

Phenolic Compounds of Propolis from Central Chilean Matorral

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Pinocembrin, acacetin, galanguin, izalpin, kaempferide, prenyletin and diarylheptane were isolated from propolis from Central Chile.

Introduction

Propolis is an important product of the beehive produced by the activity of honey bee (*Apis mellifera*), gathering and transforming the bud exudate, by mixing it with waxy substances (Serra Vonhevi and V. Coll, 1996).

The mean composition of propolis is 55% resins and balsam, 30% waxes, 10% essential oils and 5% pollen. The chemical pattern of resin or balsam fraction varies considering the botanical and geographical origin, likewise the pattern of pollen grains (Brown, 1995; Walker and Crane, 1987).

This substance, propolis, is employed by the bee in the protection of beehive. It is used to fill cracks, reduce or close openings, strengthen and join cells to seal their hive from the penetration of water, thus creating an unfavorable environment for the development of microorganisms. Propolis also is used as an “embalming” substance to cover hive invaders which bees have killed but cannot transport out the hive (Ghisalberti, 1979). Chemical composition of Chilean propolis shows substances in the series lignane (Valcic *et al.*, 1998). Recently we isolated further known compounds (Muñoz *et al.*, 2001) from material of Cuncumen, an inland Mediterranean type climate site.

Chemical composition of the propolis appears close to the chemical composition of plants located near to the beehive. For example, the plant species, *Baccharis linearis*, shows five phenolic compounds found in the propolis obtained in the study site (Valcic *et al.*, 1999).

The site is located in Colliguay a property apart of main highroad in sclerophyllous shrubland of coast. Nevertheless, it is an area where the forest become a matorral (shrubland), with many regenerative states. The following representatives are found: *Cryptocarya alba*, *Peumus boldus*, accompanied with minor ones *Escallonia pulverulenta*, *Eupatorium glechonophyllum*, *Maytenus boaria*, *Quillaja saponaria*, *Salix humboldtiana* and spots of *Nothofagus dombeyi* (coihue) and *N. obliqua* (Gajardo, 1993).

In this work, we investigate the botanical origin of samples of propolis by microscopic analysis of the pollen grains and leaf fragments found in the sample, in order to traced native species with chemical as drug potentiality. As part of a research program on the chemistry and botany of propolis from Central Chile, we report the isolation and structure elucidation of seven phenolics.

Material and Methods

General experimental procedures

¹H and ¹³C NMR were acquired on a Varian United –300 (300, 75 MHz) Spectrometer. All proton and carbon assignments are based on HMQC and HMBC experiments.

FAB-MS and high resolution were recorded on a JEOL HX 110. Negative ESI – MS was recorded on a Finnigan MAT TSQ7000. The HPLC system used was equipped with a Varian 9002 pump, a Varian Star 9040 RI detector. HPLC column used were All-



tech (Adsorbosil Silica 5 μm 4.6×250 mm). Analytical TLC was performed on silica gel GF₂₅₄ (Merck 5554) and Macherey-Nagel silica gel plates Polygram SIL G/UV₂₅₄. Spots were detected by UV light and anisaldehyde sulfuric acid as the spray reagent. Column chromatography (cc) was performed with Macherey-Nagel silica gel 60, 50–200 μm .

Biological material

The propolis samples obtained of Colliguay hives, Central Chile, (33° S 71° W) underwent several chemical analyses in the laboratory. The semi-solid sediment that originated was used to find out the botanical origin, by the analysis of the pollen grains and the morphological structures retained in propolis. Five preparations for optical microscopy were made from each sediment, where the different pollen grains were counted and identified, as well as the remains of anatomical structures such as epidermal tissues and trichomes. For this identification there is a reference palinothèque at the Pontificia Universidad Católica de Chile, permanent microscopic preparations and pertinent bibliography (Heusser, 1971; Montenegro, 1984). Preparations for scanning electron microscopy were also made, so as to corroborate the identification of the plant species, observing in greater detail the pollen grains and anatomical structures, and identifying them by comparison with permanent preparations.

Extraction and isolation

Propolis (80 g) was cut into small pieces and extracted three times with EtOAc (3×0.6 l) at room temperature for 24 h. After filtration through a paper filter, the filtrates were combined and the solvent evaporated *in vacuo*. The dried extract was applied directly on a column chromatography (150 \times 0.4 cm) on silica gel with an *n*-hexane/EtOAc gradient (0, 2, 5, 10, 20, 50, 100% EtOAc) yielding five fractions of increasing polarity. Fraction 2 was separated into four subfractions 2.1–2.4 by column chromatography on Silica gel with an *n*-hexane/EtOAc 8:2. Fraction 2.1 yielded direct **1** (34.3 mg). Fractions 2.3 was applied to a column chromatography on silica gel with hexane/EtOAc gradient (5, 10, 20, 50 and 100% EtOAc) yielding crude **2**. Additional purified by crystallization gave **2** (17.9 mg). Fraction 2.4 was applied to column

chromatography on silica gel with hexane/EtOAc gradient (5, 15, 20, 25, 35, 50, 100% EtOAc) yielding crude **3**, **4** and **5**.

