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Exotic flora dependence of an unusual Brazilian propolis: The pinocembrin biomarker by capillary techniques

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Abstract

Significant amounts of pinocembrin (>10%), a dihydroxy-flavanone, was found in the composition of an unusual brand of a subtropical Brazilian propolis. Incidentally, this sealing material was obtained from hives surrounding a large forestry site based on a single exotic flora, namely poplar (*Populus* sp.). Examination of the different botanical parts of poplar revealed the buds as the main source of the flavanone. Techniques used for the establishment of the chemical correlation between the propolis brand and the poplar buds were TLC/densitometry, capillary GC–MS in the e.i. mode, and CZE with DAD monitoring. Since color enhancement after Al^{3+} complexation applies just for more hydroxylated flavonoids, the alternative techniques herein applied were of value for pinocembrin detection and estimation. Analytical data indicated the dominance of the main phenolic pinocembrin biomarker as well as the presence of other related flavonoids in the botanical source and in the propolis derived thereof.

Keywords: Propolis; Pinocembrin; Flavonoids; CZE; GC–MS; TLC-densitometry; Chromogeny

Propolis, the beehive sealing material, is one of the most heterogeneous natural cocktail built from plant sources. Hundred(s) of components – some of them like the flavonoids and modified phenol-carboxylic acids bioactive against human pathogens – can be detected in propolis following chemical TMS-derivatization and capillary GLC inspection [1]. Other biological activities such as against inflammations, ulcers, and tumors may also correlate with the antioxidant activity of these phenols [2]. The flavonoids encompass a rather complex family of natural compounds since more than 4000 were described [3]. Naringin, a glycosylated form of the flavanone naringenin, when associated to food colorants like anthocyanin or carmin, displays a marked reduction of induced hyperlipidemia [4].

Although surrounding flora to beehives is the main source for propolis components, the chemical correlation between donor plants and propolis is a hard task since bees feed on a lot of different botanical sources to produce the sealing material. Several literature reports deal with the similarity or identity of a few plant natural substances to the same components found in propolis but no report had yet established complete identity between a defined plant source and the resulting propolis whole composition. Reason for this is simply the flora diversity found close to the hives as well as, to some extent, the biochemical ability of bees altering the native composition or adding own components to propolis (e.g., bee wax). Apiculture activity in the vicinity of a homogeneous forest can facilitate this kind of search and chemical correlation. This was the purpose of the present report considering the availability of an intense forestry on *Populus deltoides* in the mid-South geographical region of Paraná State, Brazil. The European plant was brought to Brazil by Swedish entrepreneurs about a century ago. The exotic poplar timber from *P. deltoides* (also from *P. euro-americana*, in a minor extent) is exclusively used for a local matches manufacture industrial plant.

Propolis, mainly under the form of hydroethanolic extratcts, is finding increase room in the pharmaceutical market due to the use in creams, tablets, shampoos, and toothpastes [5]. In fact more than a 10th of bioactivities are reported including inhibition of fungi, bacteria and viruses [6].

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The inhibition power is very often correlated with the occurrence of flavonoids and/or phenol-carboxylic acids or their esters. For instance, a pinocembrin content of 4.1–4.5% was reported for propolis samples from five different countries [7]. Due to its antiandrogenic activity, this 5,7-dihydroxy-flavanone has potential application in cases of androgen-dependend hyperplasia of prostate, hair loss, and even cancer. Other inhibitory actions of pinocembrin were reported for testosterone reductase and sarcoplasmic reticulum Ca^{2+}-ATPase [8,9]. *Staphylococcus aureus* is involved in nosocomial infections. Concerning the flavonoid antibacterial activity against this particular pathogen, *inter alia*, pinocembrin is reported as one of the most active [10]. Pinocembrin was also indicated as one of the propolis components responsible for the inhibition of glucosyltransferases of *Streptococcus mutans*, an oral microorganism involved in the cariogenic process [11].

Gas chromatographic analysis of 10 propolis samples arising from European countries have appointed, in two cases, unexpected composition due to the enrichement in phenolic glycerides and diterpenic acids [12] instead of the expected flavanone poplar profile.

Since propolis collected from homogeneous reforest regions may be valuable for pharmaceutical formulations enriched in flavonoids, the confirmation of the chemical relationship between the flora available to bees and propolis as final product is desirable.

