

Phytochemical Evidence for the Plant Origin of Brazilian Propolis from São Paulo State

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Botanical Origin

Propolis and plant secretions from three species, most frequently mentioned as botanical sources of the bee glue in Brazil (*Baccharis dracunculifolia*, *Araucaria angustifolia* and *Eucalyptus citriodora*) have been investigated using GC-MS. Based on chemical evidence, *B. dracunculifolia* was shown to be the main propolis source in São Paulo state. The antibacterial and antifungal activities of all four materials were also tested, the most active being propolis and *Baccharis* leaf exudate.

Introduction

Propolis (bee glue) is a sticky dark-coloured material that honeybees collect from living plants, mix it with wax and use in construction and adaptation of their nests. It has been known as a remedy since ancient times and is still used in folk medicine (Ghisalberti, 1979), in "bio-cosmetics", "health foods" and for numerous further purposes (Matsuda, 1994; Wollenweber and Buchmann, 1997). Many studies have shown that in the temperate zones bees almost exclusively collect this material from the bud exudate of poplar trees. This is true for Europe (Tamas *et al.*, 1979; Popravko and Sokolov, 1980; Papay *et al.*, 1986; Greenaway *et al.*, 1987; Bankova and Kuleva, 1989), North America (Garcia-Viguera *et al.*, 1993), the non-tropical regions of Asia (Bankova *et al.*, 1993; Chi *et al.*, 1996) and even New Zealand (Markham *et al.*, 1995); in North Russia however, birch buds (*Betula verrucosa*) supply bees with the glue. In the last few years, tropical and especially South American propolis has become a subject of increasing interest, for both commercial and scientific reasons (Aga *et al.*, 1994; Tomas-Barberan

et al., 1993; Matsuda, 1994; Wollenweber and Buchmann, 1997). Since in tropics there are no poplar trees, the origin of tropical propolis is still an open question. Vegetable propolis sources have been reported from tropical countries (Crane, 1988); however, most of the reports are based on bee observations only and not on chemical analyses of propolis.

The best indicator for the origin of propolis is its chemical composition, compared to the composition of the hypothetical source plant material. In this work, we report a comparative chemical investigation of propolis from Brazil, São Paulo State, and some plant secretions (collected in the vicinity of the hives), that have been reported to be potential propolis sources: from *Araucaria* (Bankova *et al.*, 1996; Miyataka *et al.*, 1997), *Baccharis* (Bankova *et al.*, 1995; Wollenweber and Buchmann, 1997) and *Eucalyptus* (Crane, 1988; Miyataka *et al.*, 1997).

Experimental

Propolis

Propolis was harvested in the bee keeping Section of the School of Veterinary Medicine and Animal Husbandry of Botucatu, UNESP, in January 1998, from the hives of cultivated honey-bees (*Apis mellifera*).

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Plant material

Leaves from *Baccharis dracunculifolia* DC and *Araucaria angustifolia* (Bert.) O. Kunt, and trunk from *Eucalyptus citriodora* Hook were collected at the same location and the same time as the propolis and identified by Dr. R. C. S. Maimoni-Rodella, UNESP.

Extraction

35.4 g propolis was ground and extracted with 360 ml 70% ethanol at room temperature for 24 h (yield of dry extract 12.4 g, 53%). Fresh plant material was briefly extracted with acetone to dissolve the lipophilic surface material (exudate). *A. angustifolia*: 750 g leaves with 750 ml acetone, 2.54 g dry extract (0.3%); *B. dracunculifolia*: 245 g with 500 ml acetone, 22.5 g dry extract (9.1%), *E. citriodora*: 1215 g with 1500 ml acetone, 39.5 g dry extract (3.2%).

TLC analysis

TLC was carried out on silica gel Alufolien Kieselgel Merck F₂₅₄, mobile phases hexane/ethyl acetate 7:3 v/v; chloroform/ethyl acetate 7:3 v/v; chloroform/methanol/water 60:22:4 v/v/v. The spots were visualized by spraying with 60% sulfuric acid in ethanol and heating to 120°, or for phenolics by spraying with 20% ferric chloride in methanol.

GC-MS analysis

The GC-MS analyses were performed after silylation of the dry extracts (2.5 mg extract, 20 µl dry pyridine, 30 µl N,O-bis(trimethylsilyl)trifluoroacetamide, 80 °C, 20 min). A 30 m × 0.25 mm i. d. SPB-1 fused silica capillary column was used in a HP5890 gas chromatograph with a HP 5972 MSD detector. The linear velocity of the carrier gas (helium) was 38.2 cm. sec⁻¹. The samples were introduced *via* an all-glass injector working in the split mode, with a temperature program 80–240 °C at 8 deg. sec⁻¹, 240–300 °C at 121 deg. sec⁻¹. The identification was accomplished using computer search of commercial libraries. In some cases, when identical spectra were not found, only the structural type of the corresponding component was proposed, based on its mass spectrum. Reference compounds were co-chromatographed where possible to confirm GC retention times.

