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Artigo

CHEMICAL PROFILING OF SIX SAMPLES OF BRAZILIAN PROPOLIS

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Six samples of Brazilian propolis from Minas Gerais and Paraná states were analyzed to identify the constituents (GC/MS and HPLC/MS) and to determine their contents (HPLC and external standardization). All samples contained characteristic constituents of green propolis, but the samples from Minas Gerais had higher contents of prenylated phenylpropanoids and caffeoylquinic acids. Kaempferide and two other flavonoids were among the major constituents of the samples from Minas Gerais. Luteolin 5-*O*-methyl ether was detected only in samples from Paraná. *Baccharis dracunculifolia* was a source of resins for all samples analyzed, but the samples from Paraná had more complex plant origin.

Keywords: Brazilian propolis; caffeoylquinic acids; phenylpropanoids.

INTRODUCTION

The word propolis derives from the Greek *pro* (in defense of, or in front of) and *polis* (city), implying a product useful for the defense of the hive.[1] It is a complex mixture of compounds with resinous aspect, made by *Apis mellifera* bees from plant resins and beeswax. Its chemical composition depends on the plant or plants from which the resin is collected and, consequently, on the geographic location of the hive.[1-3] Propolis samples have been shown to exhibit many biological activities, such as antimicrobial,[4] anviral,[5] antiinflammatory,[6] antiprotozoan,[7] antitumoral,[8,9] and antioxidant actions.[10,11]

Many types of propolis, comprising a wide diversity of botanical sources, have been described. The main sources of European propolis are buds of poplars (*Populus* spp.).[2] In Cuba and Venezuela bees collect resins from *Clusia* spp.[12] In Brazil, there are at least four distinct resin sources for propolis production: *Baccharis dracunculifolia*, the alecrim-do-campo plant (Brazilian green propolis),[3,13] *Dalbergia ecastophyllum* (Brazilian red propolis),[14] *Hyptis divaricata* (Brazilian brown propolis) and *Populus alba* (poplar type propolis).[15] Recently, new propolis types and botanical sources have been described, such as Pacific propolis (plant source: *Macaranga* spp.),[16] Mediterranean propolis (plant source: conifers)[17] and Brazilian Amazonian propolis (plant source: probably *Clusia* sp.).[18,19]

Among Brazilian propolis, the most exported and intensively studied is the green type.[3] It is composed mainly of prenylated phenylpropanoids, such as artepillin C (3,5-diprenyl-4-hidroxycinnamic acid)[3,20] and 3-prenylcinnamic acid allyl ester, both compounds assumed as markers of green propolis.[19] Flavonoids, such as kaempferide, are present, although not as major constituents.[1,15,21] Terpenoids and benzoic acids may also be found in green propolis.[3,22] Propolis from the south of Brazil has been regarded as derived either from poplars[14] or *Araucaria*.[23] Amounts of propolis constituents have rarely been investigated using standardization, either internal or external. The determination of major propolis constituents may be crucial for standardization and chemical quality control.[24]

The aim of the present study was to compare the chemical composition of four samples of Brazilian green propolis from Minas Gerais (southeast) and two from Paraná (south of Brazil). The former state is the geographical center of distribution of green propolis, while Paraná state lies on the south border of this zone of distribution. This study also sought to determine the contents of relevant constituents of the six samples, using external standardization.

EXPERIMENTAL

Propolis samples and extraction

Samples A and B were produced in the municipality of Esmeraldas, state of Minas Gerais. Samples C and D came from the municipality of Três Pontas, state of Minas Gerais. Samples E and F came from the municipality of União da Vitória, state of Paraná. Successive extractions were carried out in Soxhlet with 5 g of each sample and the solvents hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc) and methanol (MeOH). The extracts were concentrated to dryness under reduced pressure.

Derivatization

A 30 μL volume of a 10 mg/mL $CHCl_3$ solution of the $CHCl_3$ extracts was treated with 4 mL of a 5% MeOH solution of H_2SO_4 and 2 mL of toluene. The mixture was left standing in a steam bath at 80 °C for 4 h. Extraction was then performed with 2 mL of 0.5 M NaCl solution and 1 mL of methylene chloride. The mixture was vigorously stirred and then centrifuged at 5000 rpm for 5 min. The organic phase was collected and the residue extracted with methylene chloride twice using the same procedure. The pooled organic phases were washed 3 times with 0.5 M NaCl and the aqueous phase was discarded. The extract containing the derivatized products was treated with anhydrous Na_2SO_4 and concentrated under a N_2 flow.

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Chemical composition

Hexane and derivatized $CHCl_3$ extracts were analyzed by GC/MS according to Negri *et al*..[22] Identification of the compounds was accomplished using computer-based searches of commercial libraries and literature data.