All fractions were directly crystallized obtaining **3** (13 mg), **4** (24 mg) and **5** (27 mg).

Fraction four, was applied to column chromatography on silica gel with CH₂Cl₂–MeOH gradient (0.5, 1, 2 and 5% MeOH) yielding crude 12 mg of **6**. HPLC with CH₂Cl₂ MeOH (99.5:0.5 MeOH v/v) yielded 7.1 mg of **6**. Repurification of this fraction by HPLC (with CH₂Cl₂ MeOH 0.75:0.5 MeOH) yielded 5.1 mg of **6**. Fraction five was applied to column chromatography on silica gel with CH₂Cl₂–MeOH gradient (0, 0.5, 1, 2, 3%) yielding crude 12 mg of **7**. HPLC with MeOH–H₂O 4:6 yielded 4.0 mg of **7**.

Identification

Compound **1** (pinocembrin) positive high resolution FAB-MS (mNBA) *m/z* 256.1498 (calc. for C₁₅H₁₂O₃, 256, 1481), ¹H NMR (COCl₃) δ : ¹H RMN (CDCl₃) δ : 2.75 (1H, dd, *J* = 2.8, 17.1 H-3 ec), 3.05 (1H, dd, *J* = 2.8, 17.1 H-3 ax), 5.42 (1H, dd, *J* = 2.8, 12.7, H-2), 5.90 (1H, d, *J* = 2.0, H-6), 5.92 (1H, d, *J* = 2.0, H-8), 7.35 (1H, m, H, 2'), 7.43 (2 H, m, H-4', H-5'), 7.50 (H-6'm) ¹³C NMR (CDCl₃) δ : 44.30 (C-3), 80.56 (C-2), 96.38 (C-8), 97.33 (C-6), 103.52 (C-10), 127.47 (C-2'), 129.75 (C-6'), 129.83 (C-3', 4', 5') 140.53 (C-1'), 164.79 (C-9) 165.61 (C-5), 168.53 (C-7), 197.42 (C-4).

Compound **2** (acacetin) the compound was identified by co-chromatography and comparison of its NMR data with those of a standard sample. APCI-MS *m/z*: 285 [M+H]⁺ corresponding to molecular formula (C₁₆H₁₂O₅); ¹H NMR (py-d₅) δ : 3.75 (3H, s, CH₃O), 6.60 (1H, d, *J* = 2.0 Hz, H-6), 6.70 (1H, d, *J* = 2 OHZ, H-8), 6.91 (1H, s, H-3), 7.22 (1H, d, *J* = 9, H-3'), 7.12 (1H, d, *J* = 9, H-5'), 7.95 (1H, d, *J* = 9, H-2'), 7.95 (1H, d, *J* = 9, H-6'). ¹³C NMR (CDCl₃) δ : 56.9 (MeO), 93.8 (C-8), 99.3 (C-6), 105.0 (C-3), 107.0 (C-10), 128.1 (C-3', 5'), 123.5 (C-1'), 129.9 (C-2', C-6'), 158.0 (C-9), 163.5 (C-5), 163.6 (C-4'), 165.2 (C-2), 166.0 (C-7), 184.7 (C-4).

Compound **3** (galangin) APCI-MS *m/z* corresponding to molecular formula (C₁₅H₁₀O₄). ¹H NMR (py-d₅) δ : 6.76 (H, d, *J* = 2, H-6), 6.84 (1H, d, *J* = 2, H-8), 7.44 (1H, m, H-3'), 7.49 (1H, m, H-4'), 7.55 (1H, m, H-5'), 8.51 (1H, dd, *J* = 1.7, 8.1)

(H-2'), 8.51 (1H, d, d, $J = 1.7, 8.1$, H-6'). ^{13}C NMR (CDCl_3) δ : 94.74 (C-8), 99.73 (C-6), 104, 94 (C-10), 123.67 (C-2'), 123.86 (C-6'), 124.07 (C-3), 124.15 (C-5'), 128.72 (C-4'), 130.23, (C-1'), 136.63 (C-3), 146.61 (C-2), 157.98 (C-9), 162.84 (C-5), 164.3 (C-7), 178.02 (C-4).

