Original Article

Brazilian Red Propolis—Chemical Composition and Botanical Origin

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Propolis contains resinous substances collected by honey bees from various plant sources and has been used as a traditional folk medicine since ca 300 BC. Nowadays, the use of evidence-based complementary and alternative medicine (CAM) is increasing rapidly and so is the use of propolis in order to treat or support the treatment of various diseases. Much attention has been focused on propolis from *Populus* sp. (Salicaceae) and *Baccharis dracunculifolia* (Asteracea), but scientific information about the numerous other types of propolis is still sparse. We gathered six samples of red propolis in five states of Northeastern Brazil. The beehives were located near woody perennial shrubs along the sea and river shores. The bees were observed to collect red resinous exudates on *Dalbergia ecastophyllum* (L) Taub. (Leguminosae) to make propolis. The flavonoids of propolis and red resinous exudates were investigated using reversed-phase high-performance liquid chromatography and reversed-phase high-performance thin-layer chromatography. We conclude that the botanical origin of the reddish propolis is *D. ecastophyllum*. In areas where this source (*D. ecastophyllum*) was scarce or missing, bees were collecting resinous material from other plants. Propolis, which contained the chemical constituents from the main botanical origin, showed higher antimicrobial activity.

Keywords: *Apis mellifera*—botanical origin—*Dalbergia ecastophyllum*—flavonoids—propolis

Introduction

Propolis is a resinous mixture of substances collected by honey bees (*Apis mellifera*) from various plant sources. It is used by the bees e.g. to seal holes in their honeycombs and protect the hive entrance (1,2). Due to its large variety of biological activities, it has been successfully used in balsam and ointments to treat battle wounds (3). It has been used as a traditional folk medicine since ca 300 BC. Numerous biological properties have been reported including cytotoxic (4), antiherpes (5), antitumor (6), radical scavenging (7), antimicrobial (8), antifungal (9), anti-HIV (10) and suppressive effects of dioxin toxicity (11). As a result of this wide range of

biological activities, propolis is now increasingly being used as a health food supplement and in beverages (12).

Previously, we reported on the classification of Brazilian propolis into 12 groups, based on physiochemical characteristics, five in southern Brazil, one in southeastern Brazil and six in northeastern Brazil. It was also reported that the main botanical origin of propolis group 3 was the bud resin of *Populus* (Salicaceae). The botanical origin of propolis group 6 and 12 were resinous coatings from young leaves of *Hyptis divaricata* (Lamiaceae) and *Baccharis dracunculifolia* (Asteracea), respectively (13).

Propolis normally is a dark yellow or brownish resinous material. Recently, we found reddish propolis in beehives located along the sea and river shores in northeastern Brazil.

Previously, Trusheva *et al.* (14) reported bioactive constituents of Brazilian red propolis, but they did not

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describe the botanical origin of red propolis. We observed that bees kept in that area were collecting the reddish exudates on the surface of *Dalbergia ecastophyllum* (L) Taub. (15,16), it was assumed that this was the botanical origin of the reddish propolis. We therefore analyzed comparatively samples of the plant exudates as well as of this special propolis.

Materials and Methods

Propolis and its Botanical Origin

As indicated in the introduction, the reddish propolis was collected from beehives located in woody perennial shrubs along the sea and river shores in the states of Bahia, Sergipe, Alagoas, Pernambuco, and Paraíba in northeastern Brazil. The red resinous exudates secreted from a hole in a branch of *D. ecastophyllum* that was made by tree-boring-insects as shown in Fig. 1A. It was observed that the bees visited mainly *D. ecastophyllum* to collect the resinous exudates on its surface and from holes in its branches (Fig. 1B). The resin issued from these holes was collected and then passed to the hind leg (Fig. 1C). Samples of the red exudates (Fig. 1A and B) were collected for analysis and compared with samples of propolis collected from a beehive that was located in the same area. The red resinous exudates were dissolved in 80% ethanol. In the case of propolis, approximately 50 g of the red propolis were collected from one beehive that was located in the same area. We collected six same samples of red resinous exudates from the botanical origin and six samples of red propolis from respective states to examine the quality of the propolis.