1. Experimental

1.1. General procedures

Spectrophotometric quantitation of total flavonoids followed the aluminum nitrate/potassium acetate procedure [13].

In order to detect flavonoids among the propolis components turned volatile under moderately high temperatures GC–MS analyses were performed with a GC 17/GC/MS QP-5000 module from Shimadzu using non-derivatized ethanol-extracted samples and a 30 m capillary column HP-5 with 0.2 mm int. diam., 30 μm film of 5% phenyl–95% poly-dimethylsiloxane from 100 °C (2 min hold time) to 300 °C (15 min hold time) at 10 °C/min and a FID detector.

Capillary electrophoresis of buffered samples of weakly ionized phenols filtered by a 0.22 μ Millipore membrane was carried out in a 65 m fused silica capillar (i.d. of 50 μ) with a sodium tetraborate (30 mM)/sodium phosphate (50 mM) buffer (pH 8.5) modified with the inclusion of 12% of methanol in order to improve flavonoids solubility. The routine voltage was 20 kV generating a current of 53 μA and the flavonoids were monitored by a DAD device at 220, 250, and 320 nm as the main absorption wavelengths for pinocembrin.

1.2. Propolis sampling and processing

Propolis samples (0.5 kg each) were collected by Breyer Ltd. Co. at the geographical region around the town of União da Vitória in the mid-South of State of Paraná, Brazil (around latitude 51° and altitude 26° 2″). Sample PP-2-WDC was from *Apis mellifera* hives close to a heterogenous native flora (mainly angyosperm) and sample PP-4-AL from a more homogeneous flora mainly composed by a forestry with exotic poplar (gymnosperm). The later was also the source for buds and other aerial botanical parts which were lyophilized just after the crop. The biological materials were extensively extracted with warm ethanol in order to provide the initial crude organosolvent extracts. These were fractionated in columns of silicic acid (or of silica gel G as a preliminary batch procedure) using a progressive polarity-gradient from hexane:ethyl acetate from 99:1 to 1:99, the final columns washing being carried out with methanol. Fractions enriched in components reacting to hot sulfuric anisaldehyde (105 °C; 3–5 min) as strong orange colors like those obtained with hexane-ethyl acetate 90:10–50:50 fraction were selected as enriched preparations in pinocembrin and related flavonoids.

1.3. Standard substances

Pinocembrin standard and related flavonoids were purchased from Extrasynthese (Genay, France) and the standards of phenol carboxylic acids were obtained from Sigma–Aldrich Co (St. Louis, MO, USA).

1.4. TLC and densitometry

Analytical TLC was made on silica gel plates (Merck, art. 1.05553) using 1.5 successive developments of the plates with hexane: ethyl acetate (3:2, v/v) as mobile phase. Densitometry with a Shimadzu CS-9301PC flying spot-densitometer of the selected TLC lanes following the hot spray with 0.5% anisaldehyde–5.0% sulfuric acid in methanol was obtained from the colored pictures taken under visible or UV (365 nm) light with a digital camera.

2. Results and discussion

2.1. Ecological aspect

Visit of bees to poplar trees was often observed and a photographic shoot was taken for *A. mellifera* feeding on poplar buds (Fig. 1).

Fig. 1. *A. mellifera* visit to a *P. deltoides* bud.

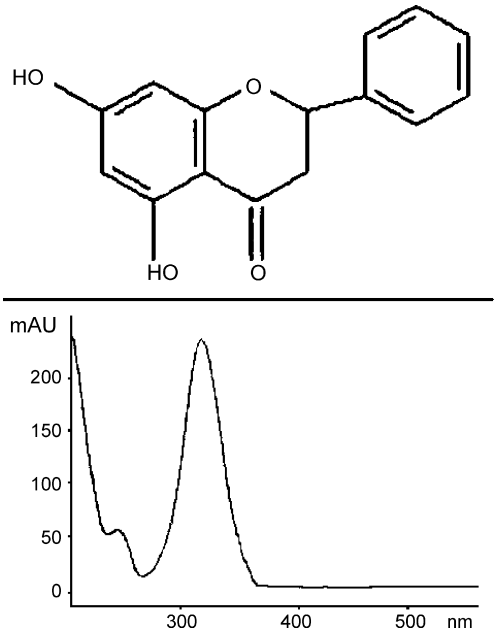

Fig. 2. The flavanone pinocembrin or dihydrochrysin or 4*H*-1-benzopyran-4-one, 2,3-dihydo-5,7-dihydroxy-2-phenyl and its UV–vis spectral profile in the alkaline buffer used for capillary electrophoresis.