Isolation of compounds from plant exudates

Using column chromatography (CC) on silica gel with mobile phases chloroform–acetone with increasing polarities, from *B. dracunculifolia* exudates the flavonoids kaempferid (**1**), aromaden-drine-4'-methyl ether (**2**) and 5,6,7-trihydroxy-3,4'-dimethoxyflavone (**3**) were isolated and identified by comparison of spectral (UV, ¹H NMR, ¹³C NMR) and chromatographic properties with authentic samples. From *A. heterophylla* exudates, by CC on silica gel, mobile phase hexane-acetone with increasing polarity, *E/Z* communic acid (**4**) was isolated as the main component and identified by comparison of spectral (¹H NMR, ¹³C NMR) and chromatographic properties with an authentic sample.

Antibacterial tests

For the investigation of the antibacterial activity we used a modification of bioautography developed in our laboratory (Kujumgiev *et al.*, 1993). The test micro-organism was *Staphylococcus aureus* 209. The activity was measured as diameter of the inhibitory zones in the soft agar layer stained after a 72-h incubation at 37 °C with methylene blue according to Loeffler (Doetsch, 1981). An inhibitory zone of 5 mm corresponds to a lack of activity (5 mm is the diameter of the spot). Control experiments with solvent (ethanol) showed that the solvent did not have any activity. The inhibitory zones of 0.4 mg of each extract were measured.

Antifungal tests

The agar cup method was used (Spooner and Sykes, 1972), with *Candida albicans* 562 as the test strain. The activity was measured as a diameter of the inhibitory zones after 96 h incubation. The inhibitory zones of 0.5 mg of each extract were measured. An inhibitory zone with a diameter less than 10 mm corresponds to lack of activity (10 mm is the diameter of the agar cup). Control experiments with solvent (ethanol) showed that the solvent did not have any activity.

Results and Discussion

The preliminary investigations showed the striking similarity between TLC patterns of propolis

Table I. GC-MS data about composition of propolis and plant exudates.

| Compound | RRT ^a | Propolis | <i>Baccharis</i> % of TIC ^b | <i>Araucaria</i> % of TIC ^b | <i>Eucalyptus</i> |
|--|------------------|----------|---|---|-------------------|
| <i>p</i> -Coumaric acid ^c | 1.00 | 2.6 | 2.3 | 0.1 | 0.4 |
| Dihydrocinnamic acid ^d | 0.58 | 1.9 | 3.6 | — | — |
| Cinnamic acid ^d | 0.73 | — | — | — | 1.1 |
| Prenyl- <i>p</i> -coumaric acid ^e | 1.24 | 1.6 | 1.2 | — | — |
| Diprenyl- <i>p</i> -coumaric acid ^c | 1.38 | 18.5 | 37.0 | — | — |
| Aromadendrine-4'-methyl ether ^c | 1.46 | 2.8 | 1.5 | — | — |
| Kaempferid ^c | 1.74 | 1.8 | 1.1 | — | — |
| 3 ^c | 1.76 | 1.0 | 1.0 | — | — |
| β-Amyrine ^d | 1.78 | 2.8 | — | — | — |
| Cycloartenol ^d | 1.82 | 6.0 | — | — | — |
| Gallic acid ^c | 1.21 | — | — | — | 30.4 |
| <i>E/Z</i> Communic acid ^{c,f} | 1.22, 1.23 | — | — | 10.2 | — |

^a Relative retention time (to *p*-coumaric acid).^b Total ion current.^c Compounds identified by comparison with authentic samples.^d Compounds identified by comparison of the mass-spectra with library data.^e Compound identified on the basis of its mass spectrum.^f Both *E*- and *Z*-isomers, the % of TIC concerns both peaks (reference substance was a mixture, see Experimental).

and *B. dracunculifolia* leaf exudate. The GC-MS analyses confirmed this observations (Table I). The main components identified in *B. dracunculifolia* exudates and in bee glue, according to GC-MS, were almost the same: dihydrocinnamic acid, *p*-coumaric acid, prenyl- and diprenyl-*p*-coumaric acids and flavonoids in similar concentrations. The main flavonoids from *B. dracunculifolia* leaf exudate were then isolated using CC and identified

as kaempferid **1**, aromadendrine-4'-methyl ether **2** and 5,6,7-trihydroxy-3,4'-dimethoxyflavone **3** by comparison of their chromatographic and spectral (UV, ¹H NMR, ¹³C NMR) properties with those of authentic samples, previously isolated from Brazilian propolis in our laboratory (Boudourova-Krasteva *et al.*, 1997). Contrary to the prenylated coumaric acids, these flavonoids have not been found earlier in *Baccharis dracunculifolia*. This facts present unambiguous evidence that at this location *B. dracunculifolia* leaf exudate is the main propolis source, which is in accordance with our earlier hypothesis (Bankova *et al.*, 1995). Other propolis samples originating from São Paulo, claimed to contain *p*-coumaric acid and its prenylated derivatives (Aga *et al.*, 1994; Marcucci *et al.*, 1998) obviously have the same plant precursor. On the other hand, using GC-MS, in propolis we identified some components which were entirely absent in *B. dracunculifolia* exudates. These were not only the typical ones for bee glue fatty acids (C₁₆, C₁₈) and sugars but also some amounts of the triterpenes β-amyrin and cycloartenol. Surprisingly, according to GC-MS the latter compounds were not present in *A. angustifolia*, neither in *E. citriodora* (Table I). Their plant origin remains unclear. They are an indication that there is a second plant source, playing a minor role in propolis

