

Phytochemical, Morphological, and Biological Investigations of Propolis from Central Chile

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Propolis from Central Chile was investigated for its plant origin by microscopical analysis of pollen grains and leaf fragments found in the sample. The pollen grains that appear with significant higher frequency in the sample corresponded to four native and two introduced species, whereas leaf fragments corresponded to four native species. Seventeen phenolic compounds that belong to the phenylpropane, benzaldehyde, dihydrobenzofuran, or benzopyran classes, were isolated from an organic extract that was found to have a moderate growth inhibitory activity against *Mycobacterium avium*, *M. tuberculosis*, and two strains of *Staphylococcus aureus*. The components responsible for activity were determined.

Introduction

Propolis is a complex mixture of beeswax, small amounts of sugar and plant exudates collected by honeybees (*Apis mellifera*) from various trees, shrubs, and herbs (Marcucci, 1995; Greenaway *et al.*, 1990). The percentage of bees foraging for propolis is very low in comparison to those collecting nectar or pollen and the amount of propolis in the beehive differs between the colonies (Gojmerac, 1980). The word propolis is derived from the Greek “pro”, for or in defense of, and “polis”, the city, that is defense of the city (or the hive). Bees use the sticky, resinous, dark-yellowish to light-brownish material, also called “bee-glue”, to fill cracks and crevices; reduce or close openings to the outside (Gojmerac, 1980); to strengthen and join the cells, and to seal their hives from penetration of water. These properties create an unfavorable environment for microorganism development (Serra

Bonvehí and Ventura Coll, 1994) and prevent the decomposition of creatures (such as mice and beetles) which have been killed by the bees after an invasion of the hive (Greenaway *et al.*, 1990; Brumfitt *et al.*, 1990). Propolis is known for its antiseptic, antimycotic, antibacterial, antiviral, antiprotozoal, and antiinflammatory properties (Marcucci, 1995). Since ancient times, propolis has been employed in folk medicine for the treatment of malignant tumors and wounds (Ioirish, 1978) and is still widely used in Europe as a component in pharmaceutical and cosmetic products, such as anti-acne preparations, facial creams, ointments, and lotions (Marcucci, 1995; Lejeune *et al.*, 1988). The bud exudates of poplar (*Populus* spp., Salicaceae) and horsechestnut (*Aesculus hippocastanum* L., Hippocastanaceae) trees are mentioned as the main sources of European and North American propolis (Greenaway *et al.*, 1987) which are known to consist of volatile oils and phenolics mostly flavones, flavanones and flavonols (Greenaway *et al.*, 1990; García-Viguera *et al.*, 1993; Bankova *et al.*, 1994).

In contrast to propolis of the Northern Hemisphere, Chilean propolis should have a different

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botanical origin due to Chile's unique flora that has developed as a result of its geographical isolation between the Pacific Ocean to the West and the Andes Mountains to the East. Chile's flora consists of many endemic plant species. Poplar or horse-chestnut trees are not native to Chile (Hoffmann, 1979) and, although introduced to certain parts of the country, they do not occur in the area where our beehives are located.

In order to determine the plant origin of propolis, the microscopic analysis of pollen grains, which are frequent contaminants in this material, are commonly used (Ricciardelli d'Albore, 1979). Since the surface structure of pollen grains is characteristic for the originating plant species, the microscopic analysis of these structures is, therefore, of great systematic significance (Montenegro *et al.*, 1992, 1997; Varela *et al.*, 1991; Iturriaga *et al.*, 1992).

In addition to pollen grains, propolis is also contaminated with leaf fragments nipped by bees during the collection of plant exudates (Montenegro, pers. commun.). Therefore, both, pollen grains and leaf fragments are of great systematic value in the analysis of the botanical origin of propolis.

Because of the different plant source as compared to propolis from the Northern Hemisphere, it was most likely that Chilean propolis had a different chemical composition. Based on this assumption, we investigated its chemistry and recently reported five lignans, three of which are new natural products (**12**, **16**, and **17**) (Valcic *et al.*, 1998).

In this work, we continued to study the chemistry of propolis from central Chile and were able to isolate and characterize 12 additional phenolic compounds. We also investigated the plant origin of this material by microscopical analysis of the pollen grains and leaf fragments found in the sample. In addition, we studied its antimicrobial activities and determined the components responsible for biological activity.