Preparation of Ethanolic Extracts of Red Resinous Exudates and Propolis

500 mg of red resinous exudates were mixed with 5 ml of 80% ethanol and the mixtures were shaken for 10 min at 70°C. After centrifugation, the supernatant was used for analysis. Propolis samples (~50 g) were frozen in a freezer and then immediately grounded to a fine powder with a blender. Then, 2 g of the powder were mixed with 25 ml of 80% ethanol and shaken at 70°C for 30 min. After extraction, the mixtures were centrifuged and the supernatants used for analysis.

Reversed-Phase High-Performance Thin-Layer Chromatography (RPHPTLC)

Portions of 3 µl of the ethanolic extracts of propolis and resinous exudates solutions were plated on pre-coated plates of silica gel RP-18F₂₅₄S for RPHPTLC purchased from Merck Co. and were chromatographed using

Figure 1. Collection of propolis from reddish resinous exudates of *D. ecastophyllum* by africanized *Apis mellifera*. (A) Secrete reddish exudates from a hole in a branch of the tree. (B) Bee is collecting the reddish exudates. (C) The collected exudates passed to the hind leg to make propolis.

ethanol/water (55:45, v/v) as solvent. The detection of flavonoids was carried out using UV-visualization at 366 nm.

Reversed-Phase High-Performance Liquid Chromatography (RPHPLC)

Analysis of flavonoids and other phenolic compounds from ethanolic extracts of propolis and red resinous

Figure 2. RPHPTLC of the ethanolic extracts of propolis and reddish exudates from *D. ecastophyllum*. 3 μ L of respective solution described in method were applied. (A) Ethanolic extracts of propolis; (B) Ethanolic extracts of reddish exudates from sample of Fig. 1A; and (C) Ethanolic extracts of reddish exudates from sample of Fig. 1B.

exudates were performed by RPHPLC with a chromatograph equipped with an YMC Pack ODS-A column (RP-18, column size 4.6 $\times$ 250 mm; particle size 5 μ m) 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 and other chemical compounds were purchased from Extrasynthese Co. France.

Figure 3. RPHPLC of ethanolic extracts of propolis and reddish resinous exudates from *D. ecastophyllum*. Respective numbers of peak represent chemical constituents that were described in Table 1.

Antimicrobial Activity of Ethanolic Extracts of Propolis and Resinous Exudates

Examination of antimicrobial activity of propolis to *Staphylococcus aureus* ATCC 25923 was determined according to the method described in (17). Actively growing nutrient broth cultures of *S. aureus* were inoculated in nutrient agar plates with sterile swabs, which were dipped in broth culture. On the inoculated plate, disks with extracts of propolis were placed and incubated overnight at 37°C. The extracts of propolis and resinous exudates were prepared by submerging 10 μ L into Whatman filter paper no. 3 disks (5 $\times$ 1 mm) and dried under low vacuum at room temperature overnight and then incubating at 60°C for 4 h.

Results

Red Propolis and Its Botanical Origin

We observed that bees were collecting the red resinous exudates on surfaces of *D. ecastophyllum* to produce propolis as shown in Fig. 1. The samples of both propolis and resinous exudates were analyzed by RPHPTLC and RPHPLC.

RPHPTLC (Fig. 2) revealed that chromatographic profiles of propolis (Fig. 2A) showed the same profile as the red resinous exudates (Fig. 2B and C) from the

Table 1. Flavonoids and other chemical constituents of propolis and *D. ecastophyllum*

