

## Original Article

## Brazilian Propolis: Correlation Between Chemical Composition and Antimicrobial Activity

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The chemical composition of ethanol extracts from samples of Brazilian propolis (EEPs) determined by HPLC and their activity against *Trypanosoma cruzi*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Candida albicans*, *Sporothrix schenckii* and *Paracoccidioides brasiliensis* were determined. Based on the predominant botanical origin in the region of samples' collection, the 10 extracts were separated into three groups: A (*B. dracunculifolia* + *Araucaria* spp), B (*B. dracunculifolia*) and C (*Araucaria* spp). Analysis by the multiple regression of all the extracts together showed a positive correlation, higher concentrations leading to higher biological effect, of *S. aureus* with *p*-coumaric acid (PCUM) and 3-(4-hydroxy-3-(oxo-butenyl)-phenylacrylic acid (DHCA1) and of trypomastigotes of *T. cruzi* with 3,5-diprenyl-4-hydroxycinnamic acid derivative 4 (DHCA4) and 2,2-dimethyl-6-carboxyethyl-2H-1-benzopyran (DCBEN). When the same approach was employed for each group, due to the small number of observations, the statistical test gave unreliable results. However, an overall analysis revealed for group A an association of *S. aureus* with caffeic acid (CAF) and dicaffeoylquinic acid 3 (CAFQ3), of *S. pneumoniae* with CAFQ3 and monocaffeoylquinic acid 2 (CAFQ2) and of *T. cruzi* also with CAFQ3. For group B, a higher activity against *S. pneumoniae* was associated DCBEN and for *T. cruzi* with CAF. For group C no association was observed between the antimicrobial effect and any component of the extracts. The present study reinforces the relevance of PCUM and derivatives, especially prenylated ones and also of caffeoylquinic acids, on the biological activity of Brazilian propolis.

**Keywords:** bactericidal activity – chemical composition – fungicidal activity – propolis – statistical analysis – *Trypanosoma cruzi*

### Introduction

Propolis presents a complex composition depending basically on the plant sources accessible to the bees, possessing a variety of biological and pharmacologic

activities, attracting the interest of an increasing number of researchers (1). Brazilian samples present striking differences in their chemical composition when compared with samples from temperate zones (2). Besides, differences are also found among tropical samples depending on the local flora at the site of collection (3). As examples we found the Brazilian green or Alecrim propolis, originated from *Baccharis dracunculifolia* (Asteraceae) (4–6), the Cuban red propolis from *Clusia nemorosa* (Clusiaceae) (7) and more recently the red propolis

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collected in the North region of Brazil (8). While the microbicidal activity of European propolis has been associated with the presence of flavonoids and derivatives of caffeic acid (9,10), in the case of tropical samples, the main bioactive compounds are phenolic acids and prenylated derivatives (11).

Due to the increasing interest in the characteristics of Brazilian propolis, we undertook a study using samples collected in different regions, aiming to determine their effect against *Trypanosoma cruzi* and different species of bacteria and fungi. *Trypanosoma cruzi* is the etiologic agent of Chagas' disease, an important Public Health problem in Latin America, the treatment of which is still inadequate since the available drug benznidazole, a nitroderivative, causes severe side effects and its efficacy for chronic patients is questionable (12,13). *Streptococcus pneumoniae* and *Staphylococcus aureus* were assayed since they can colonize the human nasopharynx and represent potential agents of several diseases (14,15). *Klebsiella pneumoniae* was also included in the present work. *Candida albicans*, *Sporothrix schenckii* and *Paracoccidioides brasiliensis*, was selected due to their importance as etiologic agents of mycosis in Brazil (16,17).

## Materials and Methods

### Propolis Extracts

Eleven samples were collected in different regions in Brazil and 30 g of each resin were triturated and extracted with 100 ml ethanol (Merck Darmstadt, Germany) for 20 days in the dark. Afterwards, the material was cooled for precipitation of waxes, which were removed by filtration and the extract was dried under reduced pressure at 60°C. Their yield varied between 40 and 60%. Stock solutions of the eleven extracts were prepared in dimethylsulfoxide (DMSO). Based on the predominant botanical origin in the region of samples collection they were separated as: group A (*B. dracunculifolia* plus *Araucaria* spp, State of Paraná): EEP-01 to EEP-04; group B (*B. dracunculifolia*, State of Minas Gerais): EEP-05 to EEP-07 and, group C (*Araucaria* spp, State of Paraná) EEP-08 to EEP-10. Another extract obtained from a red propolis collected in the State of Alagoas, EEP-11 (*Schinus terebinthifolius*, *Diploptropis incexis* and *Manilkara huberi*) was also included for the biological assays (8).

