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Botanical Origin and Chemical Composition of Brazilian Propolis

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Brazilian propolis has been classified into 12 groups based on physicochemical characteristics: five in the southern Brazil group (group 3), one in the southeastern Brazil group (group 12), and six in the northeastern Brazil group (group 6). The plant origins of these groups were investigated using reversed-phase high-performance thin-layer chromatography (RPHPTLC), reversed-phase high-performance liquid chromatography (RPHPLC), and gas chromatography−mass spectrometry (GC−MS). It was concluded that the origins of propolis group 3, group 6, and group 12 are resins of the poplar tree, *Hyptis divaricata*, and *Baccharis dracunculifolia*, respectively.

KEYWORDS: Propolis; flavonoids; Africanized *Apis mellifera*; bud resin; leaf resin

INTRODUCTION

Propolis is the generic name for the resinous substance collected by honeybees (*Apis mellifera*) from various plant sources and used by bees to seal holes in their honeycombs, smooth out the internal walls, and protect the entrance against intruders (*1*, *2*). The first report on use of propolis as a folk medicine dates back to 300 B.C. (*2*), and recently propolis has also been extensively used in food and beverages to improve health and prevent diseases (*3*−*5*).

The HPLC analyses of the phenolic compounds present in *Populus nigra* bud exudates clearly support that this is the origin for propolis in continental Europe, North America, West Asia, and New Zealand (*2*, *6*−*9*). It is also reported that in areas where poplars are not native plants, such as Australia and equatorial regions in South America, bees which use propolis will seek other plants from which to gather exudates. Wollenweber and Buchmann (*10*) have also analyzed propolis samples from managed honeybees, as well as from feral bee colonies in the Sonoran Desert, AZ, and found that the propolis collected in hives out of flight reach of poplar trees contained flavonoids and other phenolics that point to specific plants as the source of propolis in this area, namely *Ambrosia deltoidea* and *Encelia farinose*. The botanical origin of the phenolic compounds present in tropical Venezuela propolis is the resins exuded by the flowers of *Clusia minor* and *C. major*. This was proved by the HPLC analyses of methanol extracts of resins and propolis collected in that region (*7*). These results demonstrated that analyses of phenolic compounds allow the clear differentiation of propolis collected in tropical Venezuela from that collected in temperate regions.

We have previously collected 500 samples (one sample from one beehive) of propolis which was obtained by Africanized *A. mellifera* in southern, southeastern, central western, and northeastern Brazil. The honeybee populations in Brazil were mainly of European origin prior to the mid-1950s. In 1956, African bees (*A. mellifera scutellata*) were introduced into southeastern Brazil, and because of the subsequent accidental escape of African queen bees, a process of Africanization occurred with the bees present in Brazil (*11*, *12*). The respective propolis samples were extracted with ethanol, and those extracts were analyzed by appearance of measurement of absorption spectra by UV-spectrophotometry, reversed-phase high-performance thin-layer chromatography (RPHPTLC), and reversed-phase high-performance liquid chromatography (RPHPLC). In

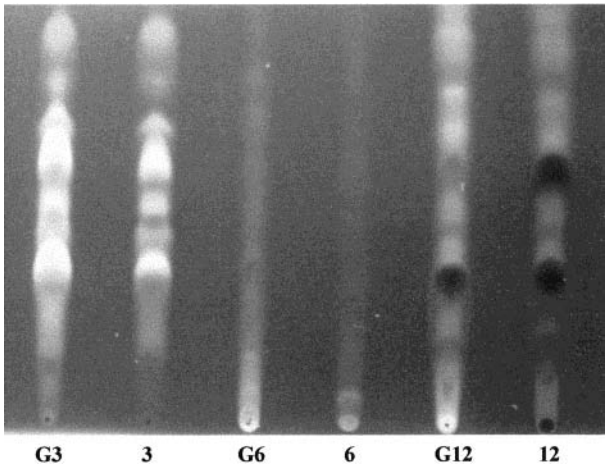

Figure 1. RPHPTLC of the ethanolic extracts of propolis and plant resins. G3 = Ethanolic extract of propolis group 3; and 3 = ethanolic extract of poplar resin from southern Brazil. G6 = Ethanolic extract of propolis group 6; and 6 = ethanolic extract of *Hyptis divaricata* resin from northeastern Brazil. G12 = Ethanolic extract of propolis group 12; and 12 = ethanolic extract of *Baccharis dracunculifolia* resin from southeastern Brazil.