Peak	Retention time (min)	Compound	Propolis [†] Content (mg/g)	<i>D. ecastophyllum</i> [‡] Content (mg/g)
1	13.42	Rutin	0.7	1.3
2	16.99	Liquiritigenin	1.8	7.1
3	20.63	Daidzein	0.3	4.3
4	22.35	Pinobanksin	1.7	6.0
5	23.84	UV λ 251, 292 nm [*]	+	+
6	24.59	Quercetin	0.5	1.9
7	28.40	Luteolin	1.2	2.1
8	30.46	UV λ 241, 272, 282 nm [*]	+	+
9	32.15	Dalbergin	0.4	0.9
10	34.62	Isoliquiritigenin	4.8	12.1
11	36.97	Formononetin	10.2	19.5
12	39.28	UV λ 235, 263 nm [*]	+	+
13	40.08	Pinocembrin	3.3	7.1
14	42.30	Pinobanksin-3-acetate	1.7	2.6
15	46.45	Biochanin A	0.5	1.5
16	55.96	UV λ 238, 260, 269 nm [*]	+	+
17	60.53	UV λ 233, 249, 329 nm [*]	+	+
18	63.43	UV λ 233, 256 nm [*]	+	+

[†]Quantity of constituents in mg/g of propolis and *D. ecastophyllum*.

Symbols: '+' means present, but not quantified.

[‡]Unidentified constituents represent only UV spectral absorption maximum.

surface of *D. ecastophyllum*. These results suggested that *D. ecastophyllum* is the botanical origin of the red propolis. Furthermore, these results were confirmed by RPHPLC as shown in Fig. 3.

Figure 3 showed profiles of qualitative and quantitative comparisons of the flavonoids and other chemical constituents in propolis and resinous exudates from *D. ecastophyllum*. The chemical constituents were quantified by RPHPLC. Identification of the chemical compounds was carried out by direct comparison with authentic standards and was based on retention time, co-chromatography and on the identity of the absorption spectra. The profiles of Fig. 3 and Table 1 indicated that the chromatographic profiles of propolis were exactly the same as those of *D. ecastophyllum*. These results clearly indicated the botanical origin of the propolis (see quantitative comparisons of flavonoids and other chemical constituents in Table 1). We also collected six further samples of red propolis in respective states. All states showed the similar results and here we demonstrate the results of Alagoas state in Fig. 4. Figure 4C showed the

Figure 4. RPHPLC and RPHPTLC of the ethanolic extracts of 6 samples of propolis (one sample from one beehive) collected in same regions.

same redness of ethanolic extracts. But the degree of redness is variable, for instance the redness of samples one and six showed a weaker redness than the others. According to Fig. 4B propolis samples two, three, four and five showed nearly identical profiles. But sample one and six appeared weaker. Finally, Fig. 4A demonstrated RPHPLC with similar characteristics in sample two, three, four and five and similar chemical constituents and quantitative amounts (Table 2). It indicates that the botanical origin of these samples is the same. But samples one and six demonstrated similar chemical constituents in extremely lower concentrations than in samples two,

Table 2. Flavonoids and other chemical constituents of red propolis

Peak	Retention time (min)	Compound	Propolis2 [†] Content (mg/g)	Propolis3 [†] Content (mg/g)	Propolis4 [†] Content (mg/g)	Propolis5 [†] Content (mg/g)
1	13.42	Rutin	0.7	1.1	0.9	0.6
2	16.99	Liquiritigenin	1.8	5.7	5.0	2.2
3	20.63	Daidzein	0.3	0.6	0.5	0.2
4	22.35	Pinobanksin	1.7	5.7	4.9	3.3
5	23.84	UV λ 251, 292 nm [‡]	+	+	+	+
6	24.59	Quercetin	0.5	3.0	4.5	2.6
7	28.40	Luteolin	1.2	2.5	2.4	1.5
8	30.46	UV λ 241, 272, 282 nm [‡]	+	+	+	+
9	32.15	Dalbergin	0.4	2.4	2.5	1.4
10	34.62	Isoliquiritigenin	4.8	9.9	8.3	5.5
11	36.97	Formononetin	10.2	10.7	10.9	10.5
12	39.28	UV λ 235, 263 nm [‡]	+	+	+	+
13	40.08	Pinocembrin	3.3	9.8	8.3	7.3
14	42.30	Pinobanksin-3-acetate	1.7	3.6	2.4	2.3
15	46.45	Biochanin A	0.5	2.1	1.7	0.4
16	55.96	UV λ 238, 260, 269 nm [‡]	+	+	+	+
17	60.53	UV λ 233, 249, 329 nm [‡]	+	+	+	+
18	63.43	UV λ 233, 256 nm [‡]	+	+	+	+

[†]Quantity of constituents in mg/g of propolis and *D. ecastophyllum*.