The EtOAc and MeOH extracts were dissolved in MeOH at the concentration of 10 mg/mL. Both extracts from all 6 samples were analyzed by injecting 10 μL of the MeOH solutions into an HPLC chromatograph equipped with a C18 RP Luna Phenomenex column (4.6 x 250 mm, 5 μm). The mobile phase used contained 0.1% acetic acid and MeOH, with a constant 0.5 mL/min flow. The HPLC-DAD-ESI-MS system was a DADSPD-M10AVP Shimadzu equipped with a photodiode array detector, coupled to an Esquire 3000 Plus, Bruker Daltonics. The mass detector was a quadrupole ion trap equipped with an atmospheric pressure ionization source through electrospray ionization interface. The mobile phase flow was 0.5 mL/min and the gradient used comprised MeOH 20 to 40%, from 0 to 10 min; MeOH 40 to 60%, from 10 to 20 min; MeOH 60 to 80%, from 20 to 30 min; MeOH 80 to 95%, from 30 to 37 min; and MeOH 95%, from 37 to 45 min. Detection was accomplished at 270 and 300 nm. Mass spectra were obtained using a negative ESI source voltage of -40 V and a capillary offset voltage of 4500 V. Nebulization was aided with coaxial nitrogen sheath gas, provided at 27 psi pressure. Temperature of the dry gas was 130 °C and the flow was 4 L/min. A counter current nitrogen flow was set at 7 L/min and capillary temperature at 320 °C, to assist desolvation. Mass spectra were recorded over the range 50-700 *m/z*. The identification of sample constituents was based on their UV absorbance band and on cross-comparison of mass spectra data with literature data.

Quantification of constituents

The contents of the main compounds were determined by HPLC analysis and external standardization, using 10 μL of MeOH solutions of the EtOAc and MeOH extracts. The solutions were injected into an HP 1090 HPLC apparatus, equipped with a reverse phase C18 column (4.6 x 250 mm, 5 μm), using the gradient described in the previous section. The amount of compounds was estimated on the basis of the areas under the corresponding peaks and standard curves prepared with quercetin (for flavonoids), *p*-coumaric acid (phenylpropanoids) and chlorogenic acid (caffeoylquinic acids). The contents of the compounds were expressed as mg per g of crude propolis.

RESULTS AND DISCUSSION

The compounds identified by GC/MS are listed in Table 1. Although with different peak intensities, all compounds were detected in the 6 samples analyzed. Compounds **3** and **6** (palmitic and stearic acids, respectively) are common constituents of natural waxes.[22] Compound **2** is a simple phenol which has been reported from Brazilian green propolis,[25] sometimes as one of the major constituents.[10] With the exception of **1** (benzenepropanoic acid), all other compounds were phenylpropanoids. Compounds **4** (an allyl ester), **5** (drupanin), as well as **10**, contain one prenyl group. The same holds for the chromanes **7** and **8**, both bearing a prenyl group involved in

Table 1. Constituents of six samples of Brazillian propolis characterized by GC-EI-MS. A-D: samples from the state of Minas Gerais (southeast Brazil); E and F: samples from the state of Paraná (south Brazil)

Peak	RT	Molecular ion and fragments	Proposed compounds	Relative amount (%)					
				A	B	C	D	E	F
1	6.78	150 (40, $C_9H_{10}O_2^+$), 104 (70), 91(100), 77(30)	benzenepropanoic acid[22,25#]	24.04	23.77	18.5	18.2	15.52	26.94
2	10.35	188 (70, $C_{13}H_{16}O^+$), 133 (100), 104 (30), 91 (30), 77 (30)	*p*-vinyl-*o*-prenylphenol[22,25#]	15.4	23.04	24.7	17.85	6.96	14.77
3	13.42	270 (1, $C_{17}H_{34}O_2^+$), 143 (30), 87(70), 74 (100), 41 (98)	palmitic acid methyl ester[22,25*]	20.99	26.90	0.1	3.22	0.1	0.1
4	15.05	256 ($C_{17}H_{20}O_2^+$), 185 (70), 145 (100), 91 (40), 77 (20), 69 (30)	3-prenylcinnamic acid allyl ester[22,25#]	23.99	20.95	32.42	22.58	4.09	9.73
5	15.46	246 (70, $C_{15}H_{18}O_3^+$), 191 (100), 171 (20), 131 (23).	4-hydroxy-3-prenylcinnamic acid (drupanin) methyl ester[8,22,25,26,27,28*]	0.1	26.94	29.59	24.54	14.84	17.57
6	16.31	298 (4, $C_{19}H_{34}O_2^+$), 143 (30), 87(70), 74(100)	stearic acid methyl ester[22,25*]	0.1	0.1	< 0.1	2.67	1.56	0.1
7	17.48	312 (14, $C_{20}H_{24}O_3^+$), 297 (100)	2,2-dimethyl-8-prenylchromene-6-propenoic acid methyl ester[8,22,25,26,27,28*]	3.38	0.1	5.13	4.96	1.53	0.1
8	19.11	330 (100, $C_{20}H_{26}O_4^+$), 297 (30), 272 (50), 225 (60), 197 (50), 171 (50).	3-hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid methyl ester[8,22,25,26,27,28*]	2.23	0.1	30.37	6.39	1.97	3.76
9	19.23	314 (68, $C_{20}H_{26}O_3^+$), 259 (100), 243 (54), 211 (38), 203 (90).	4-hydroxy-3,5-diprenylcinnamic acid (artepillin C) methyl ester[8,22,25,26,27,28*]	3.65	4.54	5.13	2.53	1.91	3.47
10	21.43	330 (100, $C_{20}H_{26}O_4^+$), 297 (50), 259 (70), 228 (30), 203 (60)	3-prenyl-4-(2-methylpropionyl-oxy)- cinnamic acid[26*]	0.1	0.1	0.1	1.91	0.1	2.66