### HPLC-MS Analysis

EEP-1 to EEP-10 were analyzed by HPLC (D-7000 Merck-Hitachi, Germany) equipped with a pump (model L-6200, Merck-Hitachi, Germany) and a diode array detector (L-3000, Merck-Hitachi, Germany) as previously

described (18). Detection of the components was monitored at 280 and 340 nm and standard compounds were co-chromatographed with the extracts. <sup>1</sup>H-NMR and <sup>13</sup>C-NMR were recorded using a Varian Gemini 300 spectrophotometer and the mass spectra obtained in a Hewlett-Packard apparatus (model 5890 Series II Plus).

### Fungicidal Activity

The agar cup method was used (19) to assay the effect of the EEPs against *C. albicans* (Ca IOC-3781), *S. schenckii* (Ss IOC-2832) and *P. brasiliensis* (Pb IOC-3698). Yeast cells of *C. albicans* were grown in Sabouraud medium (Difco Laboratories, Detroit, MI, USA) at 25°C and yeast-like cells of *S. schenckii* and *P. brasiliensis* were grown in brain heart infusion (BHI) (Difco) and peptone-yeast extract-glucose (PYG) (Difco) at 36°C. The fungal cells were washed in phosphate-buffered saline (PBS, pH 7.2) counted in a hemocytometer and the concentration adjusted to  $3.4 \times 10^7$  cells ml<sup>-1</sup>. A plate of a suitable sterile agar, poured to a depth of 4 mm, was allowed to set and a single cup (15 mm diameter), cut from the centre of the plate. The cell suspensions (50 µl) were streaked radially from the cup to the edge of the plate on the suitable agar medium surface for each fungus and the cup filled with 200 µl of EEP (2–16 mg ml<sup>-1</sup>). The plates were incubated for 3 days at 25°C for *C. albicans* and for 6 days at 36°C for both *S. schenckii* and *P. brasiliensis*. The antifungal activity was measured as the diameter of the inhibitory zones. Controls were performed with DMSO, the solvent of the stock solutions of the extracts; no inhibition zone was observed. Diameters of less than 15 mm were considered as lack of activity.

### Bactericidal Activity

The minimal inhibitory concentration (MIC) was determined by means of the broth microdilution method described by the National Committee for Clinical Laboratory Standards (20), followed by subculture. *S. pneumoniae* (ATCC 49619), *S. aureus* (ATCC 25923) and *K. pneumoniae* (ATCC 70603) were grown in Mueller-Hinton agar supplemented with 5% sheep blood and incubated for 18 h at 37°C. *S. pneumoniae* was incubated in a 5% CO<sub>2</sub> atmosphere. Bacterium inoculum was prepared in Mueller-Hinton Broth (Oxoid Ltd Basingstone, Hampshire, England), adjusted to 0.5 McFarland turbidity standard (10<sup>8</sup> CFU ml<sup>-1</sup>) and then diluted 1:10. This suspension (100 µl) was added to equal volume of the EEP, previously prepared by 2-fold serial dilutions in 96-well plates. After 18 h, subculture (10 µl) from each well was made in the same conditions. MIC was considered the lowest concentration of the extract that yields negatives subcultures. The final concentration

of DMSO in the assays did not interfere with proliferation of the bacteria.

### Trypanocidal Activity

The Y strain of *T. cruzi* was used (21). Bloodstream trypomastigotes were obtained at the peak of parasitemia from infected albino mice, isolated by differential centrifugation and resuspended with Dulbecco's modified Eagle medium (DME) to a parasite concentration of  $10 \times 10^6$  cells ml<sup>-1</sup> in the presence of 10% blood. This suspension (100 µl) was added to the same volume of the EEP, previously prepared at twice the desired concentrations also in DME (0.025–4 mg ml<sup>-1</sup>) in 96-well plates and then incubated at 4°C. Trypomastigote concentration in the wells was  $5 \times 10^6$  cells ml<sup>-1</sup> containing 5% blood (22). Cell counts were performed after 24 h of incubation and the activity of the extracts was expressed as IC<sub>50</sub> values, corresponding to the concentration that lysed 50% of the parasites. Experiments showed that in concentrations up to 0.2%, DMSO had no deleterious effect on the parasites.

