Evaluation of Antioxidant and Antimicrobial Activities and Characterization of Bioactive Components of Two Brazilian Propolis Samples Using a pK_a-Guided Fractionation

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The ethanolic extracts of two Brazilian propolis samples were submitted to a fractionation procedure based on the pK_a values of their components. The fractions obtained were evaluated for their antimicrobial activity against *Staphylococcus aureus* as well as for their antioxidant properties (reduction of DPPH radical). Their phenolic and flavonoid contents were measured spectrophotometrically, in order to establish the correlations between these contents and the measured activities. Further, the most active fractions of both extracts were analyzed by HRGC-MS and about twenty compounds could be characterized. Among them were 3-prenyl-4-hydroxycinnamic acid (drupanin) and 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C), which seem to be the major antioxidant components of the bioactive fractions.

Key words: Antibacterial Activity, Antioxidant Activity, Propolis

Introduction

Propolis is a nutritional complement produced by bees that has been widely used in folk medicine (Marcucci, 1995). Antimicrobial, antioxidant, antineoplasic, immunostimulating and wound healing activities have already been described for propolis extracts, among many others (Bosio *et al.*, 2000; Tuo and Chan, 2003; Castaldo and Capasso, 2002).

The search for the active components of propolis led to the identification of hundreds of compounds, among which aromatic acids and flavonoids are those more usually associated with the antimicrobial and antioxidant activities (Bankova *et al.*, 1995; Tazawa *et al.*, 1998). Brazilian propolis is especially rich in phenolic acids while European propolis as well as propolis from other temperate regions are described to be richer in flavonoids (Bankova *et al.*, 1995, 2002; Marcucci *et al.*, 2001). These differences are due to the local flora, and while in temperate zones the main source of propolis is poplar (*Populus* spp.), in Brazil plants from the genus *Baccharis*, *Hyptis* and *Araucaria* are believed to be the main contributors (Park *et al.*, 2002; Banskota *et al.*, 1998). This wide chemical composition leads to the fact that no individual component can be claimed to be responsible for the propolis activities; they are a result from the synergism between diverse compounds (Marcucci, 1995). Nevertheless, the identification of specific components may furnish biomarkers valuable for the quality control of propolis products. Moreover, the identification of specific structural patterns associated with the biological activities can be a source of lead compounds for further development of new drugs. One of the alternatives to achieve this goal is the bioactivity-guided chemical analysis of the extracts and their fractions, which is a successful methodology already employed for many plant extracts (Kaur *et al.*, 2005; Prabhakar *et al.*, 2006). Using TLC and HPLC as analytical tools, Santos and co-workers (2002) employed this methodology for the investigation of the chemical composition-antimicrobial activity relationships of a Brazilian propolis extract. Although they hadn't identified any individual compound, they showed the presence of phenolic derivatives in the bioactive fractions. Moreover, they proposed a fractionation methodology for propolis extracts in which the separation of the components is driven mostly due to their pK_a values.

In order to take a further insight into the antimicrobial and antioxidant activities of Brazilian propolis, we decided to explore the use of this sep-

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aration methodology in two propolis extracts and to proceed with an investigation on the chemical composition of the most active fractions by high resolution gas chromatography coupled with mass spectrometry (HRGC-MS), as well as by spectroscopic measurements of their levels of phenolic and flavonoid contents, in order to obtain more detailed composition-activity relationships data.

Material and Methods

Propolis

Crude propolis from Guarapari and Bonfim were kindly gifted by Prof. Monica Freimman de Souza Ramos (Faculty of Pharmacy, Federal University of Rio de Janeiro, Brazil). Guarapari is located in the coastal zone of the Brazilian state of Espirito Santo, possessing a tropical Aw climate and reminiscences of the original Atlantic forest. Bonfim is located in the central area of the Minas Gerais state, possessing a subtropical Cwa climate in a region of transition between the tropical forest and the Brazilian savanna (cerrado). These samples were obtained from local producers and they were 2 years old when the extracts were prepared. Voucher samples are kept refrigerated and available under request.

