Propolis from Chilean Matorral Hives

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Z. Naturforsch. 56c, 269-272 (2000); received September 8/November 16, 2000

Propolis, Structure Elucidation, Plant Sources

Viscidone (0.5%), vanillin, 3',4'-(methylendioxy)acetophenone, 3-ethoxy-4-methoxybenzaldehyde, cinnamic acid, 3-methoxy-4-hydroxymethyl ester were isolated from propolis of hives from Cuncumen. This is the first report on propolis composition of an arid and a Mediterranean type climate area.

Introduction

Propolis is a resinous substance found in Apis mellifera hives. In Central Chile is made of especially by resins from Baccharis, eucalyptus, poplars (Populus alba) and Salix humboldtiana. This substance has versatile biological activities (Ghisalberti, 1979), including antimicrobial ones, especially against Gram-positive bacteria (Vanhaelen and Valhaelen-Fastre, 1979, Focht et al., 1993; Steinberg et al., 1996, Bretz et al., 1998; Montenegro et al., 2000). Phenolic components are the main constituents of propolis, and some of those such as: pinocembrine (5, 7-dihydroxyflavanone), galangine, and the caffeic acid and ester (Kujumgiev et al., 1993, 1999; Park et al., 1998) have antibiotic properties.

Crude propolis was collected from Cuncumen hives, Central Chile. The site located in Central Region of Chile, is a typical Mediterranean type climate area with rainy winter and dry summer. The east slope of the coast range, it is a Eucalyptus forestry resource, but also includes native trees (Cryptocarya alba, Lithrea caustica, Peumus boldus, Schinus latifolius, Escallonia pulverulenta, and Maytenus boaria). This community becomes lower and more scrubby on drier slopes, and mostly arboreal in shaded canyon (“quebradas”) where moisture levels are higher (Esler et al., 1998). The site is located in a degraded area dominated by low sclerophyll forest (“matorral”).

Material and Methods

General

Column chromatography was run using Silica gel 60G (Merck® 7734), LH-20 Sephadex® (Pharmacia). TLC was performed on silica gel GF254 plates (Merck 7731). The measurements of the NMR spectra were carried out on a Bruker WP 200 spectrometer [1H NMR (200 MHz), 13C NMR (50 MHz), EJ-MS (70eV), VG Micromass LTD ZAB 2 I.

Propolis sample

647 g was collected in the hives of Cuncumen, Central Chile (33° 40' S – 71° 30' W) on July 1999. The crude propolis was extracted with ethanol for...
pollen determination. Micromorphological analysis was done using a Nikon® optical microscope and a scanning electronic microscope (SEM) JEOL JSM 25 SII. Samples for SEM were dried in acetone via CO₂ in a Polaron 33,000 critical point drying apparatus.

To analyze pollen grains in the propolis samples, the methanol insoluble part of propolis (sediment) was processed by the Erdtmann (1954) acetolysis method.

To identify the presence of epidermal annexes, such as epidermal glands, trichomes and bud tissues in the propolis samples, thin slides were fixed in a mixture of formaldehyde, ethanol and acetic acid 95:5:5 v/v, embedded in solid paraffin Paraplast® and stained with safranin and fast green, and were then observed under an optical microscope. For SEM observation, samples were fixed in the same mixture, dehydrated with an acetone series of increasing concentrations and dried from pure acetone by CO₂.

The following palynological characteristics were determined: size of the grain, number of colpi, and exine architecture, and were compared with pollen samples obtained from plants around the hive. The samples are kept in the pollen grain catalog of the botanical laboratory of the Pontificia Universidad Católica de Chile.

**Extraction and isolation**

Extraction. 647 g of propolis were cut into small pieces and then ground and extracted with methanol (3 × 21) at room temperature for 24 h. After filtration through a paper filter, the filtrates were combined and the solvent evaporated in vacuo. The dried MeOH extract was dissolved in H₂O (180 ml) and extracted with CH₂Cl₂ (3 × 200 ml). The CH₂Cl₂ extract (12 ml) was applied in succession to column chromatography on Sephadex LH-20 with an n-hexane EtOAc gradient (0, 5, 10, 20, 50, 100 EtOAc) yielding four fractions of increasing polarity. Fraction 2 was separated into five subfractions 2.1–2.5 by column chromatography on silica gel with a chloroform-acetone mobile phase with increasing polarity yielding viscidone (1) (25 mg), vanillin (2) (30 mg), 3',4'-(methyleneoxy)acetophenone (3) (12.5 mg), 3-ethoxy-4-methoxybenzaldehyde (4) (15 mg), cinnamic acid (5) (18 mg), 3-methoxy-4-hydroxymethyl ester (6) (12.5 mg). These products were desiccated and identified by comparison of spectral (UV, ¹HNM R, ¹³CNMR) and chromatographic properties with authentic samples.