2.2. Physical and chemical analyses

The main natural compound formula on focus, pinocembrin, and its UV–vis spectrum are shown in Fig. 2.

2.3. Differential features of propolis and bud samples

PP-2-WDC is a gray-greenish breakable material and PP-4-AL, conversely, is a light brown and stick mass. Their respective extracts are also clearly distinguishable by the color hues as light and deep yellowish liquids, respectively. Yields of the exhaustive warm ethanolic extracts were also quite different: 0.331 and 0.718 mg/g for PP-2-WDC and PP-4-AL, respectively. The later yield was similar to the previous one (70.2%) obtained with cumulative extractions of other propolis sample using cold and warm ethanol sequentially [1]. Dry buds, conversely, yielded only about 10% of solids, although more enriched in pinocembrin and related flavonoids (see Fig. 3A; PB sample).

2.4. Spectrophotometry of total flavonoids as Al^{3+} complexes

Spectrophotometry of the ethanol extracts in the presence of aluminum nitrate/potassium acetate and using the usual and pure quercetin for the calibration curve indicated total flavonoid contents of 0.8, 4.9, and 10.4%, respectively for PP-2-WDC, poplar buds, and PP-4-AL. No remarkable amount of pinocembrin was found in other botanical parts of poplar such as leaves, twigs, bark, and timber. It is worth of mention that flavonoid chemical structure (e.g., higher degree of hydroxylation as in quercetin, an already naturally deeply yellow penta-hydroxy-flavone) determines the color intensification as result of the complexation of Al^{3+}. Conversely, pinocembrin a simpler di-hydroxy-flavanone, naturally colorless, is almost non-reactive to this chromogenic method, reason by which we have adopted alternative procedures for the specific quantitation of pinocembrin.

2.5. Extensive chromatographic analysis

The refining of the chromatographic analyses through the comparative use of fractions obtained in silicic acid gel columns have shown the dramatic differences between the propolis samples as viewed by analytical TLC but also indicated a marked similarity between propolis sample PP-4-AL and poplar buds (Fig. 3). This correlation was not seen when comparing PP-4-AL with other aerial parts of poplar such as leaves, twigs, timber or bark (results not shown). The whole extract from poplar buds (PB; 'T') and derived fractions 3–5 as well as whole propolis PP-4-AL ('T') and their derived fractions 1 and 2 display 3 coincident strongly reactive bands to sulfuric anisaldehyde (deep orange spots at apparent $R_f = 0.56$, 0.50, and 0.44; Fig. 3A), the faster one of them co-chromatographing with the pinocembrin standard (the strongest orange spot from lane pc7/'P'). An alternative fractionation of PP-4-AL (lanes 'T′' and 't') further confirmed this finding. Pinocembrin is a hydrogenated form of chrysin at the pyranone ring (Fig. 2; [14]) but