### Statistical Analysis

The correlation of the trypanocidal (IC<sub>50</sub>) or bactericidal (MIC) activity with the composition of each extract determined by HPLC, expressed in mg g<sup>-1</sup> of dried extract (Table 1), was performed by the method of analysis of multiple regressions using the software SPSS for Windows 8.0. Positive correlation means that higher concentrations of a specific component are associated with higher antimicrobial activity, while negative correlation, means that at lower concentrations a higher activity was achieved. It is important to note that, for a given extract, lower values of the parameters IC<sub>50</sub> and MIC indicate a higher activity. The comparison between the IC<sub>50</sub> values for *T. cruzi* was performed by ANOVA followed by the Student-Newman-Keuls test ( $P < 0.05$ ).

## Results

### Chemical Composition and Fungicidal Activity of the Extracts

The chemical composition of each EEP quantified by HPLC is presented in Table 1. In order to better visualize the distribution of the components in groups A, B and C based on the predominant vegetation the concentration of each component was included in each group expressed as mean ± standard deviation (Fig. 1).

The effect of the extracts against *C. albicans*, *S. schenckii* and *P. brasiliensis* is presented in Fig. 2, being the latter species the most susceptible to propolis. No statistical correlation was performed; the values were

expressed in diameter of inhibition zone expressed in mm, for four different concentrations of each extract.

### Bactericidal Activity and Correlation With the Chemical Composition

The MIC range for *S. pneumoniae* was 0.2–0.8 µg ml<sup>-1</sup> and for *S. aureus*, 1.6–52.4 µg ml<sup>-1</sup> (Fig. 3). All the extracts were inactive against *K. pneumoniae*. Analysis by the method of multiple regression of all the extracts together showed for *S. aureus* a positive correlation for *p*-coumaric acid (PCUM) ( $P = 0.0002$ ) and 3-(4-hydroxy-3-(oxo-butenyl)-phenylacrylic acid (DHCA1) ( $P = 0.006$ ), meaning higher concentrations of each compound led to higher bactericidal effect and a negative one for 3-methoxy-4-hydroxycinnamaldehyde (G2) ( $P = 0.001$ ) and vanillin (VAN) ( $P = 0.026$ ). While for *S. pneumoniae*, a negative correlation between the activity and the concentration of G2 ( $P = 0.024$ ) was observed. When the same approach was employed for each group, since the number of extracts was four, three and three, for groups A, B and C, respectively, the multiple regression analysis gave unreliable results. However, an overall analysis revealed for group A (*B. dracunculifolia* + *Araucaria* spp) there was an association of concentrations of caffeic acid (CAF) and dicaffeoylquinic acid 3 (CAFQ3) with the activity against *S. aureus*, while for *S. pneumoniae* such association occurred and between CAFQ3 and mono-caffeoylquinic acid 2 (CAFQ2). In group B (*B. dracunculifolia*) higher activity against *S. pneumoniae* was associated with 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran (DCBEN). For group C (*Araucaria* spp) no association was observed between the bactericidal effect and any component of the extracts.

### Trypanocidal Activity and Correlation With the Chemical Composition

The activity of the extracts against bloodstream trypomastigote forms of *T. cruzi* is shown in Fig. 4. For each group the order of decreasing activity ( $P < 0.05$ ) was: EEP-01 > EEP-02, EEP-04 > EEP-03, for group A; EEP-07 > EEP-05, EEP-06, for group B and EEP-9 > EEP-8 > EEP-10, for group C.

The statistical analysis of all the extracts together and the activity against *T. cruzi* showed a positive correlation with compounds 3,5-diprenyl-4-hydroxycinnamic acid derivative 4 (DHCA4) ( $P = 0.004$ ) and DCBEN ( $P = 0.039$ ) and a negative one for 2-[1-hydroxymethyl]-vinyl-6-acetyl-5-hydroxycumarane (I) ( $P = 0.00008$ ) and L2 ( $P = 0.006$ ), a still non-identified compound. As stated earlier the multiple regression analysis could not be applied for each separated group of extracts, but there was a positive association of the trypanocidal activity with dicaffeoylquinic acid 3 (CAFQ3) for group A and