Preparation and fractionation of the extracts

The extracts from Bonfim and Guarapari crude propolis were prepared by static maceration of 6.0 g of the ground crude material with 30 mL of absolute ethanol for one week at room temperature. After filtration to remove the insoluble residues, the extracts were diluted with water (15 mL) and then submitted to the fractionation procedure described by Santos *et al.* (2002), with minor modifications. The diluted extract was partitioned with hexane (3×50 mL), and then the aqueous layer was partitioned with dichloromethane (3×50 mL). This dichloromethane layer was successively partitioned with 3×50 mL of 5% aqueous $NaHCO_3$, 5% aqueous Na_2CO_3 and 5% aqueous NaOH solutions. The alkaline fractions were then neutralized with concentrated HCl and partitioned with dichloromethane (3×30 mL), yielding the corresponding organic acidic fractions after removal of the solvent under reduced pressure (Fig. 1).

Determination of phenolic contents

The phenolic components were determined by using the Folin-Ciocalteau reagent solution (Lugasi *et al.*, 1998). Gallic acid was employed as the standard and the results were obtained as g of gallic acid per 100 mL of propolis extract (%g).

Determination of flavonoid contents

The flavonoid content was determined by the method described by Dowd (1959), employing $AlCl_3$ to form a complex, which was measured spectrophotometrically at 425 nm. Quercetin was employed as the standard and the results were obtained as g of quercetin per 100 mL of propolis extract (%g).

Microbiological assay

The antimicrobial activity of the propolis extracts against *S. aureus* ATCC 29213 was performed as described by NCCLS, using Muller-Hinton agar as culture medium (National Committee for Clinical Laboratory Standards, 1993). The minimum inhibitory concentration (MIC) was obtained as mL of propolis extract per 100 mL of culture medium (%v).

DPPH scavenging assay

This test was performed as described by Davalos *et al.* (2003). The antioxidant activity was determined as the percentage of DPPH remaining in solution, calculated according to the formula: $\%\mathrm{DPPH}_{\mathrm{rem}} = A_{\mathrm{propolis\ extract}}/A_{\mathrm{blank}} \times 100$, where $A_{\mathrm{propolis\ extract}}$ and A_{blank} are the absorbances of the propolis extract and of the blank in the assay, respectively.

HRGC-MS analysis

The HRGC-MS analyses of the fractions obtained were conduced using a gas chromatograph HP 5890-II equipped with a selective mass detector HP 5972 and an Agilent 6890 injector. The chromatographic column used was DB5-MS (internal diameter of 0.25 mm, length of 30 m and film thickness of 0.25 μm) and the data were obtained through a HP Chemstation. Helium was used as carrier gas at 14 MPa. The temperature of the injector and of the detector was 330 °C. For the fractions Guarapari B2 and Bonfim B2, the oven temperature was set to 100 °C and, after a 5 min isotherme, increased to 300 °C (rate 10 °C/

