Direct Evidence for the Plant Origin of Brazilian Propolis by the Observation of Honeybee Behavior and Phytochemical Analysis

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To identify the plant origin of Brazilian propolis directly, we observed the behavior of honeybees in Minas Gerais State of Brazil. Honeybee workers bit and chewed leaves of the plant, *Baccharis dracunculifolia*, packed the material into their pollen basket, brought it back to their nest, and used it as propolis. We collected the leaves of *B. dracunculifolia* and propolis, and compared their constituents by liquid chromatography-mass spectrometry (LC/MS) analysis. There was no difference between the chemical constituents of the ethanol extracts of *B. dracunculifolia* and those of propolis. This indicates directly that the plant origin of Brazilian propolis is *B. dracunculifolia*.

Key words propolis; honeybee; plant origin; *Baccharis*; LC/MS

Propolis, a natural resinous substance collected by honeybees from buds and exudates of plants, has been considered to be used in the beehive as a protective barrier against their enemies. Recently propolis is extensively used in food and beverages to improve health and prevent diseases.[1)]

The composition of propolis depends on the place and time of collection. Much research and development has been implemented on propolis all over the world. Most of the recent studies are related to Brazilian propolis because it has been reported to possess a characteristic biological activity.[2)] It is generally accepted and chemically demonstrated that the bud exudates of poplar trees are the main source of propolis from Europe and China.[1)] On the other hand, the plant origin of Brazilian propolis has not been clarified, since there are no poplar trees in Brazil. Recently Bankova *et al.* and Midorikawa *et al.* have reported that the plants of *Baccharis* species are important sources of Brazilian propolis.[3,4)] However their studies were not based on the observation of honeybees, and they only compared the chemical constituents of propolis with those of plants.

In this study, we observed the behavior of honeybees in Minas Gerais States of Brazil to identify the plant origin of the propolis directly. Further, we comparatively analyzed the constituents of propolis and the plant leaves by HPLC with PDA (photo-diode array) and MS detection.

Experimental

Observing the Honeybees We observed the honeybees in Minas Gerais, Carvalhopolis in Brazil (SL21.75, WL45.87), where about 20 honeybee colonies were kept. The first and second observations were made on 28—29 Jan. 1998 and on 28—29 Jan. 1999, respectively. We found honeybee workers foraging on plants and recorded the leaf-collecting behavior of honeybees on the plant *Baccharis dracunculifolia* by VTR.

Sampling of *Baccharis dracunculifolia* and Propolis We collected the 18 kinds of young leaves of *B. dracunculifolia*, which the honeybees were chewing and collecting (sample **a**), and the propolis from the neighboring honeybees' nest (sample **b**). We compared the constituents of samples **a** and **b** by the following procedure.

One hundred milligrams of the *B. dracunculifolia* leaves and propolis was cut to small pieces and extracted with 5 ml of ethanol at room temperature. After 12 h, extracts were filtered with a 0.45 μm filter prior to 10 μl injection into the HPLC system with PDA and MS detection.

Apparatus for Analysis The HPLC system consisted of a SI-1 system (Shiseido, Tokyo, Japan) with a PDA detector. For the analysis of the sample, a Capcell Pak UG 120 (Shiseido, Tokyo, Japan) C18 column (2.0×250 mm, 5 μm) was used. The mobile phase consisted of water with 2% acetic acid (A) and acetonitrile with 2% acetic acid (B). The gradient was 20—80% B in 60 min at a flow rate of 0.2 ml/min. UV spectra were recorded from 195—650 nm at a rate of 0.8 spectrum/s and a resolution of 4.0 nm.

MS was performed on an LCQ ion trap mass spectrometer (ThermoFinnigan, CA, U.S.A.) equipped with an ESI (electrospray ionization) source. The operating parameters were as follows: source voltage 5 kV; ES capillary voltage −10 V; capillary temperature 260 °C. All MS data were acquired in the negative ionization.

Results

Behavior of Honeybees on *Baccharis dracunculifolia* The leaf-collecting behavior of 10 individuals was recorded. Workers used their mandibles to bite off the margins of young leaves and chewed the pieces. Then the material was passed to the forelegs, transferred to the mid legs, and then pressed against the corbicula of the hind leg. This chain of behavior was carried out in a fraction of a second (Fig. 1a).

This piece was unloaded by another nestmate worker using its mandibles and attached to an area of the nest where similar pieces had already been placed (Fig. 1b, c). This behavior was similar to that of collecting resin but chewing was an additional behavior. Honeybee workers collected the nectar from these plants but they foraged only the margins of the leaves without stretching their proboscis for leaf collecting. After observation, we sampled these plants and identified them as *Baccharis dracunculifolia* (Compositae).