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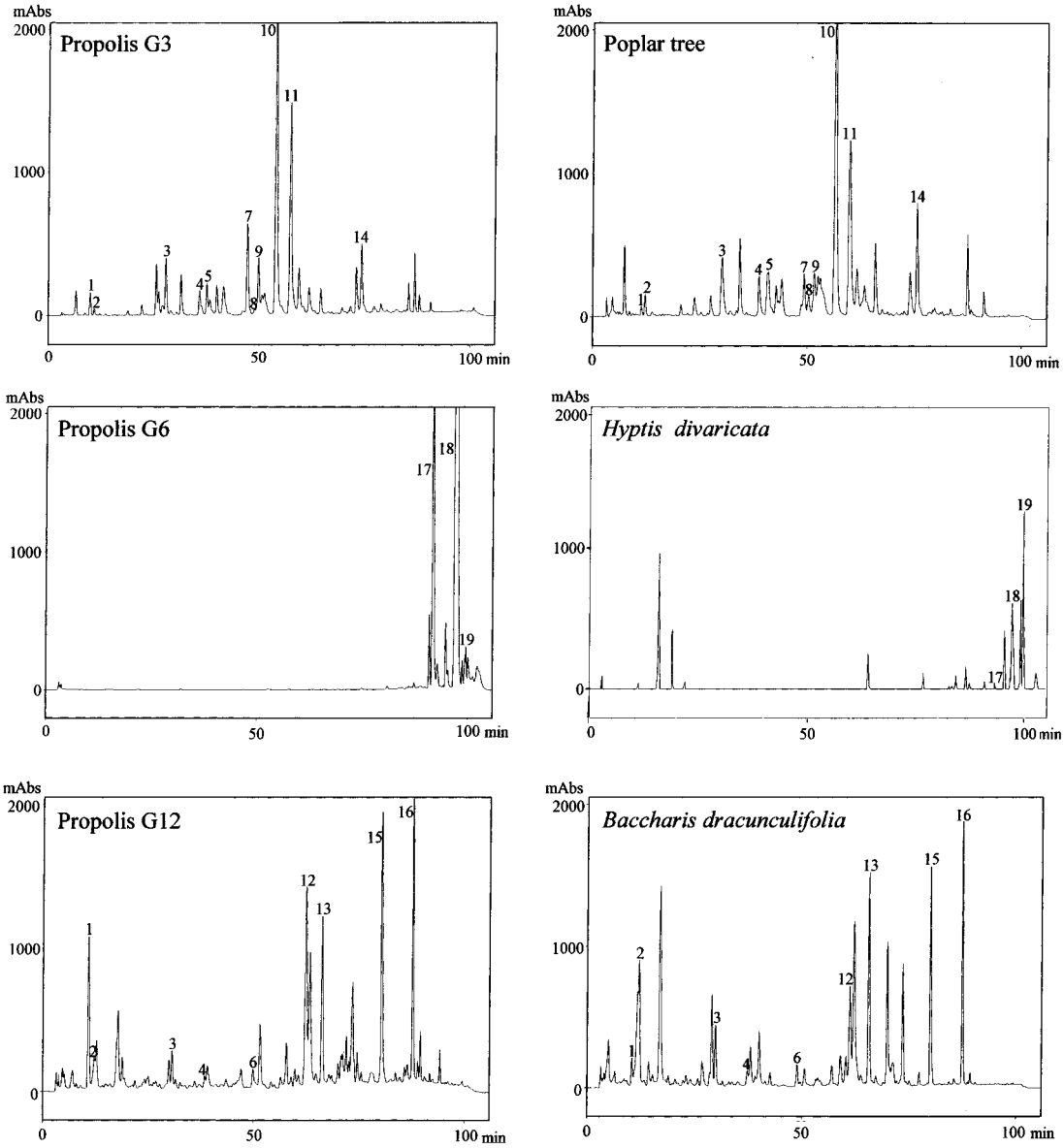