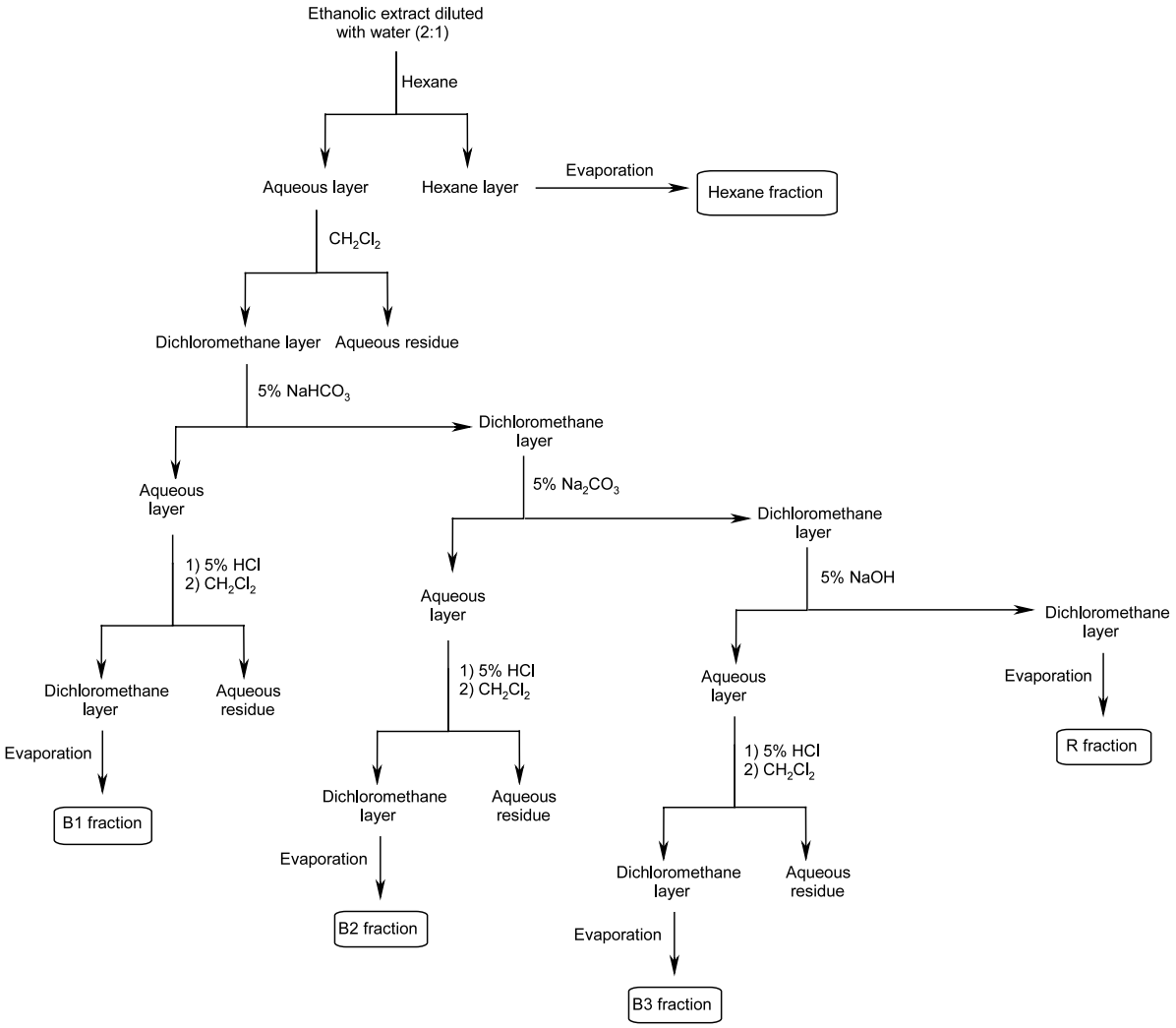

Fig. 1. Fractionation scheme for the propolis ethanolic extract.

min). For fraction Guarapari B3, the oven temperature was increased from 80 °C to 180 °C at 2 °C/min, and after 5 min at this temperature, it was raised to 300 °C at a rate of 5 °C/min. In all cases, the final temperature was kept for 10 min. All the samples analyzed were derivatized with BSTFA prior to injection (adapted from Casteele *et al.*, 1976; Popova *et al.*, 2005). The compounds were characterized by mass spectral interpretation and automatic comparison with a spectra library, and, if available, reference compounds were co-chromatographed in order to confirm the retention times. Relative retention times were calculated and are in agreement with reported values (Isidorov and Vinogorova, 2003).

Statistical analysis

All assays were carried out in duplicates. The results were analysed using ANOVA, F distribution and unpaired Student's *t*-test (p value ≤ 0.05).

Results and Discussion

The results of the determination of the phenolic and flavonoid contents as well as of the microbiological and DPPH radical scavenging assays of the propolis extracts from Bonfim and Guarapari and their fractions are described in Table I. These results show that the fractionation methodology employed is effective in separating the acidic constituents in different fractions, especially in the case

Extract/Fraction	Phenolic content (%g)	Flavonoid content (%g)	MIC (%v)	%DPPH$_{rem}$
Guarapari				
Ethanolic extract	1.5	0.3	0.4	57.5
Hexane fraction	15.4	nd	1.5	96.0
B1 fraction	27.6	0.2	1.5	92.1
B2 fraction	23.7	8.5	1.5	84.8
B3 fraction	20.0	nd	0.2	88.6
R fraction	7.7	nd	> 1.5	94.1
Bonfim				
Ethanolic extract	1.7	0.3	0.8	52.2
Hexane fraction	13.3	nd	1.5	95.7
B1 fraction	18.4	3.3	1.5	87.5
B2 fraction	25.3	15.4	1.5	81.5
B3 fraction	10.7	nd	0.6	90.0
R fraction	4.8	nd	> 1.5	98.6

Table I. Phenolic and flavonoid levels and results from the microbiological and DPPH radical scavenging activities of the propolis extracts and their fractions.

nd, not detected.

of the flavonoids, which are concentrated in the B2 fractions (medium pK_a values). It is also interesting to notice the presence of phenolic compounds in the hexane fraction, probably due to the presence of hydrophobic chains in their structures.