Compound **4** (isalpinin) positive high resolution FAB-MS (m NBA) m/z : 284.1497 (calc. for $\text{C}_{16}\text{H}_{12}\text{O}_5$, 284.488. ^1H NMR (CDCl_3) δ : 3.75 (·H, s, CH_3O), 6.35 (1H, d, $J = 2.0$, H-6), 6.45 (1H, d, $J = 2.0$, H-8), 8.15 (1H, dd, $J = 1.5, J = 8$, H-2'), 7.52 (1H, m, H-3'), 7.50 (1H, m, H-4'), 7.50 (1H, m, H-5'), 8.15 (1H, dd, $J = 1.5, J = 8$, H-6') 11.61 (OH). ^{13}C NMR (CDCl_3) δ : 55.15 (MeO), 92.01 (C-8), 97.83 (C-6), 103.21 (C-10), 126.91 (C-2', 6') 128.03 (C-3', 5'), 130.04 (C-4'), 130.09 (C-1'), 136.02 (C-3), 145.01 (C-2), 156.48 (C-9), 160.81 (C-5), 164.28 (C-7), 176.12 (C-4).

Compound **5**. APCI – MS m/z : 301 $[\text{M}+\text{H}]^+$ corresponding to molecular formula ($\text{C}_{16}\text{H}_{12}\text{O}_6$); ^1H and ^{13}C NMR in Py coincided with those reported (Urbatsch, *et al.*, 1976; Chari *et al.*, 1977).

Compound **6** (prenyletin) APCI-MS m/z : 247 $[\text{M}+\text{H}]^+$ corresponding to molecular formula ($\text{C}_{14}\text{H}_{14}\text{O}_4$); ^1H NMR (CDCl_3) δ : 1.78 (3H, s, Me), 1.81 (3H, s, Me), 4.65 (2H, d, $J = 6$, CH_2), 5.45 (1H, t $J = 1.5$ Hz H olef.), 6.28 (1H, d, $J = 9.8$ Hz, H-1), 7.58 (1H, d, $J = 9.8$ Hz, H-2), 6.95 (1H, s, Ar), 6.82 (1H, s, Ar). ^{13}C NMR (CDCl_3) δ : 151.50 (C-2), 112.09 (C-3), 142.39 (C-4), 110.85 (C-5), 66.29 (C-6), 113.73 (C-7), 113.73 (C-8), 149.29 (C-9), 100.28 (C-10), 25.79 (C-2'), 118.02 (C-3'), 140.21 (C-4'), 25.77 (C-5'), 18.32 (C-6').

Compound **7** (*trans*-3,5-dihydroxy-1,7-diphenylhept-1-ene). APCI-MS m/z : 283 $[\text{M}+\text{H}]^+$ corresponding to molecular formula ($\text{C}_{19}\text{H}_{22}\text{O}_2$). ^1H NMR (CDCl_3) δ : 1.85 (2H, m, CH_2), 2.63 (2H, m, $-\text{CH}_2-\text{Ar}$), 2.71 (2H, m, $\text{HOC}-\text{CH}_2-\text{COH}$), 4.02 (1H, m, H gem), 4.65 (1H, m, H gem), 6.25 (1H, dd, $J = 5.7, 1.5, 1.0$ Hz, H olef.), 6.61 (1H, d, $J = 5.7, 1.5, 1.0$ Hz H olef.), 7.15–7.40 (10H, m, Ar). ^{13}C NMR (CDCl_3) δ : 32.10 (C-7), 39.19 (C-6), 68.87 (C-5), 42.62 (C-4), 70.59 (C-3), 141.89 (C-2), 128.63 (C-1), 136.58 (C-1' Ar), 126.50 (C-2' Ar), 128.62 (C-3' Ar), 127.70 (C-4' Ar), 141.7 (C-1'' Ar), 128.45 (C-2'' Ar), 128.83 (C-3''), 125.90 (C-4'' Ar).

Results

Seven compounds were isolated and characterized from propolis: flavanoids, **1** pinocembrin, **2** acacetin,

3 galangin, **4** izalpinin and **5** kaempferide, the coumarin prenyletin **6** and a diarylheptene **7**, *trans*-3,5-dihydroxy-1,7-diphenylhept-1-ene. In contrast with propolis from Santa Cruz, Quebrada Yaquil (Valcic *et al.*, 1999), rich in lignane, in this study we report many flavanone, flavones, and flavonols.

Pharmacological properties of propolis of many Mediterranean and extra tropical areas of the world are well documented, (Kroll *et al.*, 1993; Marcucci, 1995; Mirzoeva and Calder, 1996; Natarajan *et al.*, 1996; Volper and Elster, 1996, Dugas *et al.*, 2000, Bankova *et al.*, 2000). Nevertheless, pharmacodynamic activity of the propolis of Chilean sources has not yet been studied, with the only exception of the isolation of antibiotic and antifungal compounds (Valcic *et al.*, unpubl. data).

Pinocembrin and galangin have antibacterial activity (Villanueva *et al.*, 1970), pinocembrin has also fungicidal activity (Metzner *et al.*, 1997) and local anesthetic activity (Paintz *et al.*, 1979) while acacetin has antiinflammatory activity (Bankova *et al.*, 1983).