Figure 2. HPLC chromatograms of the ethanolic extracts of propolis and plant resins. **1**, Coumaric acid; **2**, ferulic acid; **3**, pinobanksin; **4**, kaempferol; **5**, apigenin; **6**, isosakuranetin; **7**, pinocembrin; **8**, dimethylallyl caffeic acid; **9**, pinobanksin 3-acetate; **10**, chrysin; **11**, galangin; **12**, kaempferide; **13**, UV λ 268 nm; Rt = 66.15 min; **14**, tectochrysin; **15**, UV λ 231 nm; Rt = 80.25 min; **16**, UV λ 223, 276 nm; Rt = 87.67 min; **17**, UV λ 246 nm; Rt = 91.73 min; **18**, UV λ 241, 274 nm; Rt = 97.26 min; **19**, UV λ 247 nm; Rt = 99.58 min.

accordance with the results of these analyses, the propolis samples were classified into 12 groups: five groups from southern Brazil, six groups from northeastern Brazil, and one group from southeastern and central western Brazil (*13, 14*). It was also found that the varieties of propolis are dependent on geographical location because of differences of plant ecology. The objective of this research was to investigate botanical origins of Brazilian propolis samples and their chemical compositions.

MATERIALS AND METHODS

Propolis and Plant Resins. Brazilian propolis has been classified into 12 groups by physicochemical characteristics (*13, 14*). Among these 12 groups of propolis, three groups (group 3 from southern Brazil, group 6 from northeastern Brazil, and group 12 from southeastern Brazil) were sufficiently observed to determine which plant bud and unexpanded leaves were visited by the bees from respective honeycombs to collect resins. Thus, it was found that bees from a beehive containing group 3 propolis visited mainly the poplar tree bud, whereas bees from beehives containing groups 6 and 12 propolis visited mainly bud or unexpanded leaves of *Hyptis divaricata* and *Baccharis dracunculifolia*, respectively. The respective bud or unexpanded leaves were removed with a knife without breaking them into pieces, and immediately 2 g of respective samples (mixtures of bud and leaves) were mixed with 20 mL of 80% ethanol for 1 h and then centrifuged to separate the supernatant. The supernatants were used for chemical analysis. In the case of propolis, the ethanolic extracts were prepared as described previously (*15*). Dried propolis (approximately 50 g), was frozen in a freezer, and then immediately ground to a fine powder with a Waring blender. Then 2 g of the powder was mixed with 25 mL of 80% ethanol and shaken at 70 °C for 30 min. After extraction the mixture was centrifuged to give the supernatant, and the supernatants were used for analysis.

Reversed-Phase High-Performance Thin-Layer Chromatography (RPHPTLC). Precoated plates of silica gel RP-18 $F_{254}S$ for RPHPTLC were purchased from Merck Co. A 3-μL portion of the ethanolic extract of propolis and bud and unexpanded leaf exudates was applied to the lower edge of the plate, and ascending chromatography was run using a mobile phase of ethanol/water (55:45, v/v). The detection of flavonoids was carried out using UV−visualization at 366 nm.

