinnamic acid (D)	16.7	9.6	20.2	13.1	14.4	15.4	23.1	24.0
3-Methoxy-4-hydroxycinnamaldehyde (G2)	—	0.99	—	—	—	—	—	—
3-Prenyl-4-hydroxycinnamic acid (B)	8.2	7.3	11.4	5.5	7.4	10.1	1.3	12.7
Caffeic acid (Cf)	0.8	0.0	1.0	0.9	0.8	0.9	0.7	1.1
Caffeic acid derivative 1 (Cf1)	3.5	1.5	0.5	1.7	1.8	1.0	0.2	0.5
Caffeic acid derivative 2 (Cf2)	0.4	1.1	2.3	1.4	1.6	2.5	0.7	—
Caffeic acid derivative 3 (Cf3)	0.3	—	0.9	0.7	0.6	1.3	—	—
Caffeic acid derivative 4 (Cf4)	0.5	—	1.4	0.8	0.7	1.3	—	—
Caffeic acid derivative 5 (Cf5)	—	—	1.0	—	—	—	—	—
Cinnamic acid derivative (Cin)	24.4	17.3	24.3	18.2	20.5	22.9	—	—
Kaempferol derivative 1 (Kae)	7.8	—	17.5	11.4	13.3	16.9	13.9	14.8
p-Coumaric acid (p-Cum)	11.5	6.1	13.7	7.3	9.1	17.1	3.0	5.3
Ferulic acid (Fer)	0.8	—	0.6	0.4	0.5	0.7	0.1	0.1
Pinobanksin (Pk)	8.5	—	13.7	10.5	2.0	11.4	5.4	6.6
Total (mg/g dried extract)	215.4	183.8	257.0	213.7	95.4	245.9	86.8	96.5

a) Abbreviations in parentheses were employed in the multivariate analysis (Fig. 1). *b*) Resin extracted after trituration. *c*) Resin extracted as small pieces.

Table 3. Determination Using HPLC of the Composition of Propolis Extracts from Samples #133 and #136 (in mg/g of Dried Extract) from Different Regions of Brazil Prepared by Different Methods

	133-Et70	133-Et50	133-Wt100	136-Et100	136-Wt100	136-Mac[L+]	136- Mac[L−]
2,2-Dimethyl-6-carboxyethenyl-2*H*-1-benzopyran (DCBEN)[a)]	2.2	1.8	1.0	—	—	0.9	—
2,2-Dimethyl-8-prenyl-2*H*-1-benzopyran-6-propenoic acid (DPB)	21.0	10.1	9.6	17.8	18.8	17.0	17.8
3,5-Diprenyl-4-hydroxycinnamic acid (D)	24.7	21.2	14.5	25.5	27.2	25.0	27.0
3-Methoxy-4-hydroxycinnamaldehyde (G2)	—	—	—	—	—	—	—
3-Prenyl-4-hydroxycinnamic acid (B)	17.8	22.7	7.2	18.2	20.2	17.7	19.6
Caffeic acid (Cf)	1.1	2.5	0.6	2.0	1.8	1.4	1.4
Caffeic acid derivative 1 (Cf1)	0.8	2.3	4.6	1.0	0.1	0.9	0.1
Caffeic acid derivative 2 (Cf2)	2.6	5.2	1.8	—	0.7	0.2	0.6
Caffeic acid derivative 3 (Cf3)	1.9	0.5	—	—	—	—	—
Caffeic acid derivative 4 (Cf4)	—	0.5	—	—	—	—	—
Caffeic acid derivative 5 (Cf5)	—	—	—	—	—	—	—
Cinnamic acid derivative (Cin)	35.7	8.6	15.1	29.9	21.3	28.6	20.3
p-Coumaric acid (p-Cum)	14.7	27.3	14.8	20.2	8.3	10.5	6.7
Kaempferol derivative 1 (Kae)	16.8	14.4	6.8	11.6	19.2	19.3	20.8
Ferulic acid (Fer)	tr[b)]	tr	2.3	tr	tr	tr	tr
Pinobanksin (Pk)	3.2	2.22	—	12.4	15.1	11.8	12.8
Total (mg/g dried extract)	152.0	127.8	81.5	152.0	150.0	144.4	140.9

a) Abbreviations in parentheses were employed in the multivariate analysis (Fig. 1). *b*) Compounds found in trace amounts.