The antioxidant activities of the individual fractions are lower than that of the whole extracts, implying a synergism between different active compounds. It can also be observed that there is no linear correlation between the levels of phenolic compounds and this activity, what is an interesting point due to the fact that this property is attributed to the ability of phenolic hydroxy groups to react with free radicals and other reactive species, forming the more stabilized phenoxyl radicals (Goupy *et al.*, 2003). Moreover, the more active fractions were the richest in flavonoid compounds. Although there are innumerous reports on the high antioxidant activity of flavonoids (Heim *et al.*, 2002; Murota and Terao, 2003; Williamson *et al.*, 2005), in the specific case of Brazilian propolis they are said to play a minor role, due to their low concentrations (Bankova *et al.*, 1995). On the other hand, previous results from our group showed that, in a survey involving 49 commercial propolis extracts, a higher correlation between flavonoid contents and DPPH scavenging activity could be found than between this activity and the phenolic contents of the extracts (Silva *et al.*, 2006). These results show that we cannot rule out the contribution of the flavonoids to the antioxidant activity of Brazilian propolis extracts, although in the whole extract there seems to be phenolic compounds acting synergistically and leading to a more pronounced effect than the one of the flavonoids alone. These compounds seem to have been separated in different fractions after the partition methodology employed.

In relation to the anti-*Staphylococcus aureus* activity, the B3 fraction of both extracts showed an activity higher than that of the original extract, while the other fractions, although richer in phenolic and flavonoid components, were much less active. These results reinforce our hypothesis of a structurally related antimicrobial activity of the propolis components, and the identification of this structural pattern could furnish new lead compounds for the treatment of infections, such as those caused by oxacillin-resistent *S. aureus.*

In order to obtain more information on the structure-activities relationships, we proceeded to an analysis of the Guarapari B3, Guarapari B2 and Bonfim B2 fractions by HRGC-MS. The results obtained from the automatic comparison of the spectra obtained with those of a digital library are shown in Table II.

Most of the compounds that could be characterized at this stage were derivatives of cinnamic acid, but most of them are present in low contents. Vanillin, 4-coumaric acid, ferulic acid and caffeic acid, which are present in these fractions, have their antioxidant activities well described (Hotta *et al.*, 2002) and may have some importance for the antioxidant activity displayed by these fractions. On the other hand, hydrocinnamic acid is present in high contents but, due to the absence of phenolic groups, its relevance for the antioxidant activity displayed by these fractions is not expected to be relevant.

Table II. Compounds characterized as their trimethylsilyl derivatives by HRGC-MS in the propolis fractions analyzed.

Compound	Guarapari B2		Guarapari B3		Bonfim B2	
	%	Rt	%	Rt	%	Rt
Benzoic acid	–	–	0.4	14.4	–	–
Caffeic acid	0.5	32.6	–	–	–	–
Cinnamic acid	0.2	18.7	0.4	33.9	–	–
4-Coumaric acid	1.0	28.6	0.3	55.5	< 0.2	28.3
Ethyl 4-hydroxyhydrocinnamate	–	–	–	–	< 0.2	23.0
Ethyl hydrocinnamate	0.3	11.3	–	–	–	–
Ferulic acid	0.1	31.7	0.4	63.5	–	–
Hydrocinnamic acid	14.0	14.6	30.8	26.7	30.2	14.0
4-Hydroxyacetophenone	0.2	16.4	–	–	–	–
4-Hydroxybenzoic acid	0.1	21.3	< 0.2	38.9	< 0.2	21.0
4-Hydroxyhydrocinnamic acid	0.2	24.6	< 0.2	45.0	< 0.2	24.4
Isoeugenol	–	–	< 0.2	29.5	–	–
Linoleic acid	–	–	< 0.2	69.1	–	–
4-Methoxybenzoic acid	1.1	18.1	17.2	33.4	–	–
2-Methoxyhydrocinnamic acid	–	–	< 0.2	40.3	–	–
Myristic acid	0.2	26.6	–	–	< 0.2	26.3
Oleic acid	–	–	–	–	< 0.2	33.5
Palmitic acid	0.2	30.6	0.3	60.8	< 0.2	30.4
2-Phenylethanol	–	–	0.2	13.1	–	–
Stearic acid	< 0.2	34.3	< 0.2	70.4	< 0.2	34.0
Vanillin	–	–	< 0.2	33.1	< 0.2	17.8
3-Vanillylpropanol	–	–	1.9	49.9	–	–