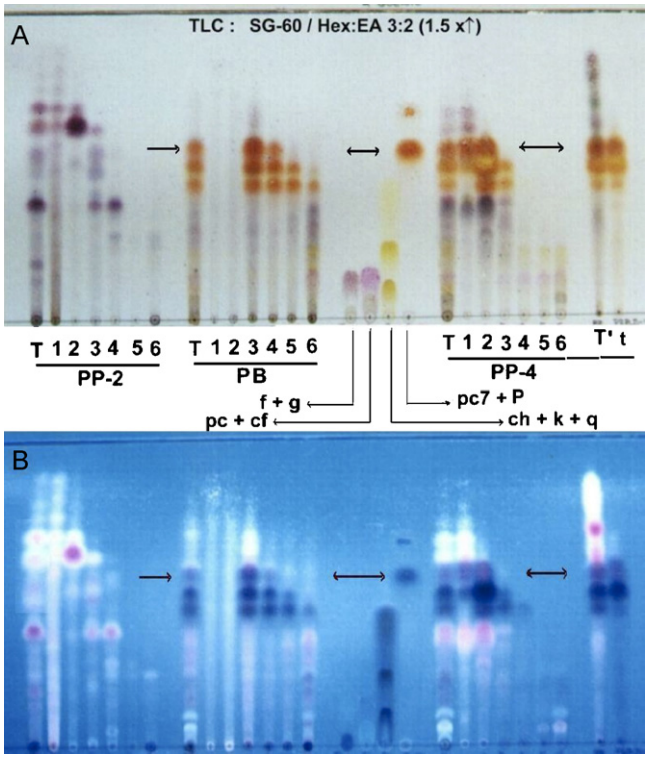

Fig. 3. TLChromatogram of propolis extracts, poplar buds, and their derived fractions in a silica gel 60 plate. Mobile phase: hexane + ethyl acetate (3 + 2, v/v), 1.5× horizontal ascendings. Chromogenic spay: hot sulfuric anisaldehyde. Color pictures, taken just after heating, with natural light (A) and under UV-365 nm (B). T and T′ refer to the crude ethanolic extracts from propolis samples (PP-2-WDC, PP-4-AL), and poplar buds (PB), respectively. Numbers refer to the sequential fractions from silicic acid gel columns. Bent arrows refer to the standard of ferulic (f) and gallic (g) acids; p-coumaric (pc) and caffeic (cf) acids; pinocembrin-7-methyl ether (pc7) and pinocembrin (P); also pointed by horizontal arrows; chrysin (ch), kaempferon (k), quercitin (q).

the latter presents only a very weak reactivity (yellow) towards anisaldehyde (the fastest spot on lane 'ch'/k/q). Although less color-specific (all flavonoids compounds display dark blue fluorescence after the anisaldehyde spray), UV-365 nm inspection of the developed plate (Fig. 3B) confirmed the identity between common compounds from propolis PP-4-AL and poplar buds chromatographing with apparent R_f from 0.40 to 0.60, including the pinocembrin standard ($R_f = 0.56$). UV inspection also confirmed the absence of pinocembrin-7-methyl ether either in propolis or buds (standard faster spot on 'pc-7'/P lane).

Densitometric analysis of UV-pictured plate from Fig. 3B, after accurate calibration of the apparatus with increasing concentrations of the pinocembrin standard in a separated plate, indicated that the flavanone content in the ethanolic extracts of propolis sample PP-4-AL was 10.1%. The equivalent figure from Fig. 3A, scanning the orange color at 440 nm, was 12.6%. Since pinocembrin is strongly reactive to hot acid/anisaldehyde these figures are more trustable than the previous aluminum cation-based method. It is worth of mention that most of the other ethanol extract components remained at the origin of the chromatographic plates.