Chemical Analysis We extracted the collected plants (*B.*

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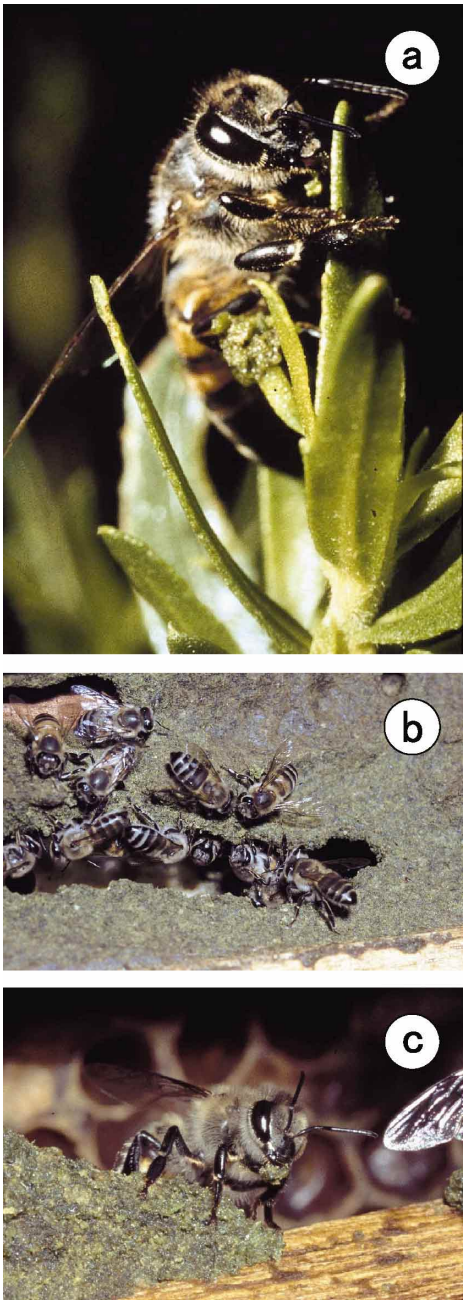

Fig. 1. Photographs of Honeybees Collecting the Leaves of *Baccharis dracunculifolia* to Bring Them Back to Their Nest as Propolis

dracunculifolia) and propolis with ethanol, and analyzed them by HPLC to compare their constituents. Fig. 2a and b show the HPLC chromatograms (280 nm) of the ethanol extracts of *B. dracunculifolia* and propolis. Peaks were assigned by comparing the retention times and UV spectra of authentic compounds by PDA detection. The HPLC chromatograms of samples **a** and **b** showed good coincidence as shown in Fig. 2. Further liquid chromatography-mass spectrometry (LC/MS) analysis was carried out to confirm the assignments of each peak.[5] Figure 3 shows the chemical structure of each compound determined by LC/MS. Negative ESI-MS of each HPLC peak corresponded to the molecular ions.

Prenylated derivatives of *p*-coumaric acid are the main components of Brazilian propolis.[2] We confirmed the existence of prenylated derivatives of *p*-coumaric acid such as

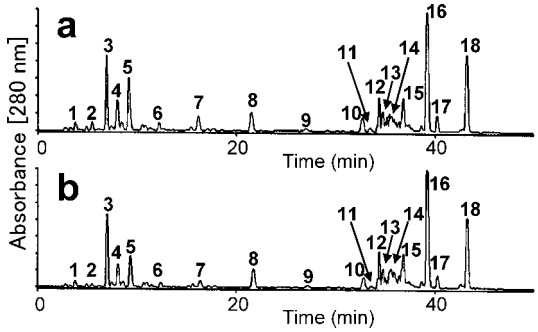

Fig. 2. HPLC Chromatograms of the Ethanol Extracts of *Baccharis dracunculifolia* (**a**) and Propolis (**b**)

drupanin (**10**) and (*E*)-3-prenyl 4-(dihydrocinnamoyloxy)-cinnamic acid (**18**) in both *B. dracunculifolia* and propolis. Artepillin C (**16**) has been reported to have antitumor activity and to be included specifically in Brazilian propolis.[2] As shown in Fig. 2, both *B. dracunculifolia* and propolis contained artepillin C.

The peak heights of 4,5-dicaffeoylquinic acid (**4**) and 3,4-dicaffeoylquinic acid (**5**) in the HPLC chromatogram of Fig. 2b were observed to be shorter than those in Fig. 2a. Compounds **4** and **5** are easily oxidized because they have antioxidant activity.[6] Thus these compounds are probably decomposed in the oxidation while they are collected as propolis.

