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Original Article

Chemical Composition and Botanical Origin of Red Propolis, a New Type of Brazilian Propolis

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Red propolis is a new type of Brazilian propolis. This material, as well as the secretions of 20 plant species that are often mentioned as its probable botanical source, have been investigated by RP-HPTLC. Phytochemical evidence based on UV-VIS spectra, RP-HPLC and GC-MS, showed *Dalbergia ecastophyllum* (L.) Taub. to be the main source of red propolis in Alagoas state. The propolis and plant resin showed high relative percentages of the isoflavonoids 3-Hydroxy-8,9-dimethoxypterocarpan and medicarpin. To our knowledge this is the first report of the secretion of a leguminous species being the source of propolis.

Keywords: *Dalbergia ecastophyllum* – isoflavonoids – plant resin – red propolis

Introduction

Brazilian propolis is a non-toxic resinous substance, collected from plant buds or exudates by *Apis mellifera* bees, which was classified into 12 types according to physicochemical properties and related to geographic locations; however, the botanical origin of only three types were identified (1). The main botanical origin of the Brazilian propolis types 3 (Southern), 6 (Northeastern) and 12 (Southeastern), has been reported to be resins from *Populus* sp., *Hyptis divaricata* and *Baccharis dracunculifolia*, respectively (1). Brazilian propolis is quite diverse in chemical composition, due to Brazil's rich biodiversity, which needs to be investigated as a source of new bioactive substances, such as cinnamic acid derivatives (2), chiefly artepillin C (3), flavonoids (4) and others with pharmacological or functional properties.

A new type of propolis, named Brazilian red propolis (BRP) because of its color, as yet not classified, was found in Maceio City (Alagoas state, Northeastern Brazil) and has attracted the attention of international business. So far, this unique propolis has not been found elsewhere in Brazil.

The best approach to finding a plant source of propolis would be by an investigation to compare its chemical composition with that of the supposed plant source and by demonstrating that the source of this propolis is a plant resin, as in the case of red Brazilian propolis.

Thus, the main objective of this study was to identify the plant source and chemical composition of the new Brazilian red propolis, by exploratory phytochemical analysis of the microflora of resins produced in the mangrove area.

Material and Methods

Plant Propolis and Resin

Apis mellifera bees from the mangrove region in Marechal Deodoro (a city in the vicinity of Maceio, capital of Alagoas State, in Northeastern Brazil, wet

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tropical climate, SL 09.40 and WL 35.41) were visually monitored and photographically recorded to observe their behavior and vegetational preference in order to determine which plant was visited to collect resin for propolis production. Twenty plant samples (*Acrostichum aureum, Anacardium occidentale, Avicennia Germinans, Avicennia schaueriana*, *Borreria verticillata L. Byrsonima crassifolia, Byrsonima verbascifolia, Canavalia obtusifolia, Cnidosculus urens L. Conocarpus erectus, Cyperus liguralis L. Dalbergia ecastophyllum*, *Hibiscus pernambucencis, Hibiscus titiaceus*, *Ipomoea prescapae, Laguncularia racemosa, Paspalum vaginatum, Passiflora subrotunda Mart. Remirea maritima, Rhizophora mangle)* were collected after monitoring the bee visits. Propolis produced in the mangrove area was sampled from a collector inside a hive, and samples from all parts of vegetation visited by bees were collected. Plant material taxonomy of the botanical source of propolis was performed in the department of Biological Science of College of Agriculture ''Luiz de Queiroz'' (University of São Paulo) and a voucher specimen (reference number ESA 96543) was deposited in the herbarium of Biological Science of College of Agriculture 'Luiz de Queiroz' (University of Sao Paulo), Piracicaba, SP, Brazil.

Propolis Extraction

Propolis was ground to a fine powder and 2 g (dry weight) were mixed with 25 ml of 80% (v/v) ethanol and shaken at 70°C for 30 min. After extraction, the mixture was centrifuged and the supernatant was evaporated under low pressure to produce the ethanolic extract of propolis (EEP), which was prepared at 1% (p/v) with ethanol 80% (v/v).

Resin Extraction

Resin was extracted immediately after it was removed from the plant shoot surface with a knife. A 10 mg of sample (dry weight) of the resin was mixed with 1 ml of 80% (v/v) ethanol to prepare the ethanolic extract of resin (EER). Both EEP and EER were used for chemical analysis.

UV-VIS Spectra

UV-VIS spectra of the EEP and EER samples were recorded from mixture of 25 µl of each extract plus 30 ml of 96% ethanol. The mixture was scanned at 200–500 nm by UV-spectrophotometer (UVMini 1240, Shimadzu Co.).

Reversed Phase–High Performance Thin Layer Chromatography (RP–HPTLC)

Precoated silica gel plates RP-18 $F_{254}S$ were purchased from Merck Co. Six µL of EEP and EER were applied to the lower edge of the plate, and ascending chromatography was run using a mobile phase of ethanol:water (55:45, by v/v). After development, the chromatograms were observed under UV light at 366 nm.