**Table 1.** Chemical composition of ethanol extracts of Brazilian propolis samples (mg g<sup>-1</sup> of dried extract)

Compounds	Group A				Group B			Group C		
	<i>B. dracunculifolia</i> + <i>Araucaria</i> spp				<i>B. dracunculifolia</i>			<i>Araucaria</i> spp		
	EEP-01	EEP-02	EEP-03	EEP-04	EEP-05	EEP-06	EEP-07	EEP-08	EEP-09	EEP-10
3-Prenyl-4-hydroxycinnamic acid (PHCA)	8.31	2.69	1.42	7.35	16.82	19.95	13.23	0.00	0.00	0.00
2,2-Dimethyl-6-carboxyethyl-2H-1-benzopyran (DCBEN)	10.02	3.48	1.54	7.62	0.00	4.80	0.00	0.00	0.00	0.00
2,2-Dimethyl-8-prenyl-2H-1-benzopyran-6-propenoic acid (DPB)	33.75	11.39	6.41	23.31	27.52	28.66	19.58	0.00	0.00	0.00
3,5-Diprenyl-4-hydroxycinnamic acid (DHCA)	20.80	5.03	2.38	26.70	36.13	38.37	33.04	0.00	0.00	0.00
3-(4-Hydroxy-3-(oxo-butenyl)-phenylacrylic acid (DHCA1)	3.10	1.67	0.00	0.95	1.35	1.30	0.83	0.00	0.00	0.00
3-Prenyl-4-(2-methoxypropionyloxy)cinnamic acid (DHCA2)	2.97	1.29	0.00	2.05	0.06	0.75	2.08	0.00	0.00	0.00
3,5-Dihydroxy-5-prenylcinnamic (DHCA3)	3.75	1.58	0.00	1.62	0.48	0.08	1.41	0.00	0.00	0.00
3,5-Diprenyl-4-hydroxycinnamic acid derivative 4 (DHCA4)	2.15	0.08	0.00	1.61	1.37	3.59	5.84	0.00	0.00	0.00
3,5-Diprenyl-4-hydroxycinnamic acid derivative 5 (DHCA5)	2.96	1.18	0.00	2.43	1.48	0.46	2.69	0.00	0.00	0.00
3,5-Diprenyl-4-hydroxycinnamic acid derivative 6 (DHCA6)	3.71	1.31	0.00	2.87	3.67	3.07	3.37	0.00	0.00	0.00
3,5-Diprenyl-4-hydroxycinnamic acid derivative 7 (DHCA7)	0.00	0.00	0.00	0.00	2.43	0.06	2.28	0.00	0.00	0.00
3,5-Diprenyl-4-hydroxycinnamic acid derivative 8 (DHCA8)	0.00	0.00	0.00	0.00	3.09	7.20	3.73	0.00	0.00	0.00
3,5-Diprenyl-4-hydroxycinnamic acid derivative 9 (DHCA9)	0.00	0.00	0.00	0.00	9.25	3.75	2.64	0.00	0.00	0.00
3-Hydroxy-2,2-dimethyl-8-prenyl-2H-1-benzopyran-6-propenoic acid (DHCA10)	0.00	0.00	0.00	0.00	4.95	5.81	0.00	0.00	0.00	0.00
3,5-Diprenyl-4-hydroxycinnamic acid derivative 11 (DHCA11)	0.00	0.00	0.00	0.00	5.86	4.85	0.00	0.00	0.00	0.00
3,5-Diprenyl-4-hydroxycinnamic acid derivative 12 (DHCA12)	0.00	0.00	0.00	0.00	5.24	0.00	0.00	0.00	0.00	0.00
3,5-Diprenyl-4-hydroxycinnamic acid derivative 13 (DHCA13)	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00
3,5-Diprenyl-4-hydroxycinnamic acid derivative 14 (DHCA14)	0.00	0.00	0.00	0.00	3.47	0.00	0.00	0.00	0.00	0.00
<i>p</i> -Coumaric acid (PCUM)	19.07	9.56	0.79	15.42	33.48	32.47	18.81	0.00	0.00	0.00
Caffeic acid (CAF)	2.39	1.54	0.00	0.00	1.35	1.55	2.94	0.00	0.00	0.00
Cinnamic acid derivative 1 (CIN1)	1.58	1.01	0.00	7.07	0.00	0.00	0.00	0.00	5.28	0.00
3-Prenyl-4-dihydrocinnamoyl-oxy-cinnamic acid (CIN2)	0.00	0.00	0.00	1.42	0.00	0.00	0.00	0.00	0.00	0.00
Benzoic acid (BENZ)	0.00	0.00	6.53	0.00	0.00	0.00	0.00	0.00	24.74	0.00
Monocaffeoylquinic acid 1 (CAFQ1)	7.35	6.35	0.00	6.05	9.79	13.59	4.31	0.00	0.00	0.00
Monocaffeoylquinic acid 2 (CAFQ2)	1.57	1.49	0.00	0.00	4.06	0.00	0.00	0.00	0.00	0.00
Dicaffeoylquinic acid 1 (CAFQ3)	2.79	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dicaffeoylquinic acid 2 (CAFQ4)	0.61	0.50	0.13	0.37	0.99	1.85	0.63	0.00	0.00	0.00
Pinobanksin (PK)	0.00	0.00	0.00	0.00	45.61	34.92	26.00	0.00	0.00	0.00
Kaempferol (KAEMP)	0.00	0.00	0.00	3.91	0.00	0.00	0.00	0.00	1.60	0.00
Kaempferide (KAEMP1)	20.80	0.00	0.00	11.76	37.05	34.08	33.04	0.00	0.00	0.00
Crysin (CRYS)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.24	0.00