Symbols: '+' means present, but not quantified.

[‡]Unidentified constituents represent only UV spectral absorption maximum.

three, four and five. On the other hand other chemical compounds were shown (Retention time 80–100 min). These are probably from other botanical origins.

Antimicrobial activity

Antimicrobial activities of six samples of propolis to *Staphylococcus aureus* ATCC 25923 were measured according to the method described above and the results are shown in Figure 5. Samples two, three, four and five demonstrated the highest inhibition of bacterial growth as compared with samples one and six, which contained lower concentrations of the chemical constituents of *D. ecastophyllum*, but also contained chemical constituents from other plants.

Discussion

As described in previous publications (13,18), Brazilian propolis has been classified into 12 groups by physico-chemical characteristics. Among these 12 groups of propolis, three (group 3, 6 and 12) were sufficiently observed to determine which plant bud and unexpanded leaves the bees visited to collect the resins. Recently, we found reddish propolis from beehives which were located along the sea and river shores in Northeastern Brazil.

Figure 5. Growth inhibition of *Staphylococcus aureus* ATCC 25923 by extracts of propolis. 10 μ L of respective solution described in method were applied to the disks.

We found that Brazilian red propolis contained liquiritigenin, daidzein, dalbergin, isoliquiritigenin, formononetin and biochanin A. Three of them (daidzein, formononetin and biochanin A) are isoflavonoids. However, previously it was reported that Cuban red

propolis also contained isoflavonoids (19). The isoflavonoids are a very restricted distribution in the plant kingdom and occur almost exclusively in Leguminosae family such as soybeans, chickpeas and lentils (19). It is interesting that the presence of the isoflavonoids in *D. ecastophyllum* was found by Donnelly *et al.* (15). It is well known that dietary consumption of food and food additives containing isoflavonoids has been associated with a variety of health benefits including relief of symptoms of menopause e.g. osteoporosis, hormonal cancer and prostate cancer.

It was already reported that the extracts of South American *D. ecastophyllum* (Leguminosae), contained liquiritigenin, daidzein, dalbergin, isoliquiritigenin, formononetin and biochanin A (15). Moreover isoliquiritigenin inhibits the growth of prostate cancer (20), whereas liquiritigenin and isoliquiritigenin inhibit xanthine oxidase. Inhibition of xanthine oxidase has been suggested for the treatment of hepatitis and brain tumor because it increased the serum xanthine oxidase levels (21).

Samples one and six also showed the presence of these compounds, but in quantitatively lower concentrations and showed some unidentified peaks that were not found in *D. ecastophyllum* exudates. We observed that the samples of propolis one and six were collected from beehives, which were located in areas where *D. ecastophyllum* was scarce, so that the bees collected from other plants. Therefore, we intend to investigate further the botanical origin of the red propolis which rarely demonstrated unknown constituents in the next project.

Conclusions

Majority samples of red propolis, which were collected from beehives located near woody perennial shrubs along the sea and river shores in Northeastern Brazil (six samples from each State), were analyzed. We observed that bees were collecting the red resinous exudates from surface of *D. ecastophyllum* to produce propolis. All samples of propolis and red resinous exudates showed very similar profiles of RPHPTLC and RPHPLC.

Therefore, the main botanical origin of the propolis is *D. ecastophyllum*. But samples of propolis collected from beehives in areas where *D. ecastophyllum* was scarce, showed lower concentrations of the chemical constituents found in *D. ecastophyllum*, instead other chemical compounds appeared (retention time 80–100 min) that were not found in *D. ecastophyllum*. Consequently, the propolis demonstrated a lower antimicrobial activity. This means that the bees collected resins from different

plants to produce propolis. Therefore, the botanical origin and its abundance are essential for the production of this type of propolis.

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