The flavanone pinocembrin, the flavone acacetin and the flavonol galangin have been previously identified in many honey samples from different geographical and botanical origins (Soler *et al.*, 1995) Prenyletin, formerly isolated from *Haplopappus baylahuen* has antiinflammatory activity (Schwenker *et al.*, 1967).

The pollen frequency pattern of Colliguay site shows the following major species as resource for propolis: *Escallonia pulverulenta* (35.19), *Salix humboldtiana* (21.78), *Eucalyptus globulus* (19.55) and minor ones *Eupatorium glechonophyllum* (7.26), *Quillaja saponaria* (6.7), *Peumus boldus* (5.02). Also were detected *Nothofagus dombeyi* (2.23), *Nothofagus obliqua* (0.55), *Cryptocarya alba* (0.55), *Maytenus boaria* (0.55) and *Pinus radiata* (0.55).

Probably honeybees obtain resins with pinocembrin from *Escallonia pulverulenta*. This flavanone was formerly reported from this source (García *et al.*, 1990). Chemistry of *Salix humboldtiana*, Chilean willow, is poor known only salicin has been reported (Gupta, 1995).

Propolis of Chile, is collected of common species of Mediterranean type climates, viz. Poplar, willows, but also from many native trees and shrubs including *Cryptocarya alba*, *Escallonia pulverulenta*, *Eupatorium glechonophyllum*, *Maytenus boaria*, *Nothofagus dombeyi*, *Nothofagus*

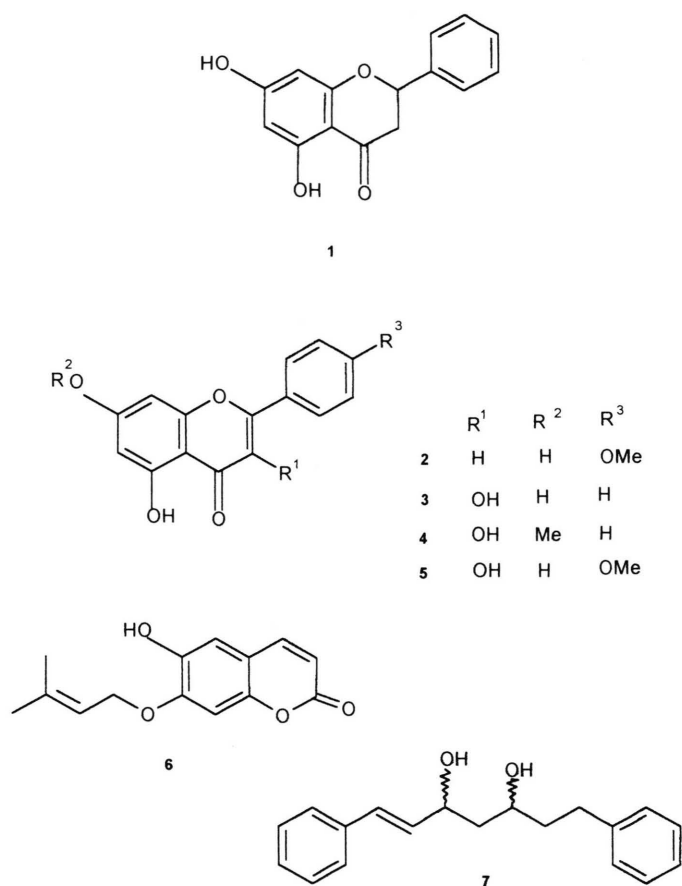


Fig. 1. Compounds **1**–**7** isolated from Chilean propolis.

obliqua, *Peumus boldus*, *Quillaja saponaria*, and *Salix humboldtiana*.

Interestingly, benzophenones are compounds isolated from Chilean sources of propolis, formerly detected in material from Venezuela, and recently from material of Cuba, three very different and isolated sites, by both climatic, and vegetation or floristic factors (Barberán *et al.*, 1993, Cuesta Rubio *et al.*, 1999). Recently, was been reported a prenylated chromane derivative from methanolic extract of Brazilian propolis. Prenylated *p*-coumaric acids and acetophenones are secondary metabolites, typical for south American *Baccharis* species (Bohlmann *et al.*, 1981). In tropical regions there are no poplars and birches, and bees have to find new plant sources of propolis. *Cistus* was identified in border areas with almost tropical climate, in Tunisia (Martos *et al.*, 1997). *Baccharis* spp. are a major source of tropical propolis, in addition to *Clusia* and *Araucaria heterophylla* (Bankova *et al.*, 1996; Ban-

skota *et al.*, 1998). *Clusia* species, were reported from tropical sources (Banskota *et al.*, 1998; Thomas Barberán *et al.*, 1993).

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