(continued)



Table 1. Continued

Compounds	Group A				Group B			Group C		
	<i>B. dracunculifolia</i> + <i>Araucaria</i> spp				<i>B. dracunculifolia</i>			<i>Araucaria</i> spp		
	EEP-01	EEP-02	EEP-03	EEP-04	EEP-05	EEP-06	EEP-07	EEP-08	EEP-09	EEP-10
3-Methoxy-4-hydroxy-benzaldehyde (VAN)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.48	8.76	22.82
3-Methoxy-4-hydroxycinnamaldehyde (G2)	0.00	0.00	1.01	5.09	0.00	0.00	0.00	0.72	0.32	2.95
2-[1-Hydroxymethyl]vinyl-6-acetyl-5-hydroxycumarane (I)	0.00	0.00	2.31	0.00	0.00	0.00	0.00	3.86	0.00	6.19
Galangin (GAL)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	11.50	0.00
Compound E (E)	48.52	15.48	0.00	43.46	78.00	77.81	56.22	0.00	0.00	0.00
Compound H (H)	0.00	0.00	20.60	0.00	0.00	0.00	0.00	11.61	97.38	17.68
Compound L2 (L2)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.67	0.00	3.61

with CAF for group B. For group C (*Araucaria* spp) no association was observed between the effect against trypomastigote forms and any component of the extracts.

## Discussion

Propolis samples from tropical zones, such as Brazil with its vast biodiversity, have become a subject of increasing scientific and economic attention. From Brazilian propolis, several bioactive derivatives of hydroxycinnamic, caffeoylquinic and diterpenic acids besides benzofuranes and benzopyranes have already been characterized (18,23–25).

As typical of Brazilian propolis (2), most of the compounds present in the EEPs were phenolics, being present in high content hydroxycinnamic acids, such as PCUM and CAF and their prenylated derivatives. For group A (*B. dracunculifolia* + *Araucaria* spp), the extracts are typified as BRP(PR) based on the main bioactive components DHCA, DCBEN, 3-prenyl-4-hydroxycinnamic acid (PHCA) and 2,2-dimethyl-8-prenyl-2H-1-benzopyran-6-propenoic acid (DPB) (26). For group B (*Araucaria* spp), they are typified as BRP-1 (26) with the main components being DHCA, PHCA, DPB, PCUM and CAF, containing also caffeoylquinic acid derivatives. The extracts originated from *Araucaria* spp (group C) are characterized as BRG, rich in coniferaldehyde compounds (VAN, G2 and I) (26). In relation to flavonoids, PK (26.0–45.6 mg g<sup>-1</sup> of dried extract) and kaempferide (KAEMP1) (33.0–37.1 mg g<sup>-1</sup>) were detected only in the three extracts of group B. KAEMP1 was also detected in two out of the four EEPs of group A.

From EEP-11, the most active extract, a red propolis collected in the State of Alagoas, has been already isolated: simple phenolics, triterpenoids, isoflavonoids, prenylated benzophenones and naphthoquinone epoxide (8).