Reversed-Phase High Performance Liquid Chromatography (RP–HPLC)

EEP and EER were performed by RP-HPLC using a chromatograph equipped with a Shimadzu ODS-A column (RP-18, column size 4.6 × 250 mm; particle size, 5 µm) and photodiode array detector (SPD-M10AVp, Shimadzu Co.). EEP and EER were filtered with 0.22 µm filter (Millipore) prior to 20 µl injected into the HPLC system. The column was eluted by using a linear gradient of water (solvent A) and methanol (solvent B), starting with 40% B and increasing to 60% B (45 min), held at 90% B (45–75 min), and decreasing to 30% B (75–85 min) with a solvent flow rate of 1 ml/min and detection with a diode array detector. Chromatograms were recorded at 260 nm as described by Park *et al.* (5). The following authentic standards of phenolic acids and flavonoids (Extrasynthese Co.) were examined: *p*-coumaric, ferulic acid, cinnamic acid, gallic acid, quercetin, kaempferol, kaempferide, apigenin, isorhamnetin, rhamnetin, sakuranetin, isosakuranetin, hesperidin, hesperetin, pinocembrin, chrysin, acacetin, galangin, myricetin, tectochrysin and artepillin C.

Gas Chromatography–Mass Spectrometry (GC-MS)

The EEP and EER samples were chemically analyzed after methylation of the extracts, as described by Markham *et al.* (6). Samples of the methylated solutions were analyzed by GC–MS by using a CP-Sil 8CB fused-silica capillary column (30 m × 0.25 mm; 0.25 µm film thickness) installed in a GC (Varian Saturn 2100D) instrument, interfaced to a Varian EM-AI mass selective detector, operated in scanning mode (m/z 40–400). The temperature program employed was 50 °C (0.3)–285 °C (15 min) at a rate of 6°C/min, with injection and detector temperatures maintained at 280 and 290°C, respectively. The split ratio was 20 : 1 with 0.5 µl of sample injected (EEP and EER). Carrier gas (He) was 1.0 ml/min. The GC–MS peaks were identified by comparison with data from literature (7) and the profiles from the Nist 98 library.

Results

Botanical Origin of Red Propolis

Twenty samples of plants were checked for the possibility of finding botanical origin and tested in our laboratory to compare the chemical profiles of propolis (EEP) and plant resin (EER) in order to select an identical

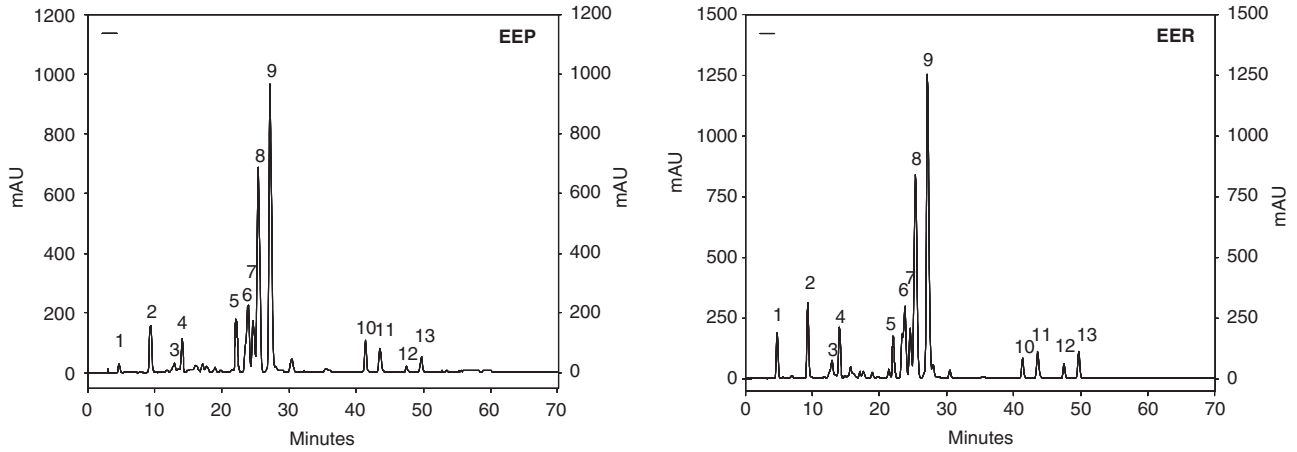

Figure 1. HPLC chromatograms of ethanolic extracts of red propolis (EEP) and *Dalbergia ecastophyllum* resin (EER). **1**, Fereulic acid; **2**, UV λ 238, 276 nm; **3**, Quercetin; **4**, UV λ 239, 246 nm; **5**, UV λ 237, 279 nm; **6**, UV λ 281, 284 nm; **7**, UV λ 237, 279 nm; **8**, UV λ 243, 320 nm; **9**, UV λ 232, 261 nm; **10**, UV λ 246, 265 nm; **11**, Chrysin; **12**, UV λ 246, 324 nm; **13**, UV λ 246, 265 nm.