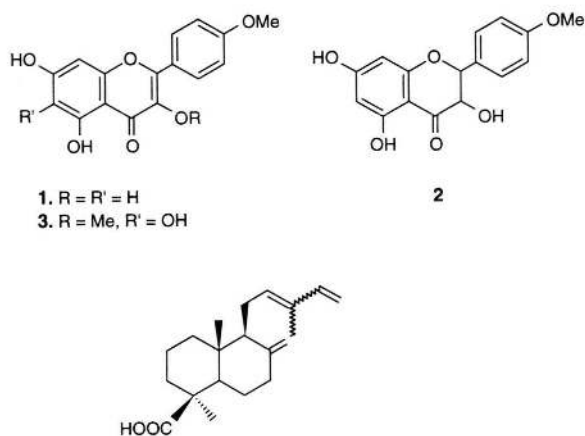


Fig. 1. Kaempferid (**1**), aromadendrine-4'-methyl ether (**2**), 5,6,7-trihydroxy-3,4'-dimethoxyflavone (**3**), *E/Z* communic acid (**4**).

Table II. Antibacterial and antifungal activity of propolis and its potential plant sources.

| Material | Antibacterial action ^a (diameter of the inhibitory zone \pm S.D. (mm)) ^c | Antifungal action ^b (diameter of the inhibitory zone \pm S.D. (mm)) ^c |
|---|--|---|
| Propolis | 10.5 \pm 0.5 | 15 \pm 1 |
| <i>B. dracunculifolia</i> leaf exudate | 9 \pm 1 | 16 \pm 2 |
| <i>A. angustifolia</i> leaf exudate | 8.2 \pm 0.3 | 0 |
| <i>E. citriodora</i> resin | 10 \pm 0.5 | 12 \pm 1 |

^a Against *S. aureus*.^b Against *C. albicans*.^c Mean of three measurements.

production (the ratio triterpenes/*Baccharis* components was about 1:7).

Eucalyptus species have been referred to as propolis sources in South America (Miyataka *et al.*, 1997; Bonvehi and Coll, 1994). Our investigation showed that the main components of *E. citriodora* resin are aromatic acids, a class of compounds that is usually found in bee glue, and sugars. However, the aromatic acid profile of propolis was different from this of *E. citriodora*. Propolis contained dihydrocinnamic, *p*-coumaric, ferulic and caffeic acids, as well as prenylated derivatives of *p*-coumaric acid, the 3,5-diprenyl derivative being one of the main components of the sample. On the other hand, *E. citriodora* resin contained cinnamic and *p*-coumaric acid (no prenylated derivatives), and, as a main constituent, gallic acid, which was entirely absent in propolis (Table I). Obviously, bees definitely prefer the compound combination offered by *Baccharis* leaf exudate!

A. angustifolia exudate contained only traces of aromatic acids, it consisted mainly of terpenes, especially diterpenic acids. The major component of this type was isolated and identified as a mixture of *E*- and *Z*-communic acid **4**, known *Araucaria* resin components. These compounds, along with other diterpenic acids, were found earlier in propolis from another location in Brazil and for this reason *Araucaria* species were proposed as possible bee glue source (Bankova *et al.*, 1996). In the propolis from Botucatu, however, no such compounds were identified.

The results obtained, as well as literature data, allow us to point out *B. dracunculifolia* as one of

the main sources of propolis in São Paulo State, Brazil. Its typical components, prenylated derivatives of *p*-coumaric acid, have been found in samples from this region investigated earlier, as already mentioned. It is not clear why the bees choose this particular plant source. On the other hand, propolis is thought to be a defence of bees against infections, so it was interesting to compare the antibacterial and antifungal activity of propolis and the plant exudates in order to establish if the bees have made a good choice. We investigated this activity, using as test strains the pathogens *Staphylococcus aureus* and *Candida albicans*. The results are presented in Table II.

The antibacterial activity of the four materials is similar, in the antifungal however, there are some differences. Propolis and *B. dracunculifolia* leaf exudate showed the highest (and practically identical) antifungal action, *E. citriodora* has lower activity and *A. angustifolia* is not active against the test strain. Obviously, the bees have chosen the best agent to protect their hives against bacterial and fungal infections.

The genus *Baccharis* is widespread in South America, so it appears meaningful to investigate some other species in order to find out if they are propolis sources, too.

Acknowledgements

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