Red colored propolis has been previously reported as typical in Cuba (7) and in Venezuela (11).

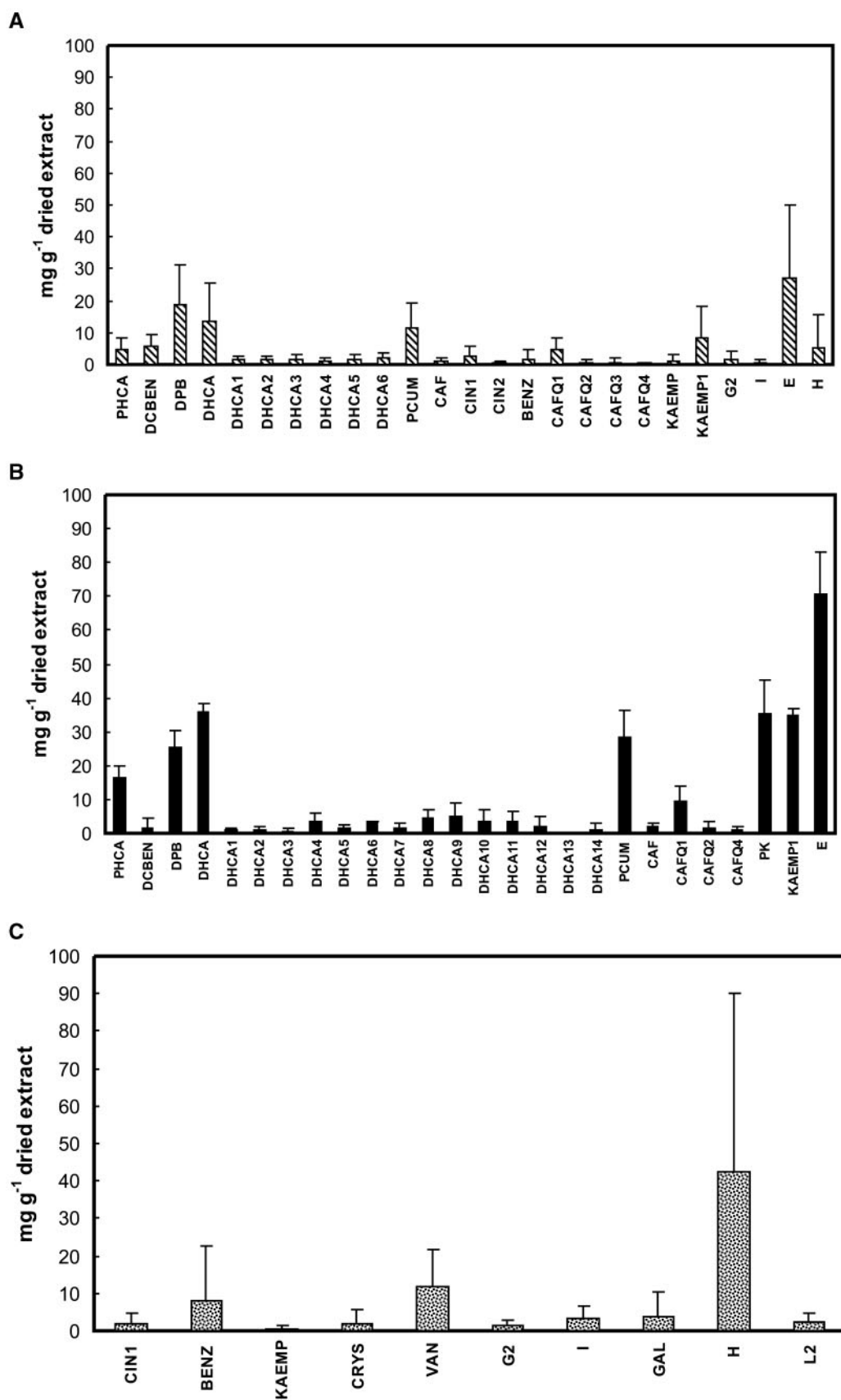
The fungicidal activity of Brazilian propolis has already been reported (27,28). All the extracts assayed presented similar activities considering a given fungus species and an overall analysis shows that *P. brasiliensis* was more susceptible to the extracts than *C. albicans* and *S. schenckii*.