Two major components of the three fractions analyzed were not characterized by the automatic comparison with a digital library; so we proceeded to analyse the fragmentation pattern observed in the mass spectra of these compounds (Figs. 2 and 3) and compared it with published spectral data (Marcucci *et al.*, 2001). These components were tentatively characterized as 3-prenyl-4-hydroxycinnamic acid (drupanin) and 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C), which had already been identified in a Brazilian propolis sample from Mandirituba [Parana state, predominant flora composed by araucaria trees (Coniferae) and temperate Cfb climate] and had their antioxidant and antimicrobial activities evaluated previously (Marcucci *et al.*, 2001). Drupanin is present in 29.4%, 26.2% and 10% in fractions Guarapari B2, Guarapari B3 and Bonfim B2, respectively, while artepillin C is present in 29.3%, 28.4% and 10.5%, respectively. Due to their high

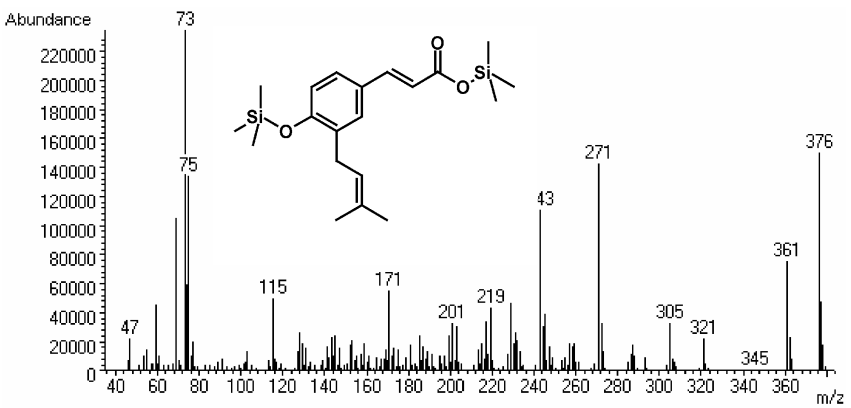

Fig. 2. Mass spectrum of 3-prenyl-4-hydroxycinnamic acid [bis(trimethylsilyl) derivative].

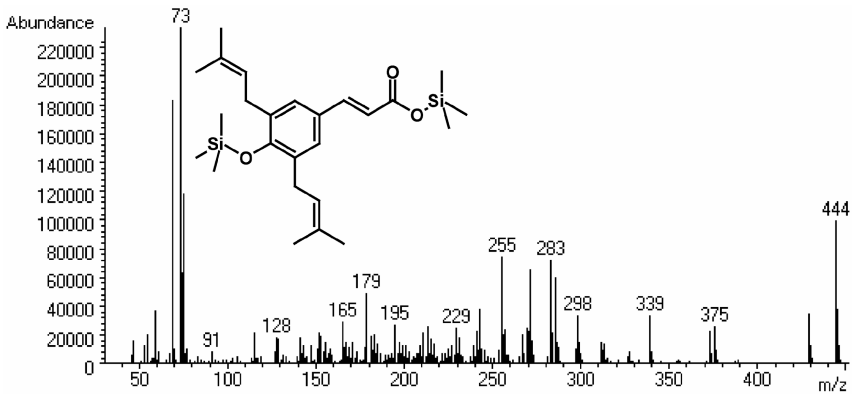

Fig. 3. Mass spectrum of 3,5-diprenyl-4-hydroxycinnamic acid [bis(trimethylsilyl) derivative].

contents in these fractions, it seems that artepillin C and drupanin have an important contribution for the antioxidant activities found, especially for the Guarapari fractions, where they seem to be determinant for this activity. The DPPH scavenging efficiency of these compounds described by Tapia *et al.* (2004) showed that drupanin has a higher activity than artepillin C in this assay (81% and 27% of decoloration of DPPH radical solution at 100 μg/mL, respectively).

In relation to the anti-*Staphylococcus aureus* activity, the concentrations of these cinnamic acid derivatives seem not to be an important factor. Artepillin C, indeed, had already been reported to show modest inhibition of the growth of *S. aureus* strains (Marcucci *et al.*, 2001). Another identified major component of the most active fraction, 4-methoxybenzoic acid, had already been shown to lack antibacterial activity (Friedman *et al.*, 2003) over *S. aureus* (Meng *et al.*, 2000). We were not able to identify the component(s) responsible for the higher antimicrobial activity displayed by fraction Guarapari B3. Anyway, it is interesting to notice that for both propolis samples analyzed the most active fractions were those obtained by extraction with 5% aqueous NaOH solution, and that in both of them the flavonoid levels were below the detection limit of the method employed.

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