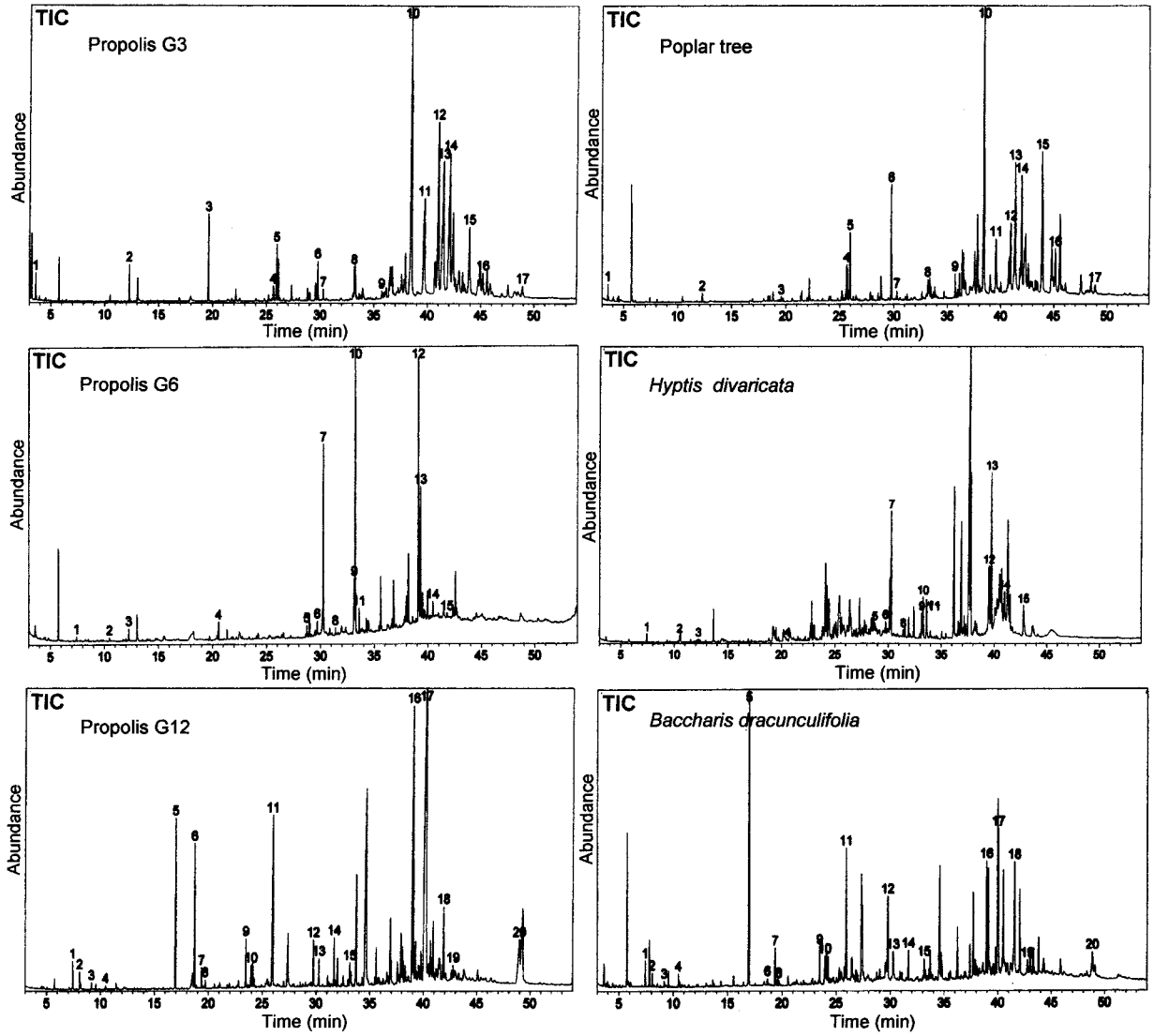

Figure 3. GC–MS profiles of ethanolic extracts of propolis and plant resins. Each line represents the total ion chromatogram (TIC) of one type of propolis (left) and its botanical origin (right). In each line the numbers of peaks represent the same compounds.