As observed with propolis from temperate regions, Brazilian samples also present a higher activity against Gram-positive bacteria than Gram-negative ones (29,30), as observed in the present work using *K. pneumoniae*. The relationship of propolis origin, chemical composition and activity against *S. aureus*, as observed in the present study, was in accordance with the reports of other investigators (31,32). The statistical analysis of all the extracts together revealed that higher concentrations of PCUM and DHCA1 are correlated with higher activity against *S. aureus*.

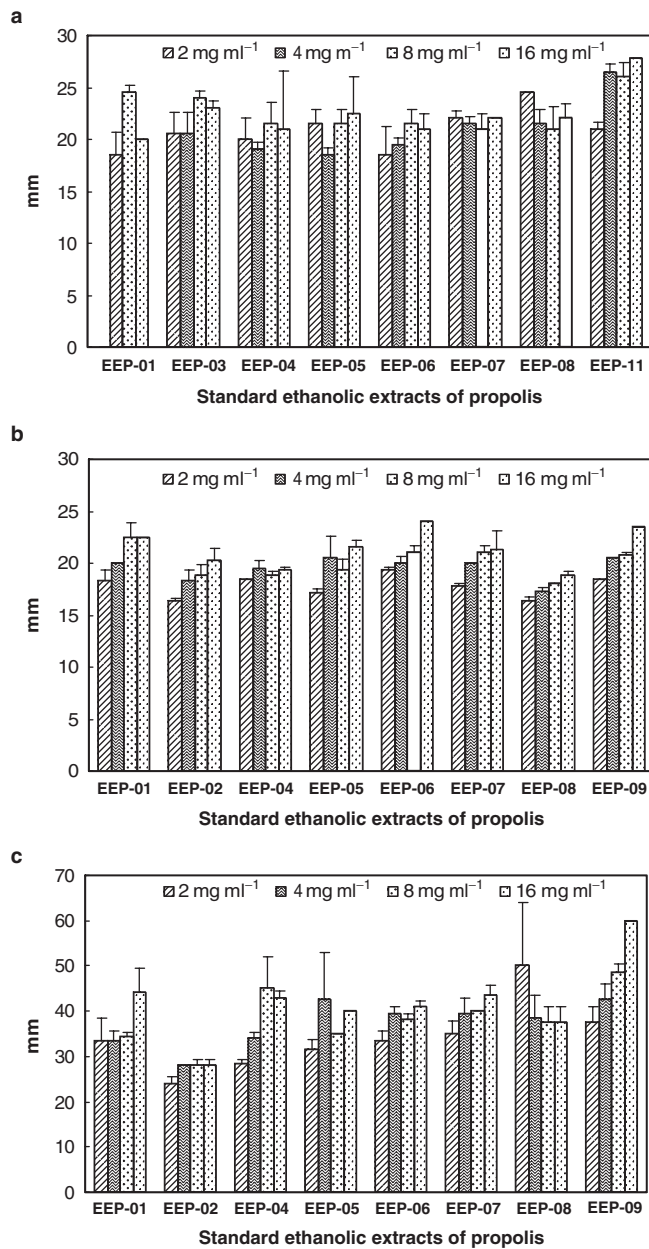
The IC<sub>50</sub> for the activity against *T. cruzi* was in the range of 200–2000 µg ml<sup>-1</sup>, while for the standard drug, crystal violet, in the same experimental conditions, the value was 187.0 ± 21.0 µg ml<sup>-1</sup> (33). Statistical analysis of all the extracts showed that higher levels of DHCA4 and DCBEN were associated with higher trypanocidal effect.

The separation of the extracts in groups A, B and C, decreasing the number of observations in each group, prevented the application of multiple regression analysis. However, for *S. aureus*, *S. pneumoniae* and *T. cruzi*, we found, always a positive association of the biological activity with derivatives of PCUM and of caffeoylquinic acids, which correspond to caffeic acids esterified with sugar residues.