[#] = compounds detected in non-derivatized hexane extracts; *= compounds detected in derivatized chloroform extracts

Table 2. Constituents of six samples of Brazillian propolis characterized and quantified by HPLC-ESI-MS. A-D: samples from the state of Minas Gerais (southeast Brazil); E and F: samples from the state of Paraná (south Brazil)

Peak	RT (min)	Fraction	UV_{max}(nm)	$[M-H]^-$	[M+H]+	Proposed compound	Quantity (mg/g of crude propolis)					
							A*	B	C	D	E	F
11	19.18	MeOH	300, 330	353	355.1	3-*O*-Caffeoylquinic acid[29,30,31]	0.16	0.38	0.11	0.17	0.05	0.25
12	21.65	MeOH	300, 330	352.9	355.2	5-*O*-Caffeoylquinic acid[29,30,31]	1.96	4.14	1.83	2.93	0.60	3.18
13	25.82	MeOH	312	nd*	355.1	4-*O*-Caffeoylquinic acid[29,30,31]	0.61	0.76	0.45	0.71	0.08	0.35
14	37.15	MeOH	290, 325	519.1	521.3	didihydrocaffeoylquinic acid[29,30,31]	0.67	0	0.92	1.50	0.21	0.32
15	39.62	MeOH	300, 330	515.1	nd	3,5-di-*O*-Caffeoylquinic acid[29,30,31]	7.41	11.96	4.78	11.38	1.98	8.24
16	34.07	EtOAc	310	162.9	nd	*p*-Coumaric acid [8,26,32]	7.43	6.20	7.67	2.89	1.41	1.96
17	50.09	MeOH	300, 330	515.1	517.2	4,5-di-*O*-Caffeoylquinic acid[29,30,31]	10.52	19.35	6.97	16.78	2.89	13.79
18	53.14	MeOH	295, 325	529.2	531.3	3-*O*-Feruloyl-5-*O*-Caffeoylquinic acid[29,30,31]	0.56	0.35	0.38	0.96	0.02	0.45
19	61.90	MeOH	300, 330	677.1	679.3	3,4,5-tri-*O*-Caffeoylquinic[29,30,31]	3.13	3.61	1.99	4.46	1.07	3.00
20	65.90	EtOAc	290	301.1	303.1	Methoxypinobanksin[26]	4.22	3.96	2.52	1.55	0	0.41
21	71.08	EtOAc	268, 365	315	317.1	Isorhamnetin[26]	6.13	4.76	2.25	2.40	0.58	0.78
22	74.35	EtOAc	265, 350	299	301.2	Luteolin-5-methyl ether[26]	0	0	0	0	2.95	2.97
5	78.43	EtOAc	315	231	nd	4-Hydroxy-3-prenylcinnamic acid (drupanin)[8,22,25,26,27,31,33]	0.73	1.15	0.58	0.17	0.04	0.32
10	81.54	EtOAc	275sh. 318	315	nd	3-Prenyl-4-(2-methylpropionyl-oxy)-cinnamic acid[26]	0.17	0.29	0.06	0.02	0	0
23	82.88	EtOAc	268, 365	299	301.2	Kaempferide[26]	15.31	10.18	11.57	5.59	1.92	3.07
8	87.08	EtOAc	320	315.1	nd	3-hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid[8,22,25,26,27,31,33]	0.13	0.19	0.16	0.05	0.09	0.10
9	91.49	EtOAc	315	299.1	nd	4-Hydroxy-3,5-diprenylcinnamic acid (artepillin C)[8,22,25,26,27,31,33]	1.02	0.86	0.35	0.08	0.15	0.30
24	97.18	EtOAc	290, 330	363.1	nd	3-Prenyl-4-(dihydrocinnamoyl-oxy)-cinnamic acid (baccharin)[8,22,25,26,33]	0.10	0	0.11	0	0	0.09

the formation of a heterocycle. Compound **9** is artepillin C, a diprenyl derived from *p*-coumaric acid, and an important marker compound of Brazilian green propolis.[19]

