Propolis from Chilean Matorral Hives

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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 dried summer. The east slope of the coast range, it is a *Eucalyptus* forestry resource, but also includes native trees (*Cryptocarya alba*, *Lithrea*, *Quillaja*, *Salix humboldtiana*) and shrub (*Baccharis linearis*). Minor species are *Colletia spinosa*, *Escallonia rubra*, *Trevoa trinervis*, *Peumus boldus*, and weeds as *Galega officinalis*, *Echium vulgare*, and *Hipochaeris radicata*.

Four vegetation types have been identified along this coastal zone (Gajardo,1994); the southernmost of these units has been termed coastal sclerophyll forest ("bosque esclerófilo costero"), a zone dominated by low sclerophyll forest with such species as *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 GF_{254} (Merck 5554). Spots were detected under UV light or by spraying with anisaldehyde reagent and heating for 5–10 min. at 120 °C. Preparative TLC was performed on 2 mm thick silica gel F_{254} plates (Merck 7731). The measurements of the NMR spectra were carried out on a Bruker WP 200 spectrometer [^{1}HNMR (200 MHz)], ^{13}CNMR (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

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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 CO_2 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 CO_2.

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 × 2 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 MeOH extract was dissolved in H_2O (180 ml) and extracted with CH_2Cl_2 (3 × 200 ml). The CH_2Cl_2 extract (12 ml) was applied in succession to column chromatography on Sephadex LH-20 with au 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′-(methylendioxy)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, ^{1}HNMR, ^{13}CNMR) and chromatographic properties with authentic samples.

Results

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

All compounds were identified by ^{1}HNMR, viscidone was further elucidated by ^{13}CNMR and confirmed by EIMS.

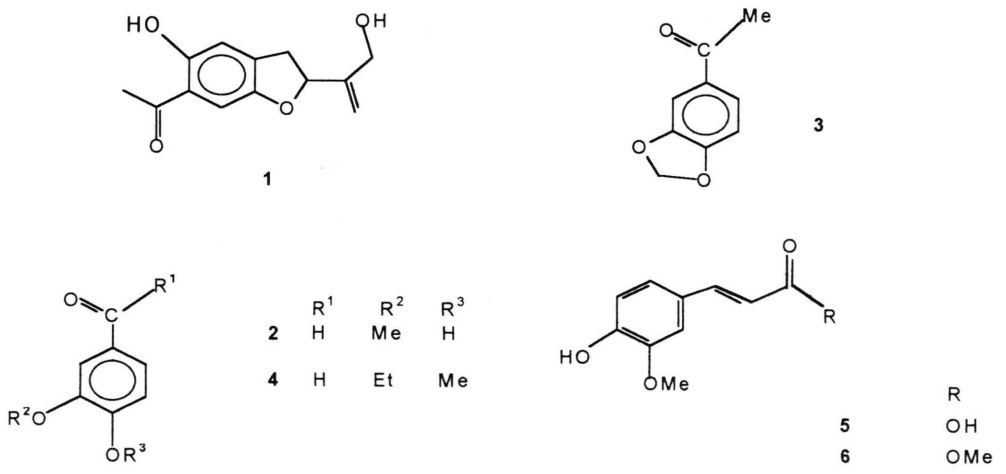

Fig. 1. Chemical structures of propolis constituents.

Compound **1** (viscidone). ^{1}H NMR ($CDCl_3$) δ: 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 ($CDCl_3$) δ: 83.00, 35.51, 107.83, 156.93, 117.58, 114.21, 151.39, 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 ($CDCl_3$) δ : 9.81 (1H, s, CHO), 7.40 (1H, dd, J = 1.71, 8.5 Hz, H-6), 7.39, (1H,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 ($CDCl_3$) δ : 191.01, 151.80, 142.20, 129.55, 127.51, 114.41, 108.83, 56.00. Compound 3. 3′, 4 – (methylendioxy)acetophenone ^{1}H NMR ($CDCl_3$+DMSO-*d6*) δ: 2.50 (3 H, s, CH_3 -), 6.10 (2 H, s,- CH_2-), 6.90 (1 H, d, aromatic) 7.38 (1 H, s, aromatic), 7.55 (1 H, d, aromatic) ^{13}C NMR ($CDCl_3$ + DMSO- *d6*) δ: 191.03, 151.83, 147.22, 129.7, 127.53, 107.31, 108.01, 101.21, 26.313. 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.