For both *S. aureus* and *T. cruzi* higher levels of 4-hydroxy cinnamic acid and derivatives were associated with a stronger biological activity, while no correlation was found between such activities and the content of flavonoids. DHCA and DCBEN, previously



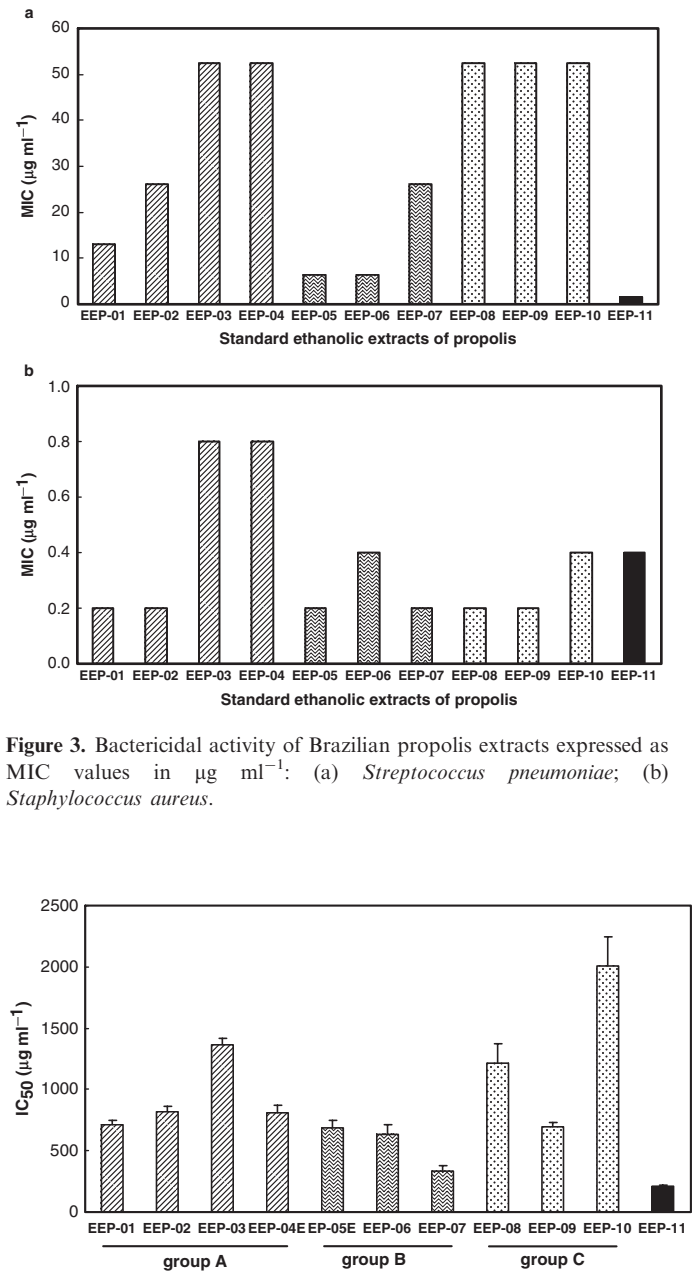
**Figure 1.** Concentration expressed in mg g<sup>-1</sup> dried extract (mean  $\pm$  standard deviation) of individual components of the ethanol extracts of Brazilian propolis samples separated by botanical origin: (A) group A; (B) group B; (C) group C.



**Figure 2.** Fungicidal activity of Brazilian propolis extracts (2, 4, 8 and 16 mg ml<sup>-1</sup>) expressed as diameter of inhibition in mm: (a) *Candida albicans*; (b) *Sporothrix schenckii*; (c) *Paracoccidioides brasiliensis*.

characterized both in Brazilian propolis and in its main plant source, *B. dracunculifolia*, were active against *T. cruzi* and *S. aureus* (18,34) and are useful markers to typify different samples (26).

The association of the chemical composition of propolis from different geographic regions with biological activities lead to the identification of active principles, a fundamental tool to achieve standardization of this bee product. The present study reinforces the relevance of PCUM and derivatives, especially prenylated ones and also of caffeoylquinic acids, on the biological activity of Brazilian propolis.



**Figure 3.** Bactericidal activity of Brazilian propolis extracts expressed as MIC values in µg ml<sup>-1</sup>: (a) *Streptococcus pneumoniae*; (b) *Staphylococcus aureus*.

**Figure 4.** Effect of the Brazilian propolis extracts against bloodstream trypomastigotes of *Trypanosoma cruzi* after 1 day of treatment at 4°C. The bars represent the standard deviation of at least three independent experiments. The activity of EEP-01 was higher than that of EEP-02, EEP-04 and EEP-03 ( $P < 0.05$ ); the activity of EEP-07 was higher than that of EEP-05, EEP-06 ( $P < 0.05$ ); the activity of EEP-9 was higher than that of EEP-8, which has higher than that of EEP-10 ( $P < 0.05$ ).

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