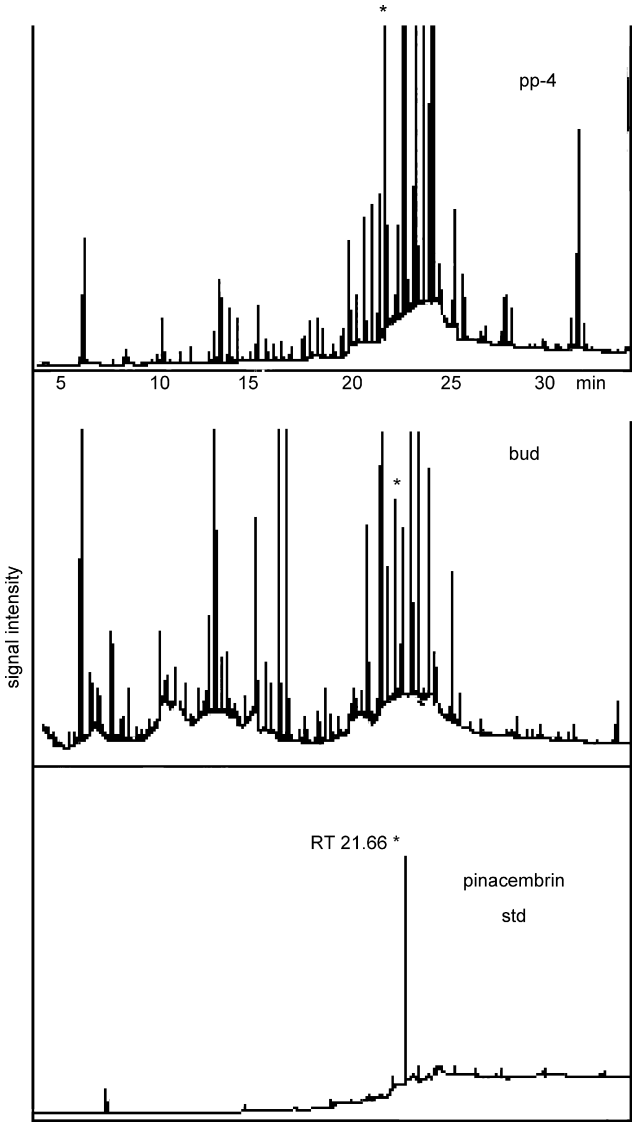

Fig. 4. Comparative gas chromatograms for underivatized ethanolic extracts from propolis PP-4, poplar buds, and pinocembrin standard (R_t at 21.66 min).

2.6. Molecular characterization

GC–MS of non-derivatized samples afforded the final confirmation for the occurrence of unusually high amounts of pinocembrin both in propolis sample PP-4-AL and in poplar buds (Fig. 4). The mass spectra for the peaks at $R_t = 21.73$ and 21.63, besides the molecular ion ($C_{15}H_{12}O_4$; 256 Da), indicated the expected fragments of 179, 152, and 124 Da. Also worth of mention is the similar chromatogram profiles obtained for PP-4-AL and poplar buds as compared to propolis samples PP-2-WDC. Quantitation based in peak areas of the ethanolic extratc indicated 13.1% of pinocembrin in propolis PP-4-AL, a result very close to that obtained with densitometry with visible wavelength.

2.7. Capillary electrophoresis

CZE of ethanolic extracts using the alkaline buffer moderated with methanol as co-solvent and monitored at the maximum wavelength for flavanones allowed the detection of >25 peaks in propolis PP-4-AL (at 130 mAU sensitivity; Fig. 5A) and of >30 peaks in poplar buds (at 35 mAU as sensitivity; Fig. 5B). Pinocembrin (peak around 22.4 min) is the major component in both samples and the dominant peak triad between 19 and

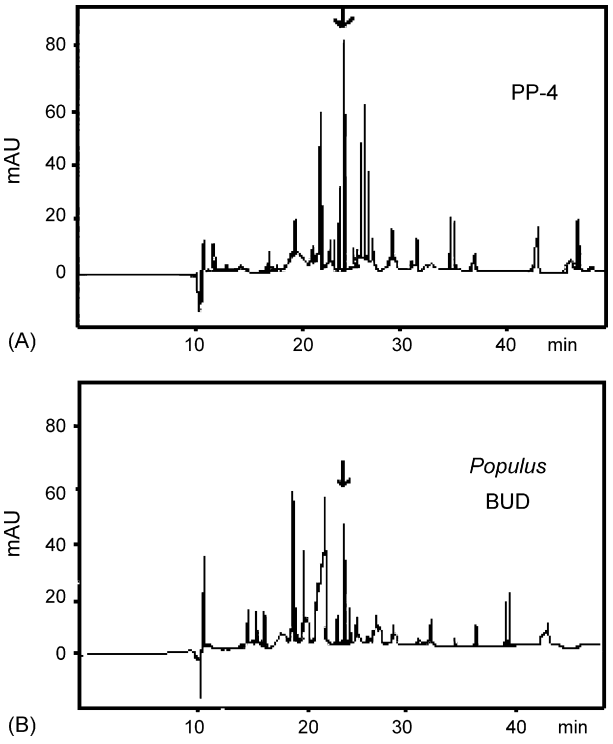

Fig. 5. Capillary eletrophoretograms for the ethanolic extracts of propolis sample PP-4-AL (A) and poplar buds (B). (↓) indicates the migration position with respect top the standard of pinocembrin ($R_t = 24$ min in the pH 8.5 electrophoresis buffer with a λ max = 320 nm).

25 min resembled the flavonoid profile previously detected by TLC.

3. Conclusion

A combination of chromatographic and electrophoretic procedures allowed to establish the chemical correlation between an almost homogeneous flora source, polpar buds, and a less usual propolis brand derived thereof regarding the content of a particular bioactive flavanone, pinocembrin, thus overcoming the limitation of the Al^{2+} salt-based spectrophotometric method for less hydroxylated flavonoids. GC–MS, CZE with alkaline buffer, and TLC/densitometry all them provided good resolution to distinguish and to quantitate this specific flavonoid as a dominant biomarker for both biological materials.

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