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

25.54	*Eucalyptus globulus* Labill.	2.49	*Colletia spinosa* Lam.
15.58	*Salix humboldtiana* Willd.	2.18	*Trevoa trinervis* Miers
14.33	*Baccharis linearis* (R. *et* P.) Pers.	1.55	*Peumus boldus* Mol.
11.21	*Quillaja saponaria* Mol.	1.24	*Echium vulgare* L.
6.23	*Lithrea caustica* (Mol.) H. Et A.	1.24	*Tristerix verticillatus* (R. *et* P.) Barlow *et* Wiens
4.98	*Galega officinalis* L.	0.93	*Cryptocarya alba* (Mol.) Looser
3.42	*Escallonia rubra* (R. *et* P.) Pers.	0.31	*Pinus radiata* D. Don
3.11	*Hypochaeris radicata* L.		

Banskota A. H., Tezuka Y., Prasain J. K., Matsushige K., Saiki I. and Kadota S. (1998), Chemical constituents of Brazilian propolis and their cytotoxic activities. J. Nat. Prod. **61,** 896–900.

Bretz W. A., Chiego Jr. D. J., Marcucci M. C., Cunha I., Custódio A. and Schneider L. G. (1998), Preliminary report on the effects of propolis on wound healing in dental pulp. Z. Naturforsch. **53c**, 1045–1048.

Erdtmann G. (1954), An Introduction to Pollen Analysis. Chronica Botanica Company, Waltham, MA., USA.

Esler K. J., Rundel P. W. and Cowling R. M. (1998), Biodiversity and conservation biology of coastal transition zones from Mediterranean to desert Ecosystems: An intercontinental comparison. In: Landscape Degradation and Biodiversity in Mediterranean-type Ecosystems (Rundel *et al.*, eds). Springer Verlag Berlin, Heidelberg.

Focht J., Hansen S. H., Nielsen J. V., van den Berg-Segers A. and Riezler R. (1993), Bactericidal effect of propolis *in vitro* against agents causing upper respiratory tract infections. Arzneimittel-Forsch. **43,** 921–923.

Gajardo R. (1994), La vegetación natural de Chile: clasificación y distribución geográfica. Editorial Universitaria, Santiago, Chile.

Ghisalberti E. (1979), Propolis: A review. Bee World **60**, 59–84.

Kujumgiev A., Bankova V. and Ignatova A. (1993), Antibacterial activity of propolis, some of its components and their analogs. Pharmazie **48**, 785–786.

Kujumgiev A., Tsvetkova I., Serkedjieva Y., Bankova V., Christov R. and Popov S. (1999), Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. *J. Ethnopharmacol.* **64**, 235–240.

Montenegro G., Timmermann B. N., Peña R. C., Mujica A. M. and Avila G. (2000), Pollen grains and vegetative structures in propolis as indicators of potential drugs in Chilean plants. Phyton **66**, 15–23.

Park Y. K., Koo, M. H., Abreu J. A., Ikegaki M., Cury J. A. and Rosalen P. L. (1998), Antimicrobial activity of propolis on oral microorganisms. Curr. Microbiol. **36**, 24–28.

Serra Bonhevi, J. and Coll V. (1996), Phenolics composition of propolis from China and South America. Z. Naturforsch **49c**, 712–718.

Steinberg C., Schneider C., Rotscheidt K. and Breitmeier E. (1996), Antibacterial effect of propolis. Am. J. Dent. **9**, 236–239.

Valcic S., Montenegro G., Mujica A. M., Franzblau S., Singh M. P., Maiese W. M. and Timmermann B. N.(1999), Phytochemical, morphological and biological investigation of propolis from Central Chile Z. Naturforsch. **54c,** 406–416.

Vanhaelen M. and Vanhaelen-Fastre R. (1979), Propolis-I. Originie, micrographie, composition,chimique et activité therapeutique. J. Pharm. Belg. **34**, 253–259.