Compounds **5**, **8**, **9** and **10** were also detected by HPLC/MS (Table 2). All four compounds stemmed from the EtOAc extract. Most compounds identified by HPLC/MS are typical of Brazilian green propolis: caffeoylquinic acids (compounds **11**-**15** and **17**-**19**), prenylated phenylpropanoids (**5**, **7**, **9**, **10 and 24**) and flavonoids (**20**-**23**).The number of caffeoyl residues in the caffeoylquinic acids detected may be one (**11**-**13**), two (**17**) or three (**19**). Compound **22** (a luteolin derivative) stands out for being exclusively from the samples from Paraná. This flavonoid has been reported as a constituent of Argentinian propolis.[26]

The detection of 3-prenylcinnamic acid allyl ester (**4**) and artepillin C (**9**) indicates that alecrim-do-campo is a source of resin for the production of all propolis samples analyzed. However, the presence of compound **22** (luteolin-5-methyl ether) suggests that other sources of resin also contribute to propolis production in Paraná, a state lying on the south border of the distribution zone of alecrim-do-campo.[34] Poplar plants (*Populus nigra*), the most common propolis source in temperate areas, has been indicated as a plant source for propolis production in the South of Brazil.[15] However, **22** is unknown as a constituent of either temperate propolis or poplar plants. This suggests that another plant is the origin of this flavonoid.

The equations and coefficients of the standard curves for the HPLC quantitative analyses are shown in Table 3. The contents of the constituents of the samples analyzed are shown in Table 2.

Relatively low contents of the phenylpropanoids drupanin (**5**), artepillin C (**9**) and baccharin (**24**) characterize the samples analyzed.

Table 3. Parameters of standard curves used for quantification of major compounds of samples from Brazilian propolis

Standard	Range (μg)	equation	R^2
Quercetin	0.1 - 2	y=0,0005x	0.9958
Chlorogenic acid	0.05 - 6.0	y=0,0002x	0.9982
*p-C*oumaric acid	0.01 - 1.5	y=0,00006x	0.9939

Other prenylated phenylpropanoids occurring as minor constituents of the samples analyzed are **7** and **10**. Among the caffeoylquinic acids, **17** (a dicaffeoylquinic acid) predominates. In addition to caffeoylquinic acids, flavonoids, such as methoxypinobanksin (**20**), isorhamnetin (**21**) and kaempferide (**23**) are also relevant constituents, mainly with respect to the samples from Minas Gerais (**A**-**D**). The simple phenylpropanoid *p*-coumaric acid (**16**) was also an important constituent of all samples from Minas Gerais (Table 2).

Flavonoids have been regarded as minor constituents of Brazilian propolis.[2,3] The data given in Table 2, however, indicate that the flavonoids **20, 21** and **23** number among the major constituents of the propolis samples from Minas Gerais (Table 3). While kaempferide (**23**) has been reported as an important constituent of Brazilian green propolis,[33] isorhamnetin (**21**) and derivatives of pinobanksin (such as **20**) have rarely or never been detected in this type of propolis. Caffeoylquinic acids have been mentioned as frequent biologically active constituents from aqueous extracts of Brazilian propolis.[19,30,35] Prenylated phenylpropanoids (e.g. **5**, **7**, **9**, **10**, and **24**) occur at relatively low levels in the samples from both Minas Gerais and Paraná. Contrary to the general assumption that prenylated phenylpropanoids

are major constituents of Brazilian green propolis,[2,3] the quantitative analysis of the present study indicates that dicaffeoylquinic acids are the most abundant compounds of this type of propolis.

CONCLUSIONS

Although both marker compounds of Brazilian green propolis were detected in the samples from Minas Gerais and Paraná, the qualitative and quantitative analyses indicated that the samples from the two localities are chemically quite distinct and that quantitative aspects should be taken into account to address the complex problem of propolis standardization.

Plants of alecrim-do-campo are not abundant in Paraná, and thus other sources (such as poplar and an unknown source of **22**) probably complement the provision of resin for propolis production in this state. Therefore, samples of propolis from Paraná likely have a more complex botanical origin than the samples from Minas Gerais.

Quantitative analysis might contribute toward a revision of the traditional concept that prenylated phenylpropanoids are the most abundant constituents of Brazilian green propolis.

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