Table 1. Relative percentages of compounds, determined by GC/MS, from ethanolic extracts of red propolis and *Dalbergia ecastophyllum* resin; compounds are referred to by their respective names or mass spectra data (molecular ion and base peak)

Retention time	Compounds	Relative%	
		Plant Resin	Propolis
20.24	208 $[M^+]$, 193 $[M\text{-}15]^+$	0.89	2.47
31.62	254 $[M^+]$, 223 $[M\text{-}31]^+$	0.71	0.80
35.64	284 $[M^+]$, 253 $[M\text{-}31]^+$	1.91	6.62
36.10	Medicarpin	22.20	6.12
37.33	3-Hydroxy-8,9-dimethoxypterocarpan	61.58	67.59
37.80	330 $[M^+]$, 300 $[M\text{-}30]^+$	1.55	2.25
38.00	330 $[M^+]$, 300 $[M\text{-}30]^+$ isomer	0.68	0.81
38.32	330 $[M^+]$, 298 $[M\text{-}32]^+$	4.01	7.93
38.53	330 $[M^+]$, 298 $[M\text{-}32]^+$ isomer	1.03	0.83

or similar profile. At first, only one plant showed an EER with profile similar to that of red propolis when compared with RP-HPTLC plate. The plant, locally and popularly identified as rabo-de-bugio (*monkey tail*), was later identified as *Dalbergia ecastophyllum* (L.) Taub. Family: Fabaceae (Leguminosae).

Chemical Assays

The results of UV-VIS spectroscopy, HPLC (Fig. 1) and GC-MS (Table 1) demonstrated that the chemical profile of *Dalbergia ecastophyllum* was similar to the chemical profile of red propolis. According to GC-MS, the main constituents of both extracts are the isoflavononoids medicarpin and 3-Hydroxy-8,9-dimethoxypterocarpan, the latter representing more than 60% of the composition in both extracts (Table 1).

Discussion

The chemical composition of propolis is variable depending on the biodiversity and the geographical origin of this natural substance (5,8).

In this study, a new type of Brazilian propolis, named red propolis, collected from Northeastern Brazil is presented. It has an intense red color and its chemical composition differs from that of the 12 types of Brazilian propolis classified by Park *et al*. (5).

From the results of UV–VIS spectroscopy, HPLC (Fig. 1) and GC-MS (Table 1), we could observe that not only did EEP and EER present a similar spectral pattern in the region between 200–500 nm, but also the same wavelength of maximum absorption at 282 nm. The EEP and EER analysis using HPLC also demonstrated an identical chemical profile. In addition, we found that the two extracts had at least 12 chemical substances in common, and in similar proportions (Fig. 1). Only two flavonoids (quercetin and chrysin) and one phenolic acid (ferulic acid), identified as standard in the 12 types of Brazilian propolis classified by Park *et al*. (5), were found in Brazilian red propolis. Absorption spectra obtained with a photodiode detector were used to compare and distinguish peaks. According to the results of the chemical profile obtained with HPLC, it was possible to state that this material proved to be a new type of Brazilian propolis.

The presence of the isoflavononoids medicarpin and 3-Hydroxy-8,9-dimethoxypterocarpan in both extracts is in agreement with Trusheva *et al*. (9) who also observed the presence of isoflavonoids, such as isosativan and medicarpin, in samples of Brazilian red propolis.

The isoflavonoids are compounds typical of the leguminosae family. Thus, these compounds may be useful as chemical markers of this new type of Brazilian propolis. Several isoflavonoids have already been found in *Dalbergia ecastophyllum*, among which are medicarpin

(10), corroborating that this species is the source of resin for the production of Brazilian red propolis.

Brazilian red propolis has a composition similar to that of a specific type of Cuban red propolis, produced in the province of Pinar Del Rio, which has various isoflavonoids, among them medicarpin and 3-Hydroxy-8,9-dimethoxypterocarpan (7). Nevertheless, Nepalese propolis also demonstrated to contain various biologically active neoflavonoids, in addition to the presence of the isoflavonoids medicarpin and (+)-vesticarpan (11). There are several studies in the literature, showing that isoflavones have antimicrobial, antifungal, anticancer, osteoporosis, antioxidant action and, relieve the symptoms of menopause. Thus, consumption of foods containing isoflavone phytoestrogens has been associated with a variety of health benefits (12–16).

From the results of UV-VIS spectroscopy, RP-HPTLC, RP-HPLC and GC/MS we conclude that *Dalbergia ecastophyllum* resin is the botanical source of Brazilian red propolis and that this can be considered a 13th type of Brazilian propolis, complementing the 12 types proposed by Park *et al*. (5). This is the first time the botanical origin of a type of propolis is reported as occurring in a species of the Leguminosae family, rich in isoflavonoids.

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