**Results**

Six compounds were isolated and characterized from propolis: viscidone (1), vanillin (2), 3’,4’-(methyleneoxy)acetophenone (3), 3-ethoxy-4-methoxybenzaldehyde (4), cinnamic acid (5), 3-methoxy-4-hydroxymethyl ester (6) (Fig. 1).

All compounds were identified by ¹HNM R, viscidone was further elucidated by ¹³CNMR and confirmed by EIMS.

![Chemical structures of propolis constituents.](image-url)
Compound 1 (viscidone). $^1$H NMR (CDCl$_3$) $\delta$: 2.58 (3 H, m, CH$_3$ - CO), 3.17 and 3.41 (2 H, dd, $J = 17.0$, 8.5 Hz, C - CH$_2$ - C), 4.27 (2 H, m, HO-CH$_2$-C), 5.31 (2H, m, CH$_2$=C), 5.32 ( 1H, s, -CH- O - ) 6.83 and 7.08 (2 H, s, aromatics), 12.2 (1H, s, OH) $^{13}$C NMR (CDCl$_3$) $\delta$: 83.00, 35.51, 107.83, 156.93, 117.58, 114.21, 137.41, 203.58, 26.38, 146.88, 111.58, 62.23. EI - MS m/z (rel.int.): 318.11 [M]+, 276 (59), 216 (28), 201 (12), 176 (25), 161 (10), 43 (100).

Compound 2 (vanillin). The compound was identified by co-chromatography and comparison of its NMR data with those of a standard sample. $^1$H NMR (CDCl$_3$) $\delta$: 9.81 (1H, s, CHO), 7.40 (1H, dd, $J = 1.71$, 8.5 Hz, H-6), 7.39, (lH ,d, $J = 1.8$ Hz, H-2), 7,09 (1 H, d, $J = 9.5$ Hz, H-5), 6.26 (1 H , brs, OH), 3.93 (3 H, s, OCH$_3$). $^{13}$C NMR (CDCl$_3$) $\delta$: 191.01, 151.80, 142.20, 129.55, 127.51, 114.41, 108.83, 56.00.

Additional compounds were established by direct comparison with authentic samples by TLC and NMR. Viscidone and vanillin, were previously found in samples of propolis from Santa Cruz, Chile (34°24' S-71°28 W) (Valcic et al., 1999). Bees use sticky, resinous, dark-yellowish to light brownish material to fill cracks and crevice; reduce or close openings to the outside; to strengthen and join the cells, and to seal their hives from penetration of water. These properties create an unfavorable environment for microorganism development and prevent the decomposition of creatures (such as mice and beetles) which have been killed by the bees after an invasion of the hive (Valcic et al., 1999).

An aldehyde related to viscidone found in Baccharis species is found in propolis from tropical sources (Banskota et al., 1998). Eucalyptus and Salix humboldtiana are the main resources of Apis mellifera in Chile. Baccharis linearis, a very common shrub in the zone, is also often visited by honeybee. Quillaja, Lithrea also are present in the pollen samples of studied propolis.

It is noticeable that with the exception of Eucalyptus all species are representative of the Chilean sclerophyll forest (Table I).

Although none of the isolated compounds in this study were new natural products, this is the first time that their occurrence is reported from material of an inland site of Mediterranean type climate site of Central Chile.

Acknowledgement

The project was carried out with the financial support of FONDECYT 1980967 (G. Montenegro) and N° UO1 TW 00316–06 from National Institute of Health (NIH) (B. N. Timmermann).

Table I. Frequency of pollen grains of propolis from Cuncumen Property.

<table>
<thead>
<tr>
<th>Percentage</th>
<th>Pollen Grain</th>
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<tbody>
<tr>
<td>25.54</td>
<td>Eucalyptus globulus Labill.</td>
</tr>
<tr>
<td>15.58</td>
<td>Salix humboldtiana Willd.</td>
</tr>
<tr>
<td>11.21</td>
<td>Quillaja saponaria Mol.</td>
</tr>
<tr>
<td>6.23</td>
<td>Lithrea caustica (Mol.) H. Et A.</td>
</tr>
<tr>
<td>4.98</td>
<td>Galega officinalis L.</td>
</tr>
<tr>
<td>3.11</td>
<td>Hypochaeris radicata L.</td>
</tr>
<tr>
<td>2.49</td>
<td>Colletia spinosa Lam.</td>
</tr>
<tr>
<td>2.18</td>
<td>Trevoa trinervis Miers</td>
</tr>
<tr>
<td>1.55</td>
<td>Peumus boldus Mol.</td>
</tr>
<tr>
<td>1.24</td>
<td>Echium vulgare L.</td>
</tr>
<tr>
<td>1.24</td>
<td>Tristerix verticillatus (R. et P.) Barlow et Wiens</td>
</tr>
<tr>
<td>0.93</td>
<td>Cryptocarya alba (Mol.) Looser</td>
</tr>
<tr>
<td>0.31</td>
<td>Pinus radiata D. Don</td>
</tr>
</tbody>
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