Reversed-Phase High-Performance Liquid Chromatography (RPHPLC). Analysis of flavonoids from ethanolic extracts of bud and unexpanded leaf exudates and ethanolic extracts of propolis was performed by RPHPLC with a chromatograph equipped with a YMC-Pack ODS-A column (RP-18, column size 4.6 × 250 mm; particle size, 5 μm) and photodiode array detector (SPD-M10A, Shimadzu Co.). The column was eluted by using a linear gradient of water (solvent A) and methanol (solvent B), starting with 30% B (0−15 min) and increasing to 90% B (15−75 min), held at 90% B (75−95 min), and decreasing to 30% B (95−105 min) with a solvent flow rate of 1 mL/min and detection with a diode array detector. Chromatograms were recorded at 268 nm. The authentic standards of flavonoids were purchased from Extrasynthase Co., France. Pinobanksin, pinobanksin 3-acetate, and 1,1-dimethylallylcaffeic acid ester were donated by Dr. E. Wollenweber, Darmstadt, Germany.

Gas Chromatography−Mass Spectrometry (GC−MS). Analyses of ethanolic extracts of propolis and plant resins were performed after methylation of the extracts as described by Markham et al. (*8*). Extract aliquots of 400 μL were introduced into glass vials. A solution of CH_2N_2 was added to each of the sample solutions. Samples were refrigerated for 4 h to allow complete methylation to occur. Samples of the methylated solutions were analyzed by GC−MS using a 30 m × 0.25 mm i.d. CBP5 column installed in a GC 17A (Shimadzu Co.) instrument interfaced to a QP 5000 mass selective detector operated in scanning mode (*m*/*z* 40−400). GC−MS analysis was temperature programmed from 50 °C (0.3 min hold) to 285 °C (15 min hold) at 6 °C min. Samples were injected using a AOC-17 autoinjector utilizing a splitless injection technique (0.6 μL injection volume). Integration was performed using QP5000 software.

Flavonoids, phenolic compounds, and 1,1-dimethylallylcaffeic acid were identified by comparing the data (retention time, molecular and fragment ions) obtained by GC−MS with those of methylated standards eluted under the same conditions. The other chemical compounds were tentatively identified by comparison with library mass spectra (Wiley 139-Shimazu).

RESULTS AND DISCUSSION

Propolis and Plant Resins. As described in previous publications (*13*, *14*), five groups of propolis were collected in southern Brazil, and we have observed in the case of propolis G3 that bees were visiting mainly a grove of poplar trees to collect resins (which are excreted on the surface of the buds and unexpanded leaves) in Santa Cruz, Santa Catarina State, where poplars are not native plants. The poplar tree buds and unexpanded leaves were collected as described in the Methods section. At the same time, the propolis collected by bees was cut from nearby beehives with a knife. Ethanolic extraction of both plant resins and propolis were carried out. In the southeastern region, such as the states of São Paulo and Minas Gerais, one group of propolis (propolis G12) was identified (*13*,

Table 1. Flavonoids and Other Constituents of Propolis and Plant Resins, Determined by HPLC.