136-Mac[L+], and 136-Mac[L−] (Table 2) shows that the use of temperature (Soxhlet method) does not destroy the phenolic components. The compounds present in higher amounts in all extracts were 3,5-diprenyl-4-hydroxycinnamic acid (D), a known propolis component with different biological activities;[10—12)] 2,2-dimethyl-8-prenyl-2*H*-1-benzopyran-6-propenoic acid (DPB); 3-prenyl-4-hydroxycinnamic acid (B); a cinnamic acid derivative (Cin), *p*-coumaric acid (*p*-Cum); a kaempferol derivative (Kae); and pinobanksin (Pk). Although the yield of 136-Et100 (53.6%) was higher than that of 136-Wt100 (14.2%), their compositions were similar with the major components in both extracts being D, Cin, B, DPB, Kae, *p*-Cum, and Pk (Table 3). Between 134-Et100 and 136-Et100 from two samples collected in the same region, but with different vegetation, the main difference was the presence of Cin only in the latter extract. Although all extracts showed lower activity against *T. cruzi* than the standard compound, those with higher activity (ED_{50}/24 h$<$0.6 mg/ml) were obtained by the different methods from samples #133, #134 and #136 with exception of extract 133-Wt100 (Table 4). The extracts with lower activity (ED_{50}/24 h$>$1 mg/ml) included those obtained in absolute ethanol from samples #118, #125, #12 and, #126 and 133-Wt100. Multivariate analysis was used to evaluate the efficiency of the different extracts and the trypanocidal activity. Two groups were clearly observed. In the first (G1), located in the negative quadrant in PC1 (Fig. 1A), most of the extracts were obtained by reflux in Soxhlet using 100% ethanol, with lower content of DPB, D, G2, B, Fer, Pk, and Cf5, with higher values of ED_{50}/24 h (Fig. 1B), meaning lower activity against *T. cruzi*. The second group (G2), located in the positive quadrant in PC1 (Fig. 1A) comprises extracts obtained from the samples 133, 134, and 136 obtained through different procedures (Table 1), which had intermediate levels of bioactive compounds (Fig. 1B) and higher biological activity than G1. No major differences in the chemical composition were ob-

Table 4. Trypanocidal Activity of Different Extracts against *Trypanosoma cruzi*

Sample No.	Extract	ED_{50}/24 h (μg/ml)	Index of variation [a]
110	110-Et100	881.1±126.1	0.212
112	112-Et100	811.8±111.7	0.230
118	118-Et100	1107.0±143.4	0.169
124	124-Et100	1437.0±43.0	0.130
125	125-Et100	1055.0±125.3	0.177
126	126-Et100	1073.3±222.5	0.174
133	133-Et70	505.8±61.6	0.370
	133-Et/Wt	608.5±13.5	0.307
	133-Wt	1149.6±216.9	0.163
134	134-Et100[b]	537.3±44.7	0.348
	134-Et100[c]	503.1±27.4	0.372
136	136-Et100	421.0±26.5	0.444
	136-Wt	595.7±33.6	0.314
	136-Mac[L+]	535.0±60.0	0.350
	136-Mac[L−]	556.0±69.5	0.336

a) Index of variation, obtained by the ratio of ED_{50}/24 h of the standard crystal violet (187.0±21.0 μg/ml) in relation to the corresponding value of the assayed extract. *b*) Resin extracted after trituration. *c*) Resin extracted as small pieces.