Discussion

Propolis is a sticky material that honeybees collect from living plants. Human beings have used it since ancient times for its pharmaceutical properties.[7]

The compounds in propolis have been considered to originate from three sources: plant exudates collected by honeybees, secreted substances from bee metabolism, and materials that are introduced during propolis elaboration.[8] Bankova *et al.* reported that *Baccharis* and *Araucaria* species are important sources of propolis in the state of Sao Paulo of Brazil.[3] More recently Midorikawa *et al*. reported that *B. dracunculifolia* is an important source of propolis not only in Sao Paulo state but also in other states of Brazil.[4] Park *et al.* reported that the HPLC profile of *B. dracunculifolia* extracts resembles to that of the propolis from southeastern Brazil.[9]

B. dracunculifolia is classified into Compositae and indigenous throughout the southeast parts of Latin America and is used by local people as traditional medicine.[10] In Brazil, the local name of *B. dracunculifolia* is "Alecrim." The plant was reported to be one of the origins of Brazilian propolis by the chemical constituent analysis as described above. However, there are two problems when only the chemical constituents of the plant and propolis are compared. One is that the method would not identify the plant even if it had the same chemical constituents as those from propolis. The other is that the chemical constituents may vary with the part of the plant or even the plant's growth or season of sampling. Therefore the honeybees should be observed directly to confirm the assumed origin plant of propolis. Even if the plant could not be identified as the origin plant from the chemical analysis, it might have had the same chemical constituents as propolis if other parts had been sampled or if the plant had been collected in another season.

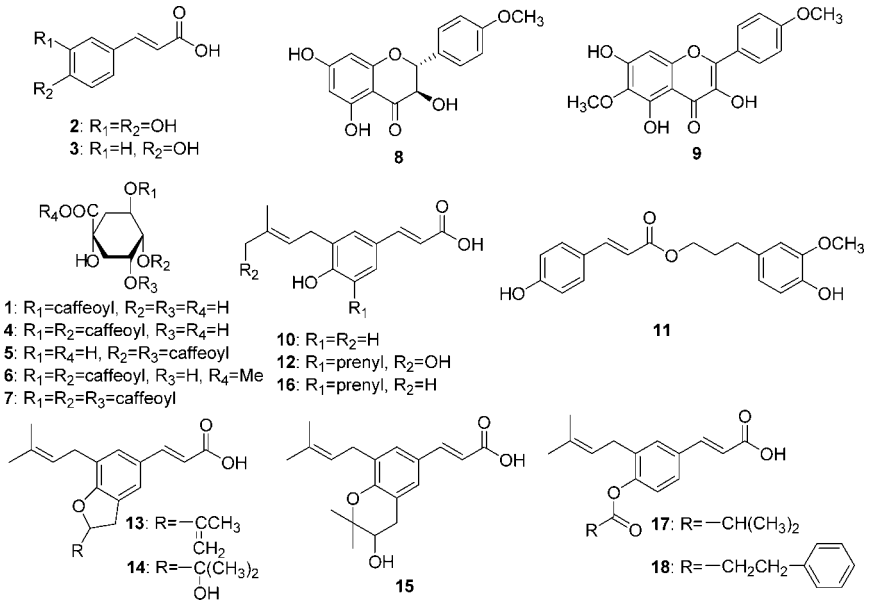

Fig. 3. Chemical Structures of Identified Compound

1: chlorogenic acid; **2**: caffeic acid; **3**: *p*-coumaric acid; **4**: 4,5-dicaffeoylquinic acid; **5**: 3,4-dicaffeoylquinic acid; **6**: 4,5-dicaffeoylquinic acid methyl ester; **7**: 3,4,5-tricaffeoylquinic acid (Compound identified on the basis of its mass spectrum.); **8**: dihydrokaempferide; **9**: 6-methoxykaempferol; **10**: drupanin; **11**: dihydroconiferyl *p*-coumarate; **12**: capillartemisin A; **13**: (*E*)-3-[2,3-dihydro-2-(1-hydroxy-1-methylethyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid; **14**: (*E*)-3-[2,3-dihydro-2-(1-methylethyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid; **15**: (*E*)-3-(2,2-dimethyl-3,4-dihydro-3-hydroxy-8-prenyl-2*H*-1-benzopyran-6-yl)-2-propenoic acid; **16**: artepillin C; **17**: (*E*)-3-prenyl-4-(2-methylpropionyloxy)-cinnamic acid; **18**: (*E*)-3-prenyl-4-(dihydrocinnamoyloxy)-cinnamic acid.

There was no difference between the chemical constituents of ethanol extracts of *B. dracunculifolia* and propolis (Fig. 2), implying that the honeybees did not add any ethanol soluble substances during the process from *B. dracunculifolia* to the propolis investigated in this study.

This is the first report directly showing that the origin plant of Brazilian propolis is *Baccharis dracunculifolia*. Further we have clarified that Brazilian propolis is made of pieces of the leaves from *B. dracunculifolia*.

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