retention time (min)	compound	propolis[a], mg/g			plant resins[a], mg/g		
		G3	G6	G12	poplar	*Hyptis divaricata*	*Baccharis dracunculifolia*
9.97	coumaric acid	4.8	-	8.5	4.2	-	0.9
10.88	ferulic acid	1.3	-	2.4	7.2	-	12.4
27.83	pinobanksin	54.9	-	8.7	70.1	-	7.8
35.83	kaempferol	3.4	-	0.4	6.1	-	0.2
37.58	apigenin	4.8	-	-	12.3	-	-
48.82	isosakuranetin	-	-	7.3	-	-	5.2
49.16	pinocembrin	54.4	-	-	40.2	-	-
50.25	dimethylallyl caffeic acid	2.2	-	-	6.3	-	-
51.56	pinobanksin 3-acetate	64.9	-	-	50.4	-	-
55.58	chrysin	54.0	-	-	69.6	-	-
58.96	galangin	35.7	-	-	36.1	-	-
62.37	kaempferide	-	-	12.3	-	-	8.1
66.15	λ 268 nm[b]	-	-	+	-	-	+
74.58	tectochrysin	11.3	-	-	22.8	-	-
80.25	λ 231 nm[b]	-	-	+	-	-	+
87.67	λ 223, 276 nm[b]	-	-	+	-	-	+
91.73	λ 246 nm[b]	-	+	-	-	+	-
97.26	λ 241, 274 nm[b]	-	+	-	-	+	-
99.58	λ 247 nm[b]	-	+	-	-	+	-

[a] Quantity of constituents is mg/g of propolis or mixture of buds and unexpanded leaf. Symbols: +, present, but not quantified; -, not detected [b] Unidentified constituents represent only UV spectral absorption maxima.

Table 2. Aromatic Compounds and Fatty Acid Esters Identified in Ethanolic Extracts of Propolis and Plant Resins by GC–MS

Rt (min)	compound	sample					
		propolis G3	poplar resin	propolis G6	*Hyptis divaricata* resin	propolis G12	*Baccharis dracunculifolia* resin
		aromatic compounds					
8.08	α-pinene	-[a]	-	-	-	+	+
9.18	β-pinene	-	-	-	-	+	+
12.29	benzoic acid	+	+	+	+	-	-
16.94	methyl hydrocinnamate	-	-	-	-	+	+
18.72	hydrocinnamic acid	-	-	-	-	+	+
19.37	benzoic acid, 4-methoxy	-	-	-	-	+	+
19.61	methyl cinnamate	+	+	-	-	-	-
23.98	benzenepropanoic acid	-	-	-	-	+	+
25.97	1,4-methanonaphthalen-9-ol	+	+	-	-	-	-
25.98	ρ-coumaric acid	-	-	-	-	+	+
29.76	ferulic acid	+	+	+	+	+	+
35.74	dimethylallyl caffeic acid ester	+	+	-	-	-	-
38.52	pinostrobin chalcone	+	+	-	-	-	-
		terpenoids					
20.58	beta-caryophyllene	-	-	+	+	-	-
23.48	farnesol	-	-	-	-	+	+
25.67	eudesmol	+	+	-	-	-	-
		fatty acid esters					
3.60	methyl lactate	+	+	-	-	-	-
10.49	butanedioic acid	-	-	+	+	+	+
30.26	hexadecanoic acid	-	-	+	+	+	+
30.30	eicosanoic acid	+	+	-	-	-	-
31.42	nonadecanoic acid	-	-	+	+	-	-
33.23	10-octadecenoic acid	-	-	+	+	-	-
33.30	9,12-octadecadienoic acid	+	+	+	+	+	+
33.60	octadecanoic acid	-	-	+	+	-	-

[a] +, Present; -, not detected.

14), but it was observed that bees visited mainly *B. dracunculifolia* buds and unexpanded leaves, and rarely visited 5 other species of *Baccharis* (*B. caprariaefolia*, *B. eriocladа*, *B. myriocephala*, *B. platipoda*, and *B. tridentate*), *Eucalyptus citriodora*, *Myrocarpus frondosus*, and *Araucaria angustifolia*. In the northeastern region, 6 groups of propolis were identified previously, but propolis G6 was collected near Salvador, Bahia State, where bees were observed visiting mainly *Hyptis divaricata* buds and unexpanded leaves. The collection and ethanolic extraction of the propolis and resins were performed as described above. All ethanolic extracts of both propolis and plant resins were analyzed by RPHPTLC, HPLC, and GC−MS.