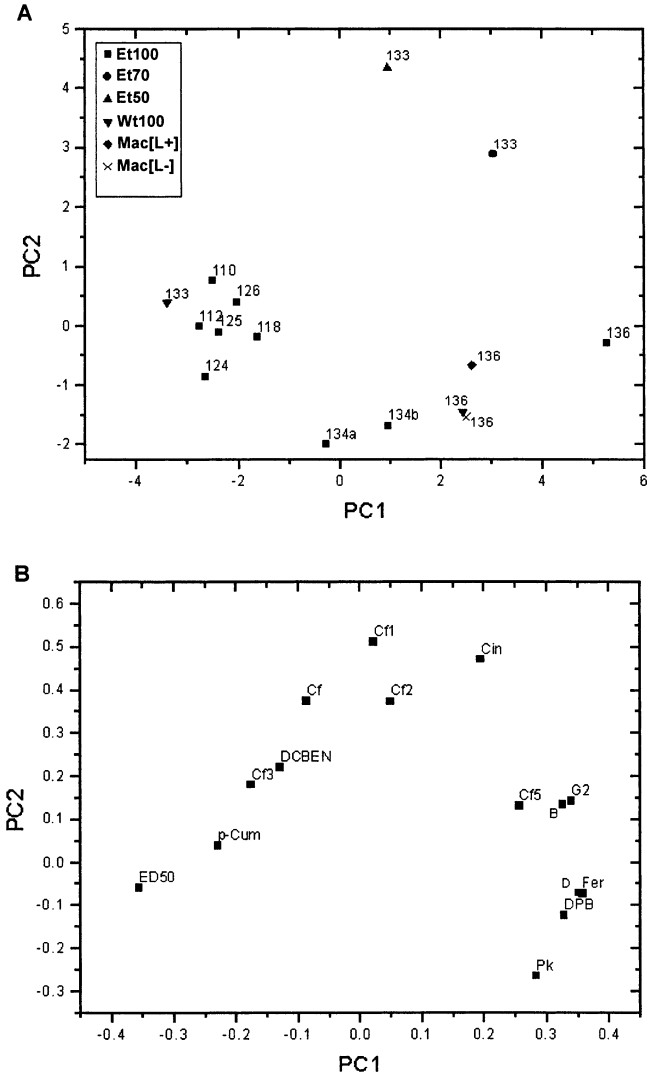

Fig. 1. Principal Components of: (A) Propolis Samples (Scores); and (B) Identified Compounds and ED_{50}/24 h in Propolis Extracts (Loadings)

For abbreviations see Tables 1, 2, and 3.

tained when different extraction methods were used. 136-Et100 was notable in G1, since it had the highest levels of these bioactive compounds together with the highest activity against the parasite when compared with all other extracts.

Experimental

Propolis Samples and Extraction Procedures The samples obtained from *A. mellifera* were submitted to two procedures: Soxhlet extraction under reflux for 1 d using absolute ethanol (Et100), at 70% (Et70) or 50% in water (Et50), or water (Wt100); and maceration at room temperature for 20 d using 96° GL grain alcohol in the presence (Mac-[L+]) or absence (Mac-[L−]) of light (Table 1). The humidity levels of the samples were determined at 60 °C in a humidity analyzer (MA30, Sartorius, Germany).

Chemical Composition of the Extracts The extracts were analyzed with HPLC (D-7000 Merck-Hitachi, Germany) on a olumn equipped with a pump and a diode array detector (L-3000, Merck-Hitachi) as previously described.[12] Detection of the components was monitored at 280 and 340 nm, and standard compounds were cochromatographed with the extracts. ^{1}H- and ^{13}C-NMR spectra were recorded using a Varian Gemini 300 spectrophotometer, and the mass spectra were obtained in a Hewlett-Packard apparatus (model 5890 Series II Plus).

Trypanocidal Activity Stock solution of the extracts was prepared in dimethylsulfoxide. This solvent at concentrations up to 4% has no deleterious effect on the parasites.[13] Trypomastigotes of the Y strain of *T. cruzi* were obtained from the blood of infected Swiss mice and were ressuspended (1×10^7 cells/ml) in Dulbecco's modified Eagle medium (DME) containing 10% blood. In 96-well microplates, 100 μl of this suspension was added to the same volume of the extracts diluted in DME at twice the desired final concentrations. After 24 h at 4 °C, the parasites in each well were counted and the ED_{50} value was calculated, corresponding to the extract concentration that lysed 50% of the parasites.

Statistical Analysis In the multivariate analysis the lines represent the propolis extracts while each column represents the variables, chemical composition, and ED_{50}/24 h value. Each extract is a point "p" in a multidimensional space (the same as the variable number), and the number of extracts is represented as "n" points in the dimensional space.

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