RPHPTLC Profile. RPHPTLC (**Figure 1**) revealed that chromatographic profiles of propolis G3, G6, and G12 showed the same profiles as 3 (poplar resinous exudates), 6 (*H. divaricata* resinous exudates), and 12 (*B. dracunculifolia* resinous exudates), respectively. These results suggested that botanical origins of propolis G3 and G12 are poplar tree and

B. dracunculifolia resinous exudates. But propolis G6 and *H. divaricata* resinous exudates showed nothing apparent here for comparison, and did not show flavonoid profiles. In the case of the southeastern region, plant resinous exudates such as the five other species of *Baccharis* as described above, *E. citriodora*, *M. frondosus*, and *A. angustifolia* were also examined by RPHPTLC, and the chromatographic profiles were entirely different from those of *B. dracunculifolia* (data not shown). Therefore, the botanical origin of propolis G12 is mainly *B. dracunculifolia* resinous exudates.

Qualitative and Quantitative Comparisons of the Flavonoid Profiles. Flavonoid profiles of plant resins and propolis were carried out by HPLC on a C-18 reversed-phase column. The absorption spectra resulting from diode-array detection were used to distinguish peaks. Identification of flavonoid and other phenolic compounds was carried out by direct HPLC comparison with authentic standards. The identifications were also confirmed by GC−MS. Furthermore, one of the authentic standards, 1,1-dimethylallylcaffeic acid, is also included because it is a common propolis constituent and has been implicated as a causative agent of contact allergies in beekeepers (*16*, *17*). As shown in **Figure 2**, chromatographic profiles of propolis G3, G6, and G12 are exactly the same as those of poplar, *H. divaricata*, and *B. dracunculifolia*, respectively. These results clearly indicate the botanical origin of propolis. Furthermore, qualitative and quantitative comparisons of flavonoid profiles are shown in both **Figure 2** and **Table 1**. It is apparent that pinobanksin, pinocembrin, pinobanksin 3-acetate, chrysin, and galangin are the dominant flavonoids in propolis G3 and poplar tree samples that were collected in southern Brazil. Qualitatively, flavonoid profiles of the propolis resemble those found in propolis from other temperate regions such as Europe, North America, and West Asia (*2*, *6*−*9*). In contrast, flavonoid profiles in propolis G12 and *B. dracunculifolia* in southeastern Brazil mainly consisted of kaempferide, isosakuranetin, and trace amounts of kaempferol. Considerable amounts of phenolic compounds such as peaks 13, 15, and 16, which are not identified, were included in this propolis and *B. dracunculifolia*. It is also noted that propolis G6 and G12 did not contain 1,1-dimethylallylcaffeic acid. Finally, both propolis G6 and *H. divaricata* did not contain flavonoids.

Profiles of Non-Flavonoid Components. Samples of the methylated extracts of propolis and plant resins were analyzed by GC−MS. The total ion current (TIC) chromatograms of the samples are illustrated in **Figure 3**. The mass spectra for methylated compounds obtained by GC−MS tentatively identified the compounds by comparison with library programs. The results are shown in **Table 2**. It is apparent that profiles of nonflavonoid components of ethanolic extracts of propolis indicated similarity with ethanolic extracts of the respective plant resins.

The preliminary investigations showed similarity between RPHPTLC profiles of propolis G3, G6, and G12 and exudates of poplar tree, *H. divaricata*, and *B. dracunculifolia*, respectively. Subsequently, RPHPLC and GC−MS profiles also confirmed similarity. It can therefore be concluded that the botanical origins of propolis G3, G6, and G12 are resins of poplar tree, *H. divaricata*, and *B. dracunculifolia* bud and unexpanded leaves. Recently, it was also reported that ethanolic extracts of propolis, collected in São Paulo (southeastern Brazil), and ethanolic extracts of resins from *B. dracunculifolia*, *A. angustifolia*, and *E. citriodora* bud and leaves were examined using GC−MS, and *B. dracunculifolia* was shown to be the main source of propolis (*18*).

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