Material and Methods

General experimental procedures

For the morphological analysis, a binocular optical microscope (Nikon, Optiphot) and a scanning electron microscope (JEOL, JSM-25-SII) were used. SEM samples were dried from 100% ace-

tone *via* CO₂ in a Polaron E 3,000 critical point drying apparatus. Optical rotations were measured on a Jasco P-1020 polarimeter, UV spectra were acquired on a Beckman DU 640 spectrophotometer, and IR spectra were obtained with a Buck Scientific Model 500 spectrophotometer using NaCl plates. ¹H and ¹³C NMR were acquired on a Varian Unity-300 (300, 75 MHz) spectrometer. All proton and carbon assignments are based on HMQC and HMBC experiments. FAB-MS and high resolution FAB-MS were recorded on a JEOL HX 110. Negative ESI-MS was recorded on a Finnigan MAT TSQ7000. Analytical TLC was carried out on Macherey-Nagel silica gel plates Polygram SIL G/UV₂₅₄. Compounds were visualized with a UV lamp and anisaldehyde sulfuric acid as the spray reagent (Krebs *et al.*, 1969). Column chromatography (CC) was performed with Macherey-Nagel silica gel 60, 50–200 µm and Pharmacia Biotech Sephadex LH-20. Flash-chromatography (FC) was performed with Lagand Chemical Co. silica gel 60, 40–63 µm and Merck silica gel 60 RP-18, 40–63 µm. The HPLC system used was equipped with a Varian 9002 pump, a Varian Star 9040 RI detector. HPLC column used were Alltech (Adsorbosil Silica 5 µm, 4.6×250 mm) and Alltech (Econosil Silica 10 µm, 10×250 mm). Solvent compositions for CC, FC, and HPLC are v/v.

Biological material

Propolis was collected in December 1995 and provided to us by Mr. Gustavo Adolfo Castillo Orozco at Rincon de Yaquil, Santa Cruz, Quebrada de Yaquil, VI Region, Chile or Orebro 485, Estación Central Santiago, Chile. Phone: 56-2-741 4883, Fax: 56-2-223 73 19. The hives and forage fields are located in a small area called Quebrada Yaquil (34° 24' LS; 71° 28' LW; altitude 160 meters above sea level) in the mediterranean semiarid region, near Pichilemu, Chile. This region's climate is characterized by hot and dry summers and cold and rainy winters. The natural vegetation in this area corresponds to the Matorral, an evergreen shrub land, dominated by evergreen sclerophyllous and summer deciduous shrubs. The herbaceous stratum is seasonal, appearing only after the first rains of the year. Dominant species in the forage fields are the sclerophyllous shrubs *Lithrea*

caustica, *Quillaja saponaria*, *Cryptocarya alba*, *Kageneckia oblonga*, *Colliguaja odorifera*, *Trevoa trinervis*, *Baccharis linearis*, and *Peumus boldus* and the herbaceous *Madia sativa*, *Helenium aromaticum*, and *Pasithea coerulea*.

Morphological analysis

To analyze the pollen grains in the propolis sample, the methanol insoluble part (sediment) of propolis was processed by the Erdtman acetolysis method consisting of treating the material with acid and alkali (Erdtman, 1954). While the internal pollen content and the cellulosic intine of the pollen wall as well as other plant elements were disintegrated by acetolysis, the external part of the pollen wall (exine), which consists of sporopollenine (polyterpenes) (Jacob *et al.*, 1987) was resistant to acid and alkali action leaving the surface structure of pollen grains intact.

In order to detect the presence of vegetative organs such as epidermis, glands, trichomes and bud tissue in propolis, thin slides of propolis were fixed in FAA, embedded in paraplast and stained in safranin and fast green, and observed under the optical microscope. For the observation under the scanning electron microscope, samples were fixed in FAA, dehydrated with an acetone series of increasing concentrations and dried from 100% acetone *via* CO₂. To identify leaf fragments such as stomata, epidermis and plant trichomes, scanning microscope control preparations of plants surrounding the beehives were performed, then measured and photographed.

Extraction and isolation

Propolis (150 g) was cut into small pieces and extracted three times with MeOH (3×0.5 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 water and extracted with CH₂Cl₂. The CH₂Cl₂ extract was tested for growth inhibition against *M. tuberculosis* and *M. avium* complex. For a bioassay-guided fractionation and isolation of compounds, the CH₂Cl₂ was applied in succession to CC on Sephadex LH-20 with CH₂Cl₂–MeOH 1:1 and CC on silica gel with a hexane/EtOAc gradient (0, 1, 2, 5, 10, 20, 50, 100% EtOAc) yielding seven fractions of

increasing polarity (fractions 1–7) which were also tested in the biological assay. Fraction 1 was applied to prep TLC yielding 327 mg of **2**, and a mixture of **6** and **3**, which were separated on HPLC (silica gel) with hexane/EtOAc 9:1 yielding 9.4 mg of **6** and 35.0 mg of **3**. Fraction 2 was separated into six subfractions 2.1–2.6 by CC on silica gel with hexane/EtOAc, 8:2. Fraction 2.3 was applied to semi-prep HPLC on silica gel with hexane/EtOAc 83:17 yielding additional **3** (9.4 mg), additional **2** (169 mg), crude **8**, **9**, and crude **11**. The latter three components were rechromatographed on HPLC with hexane/EtOAc 8:2, hexane/CH₂Cl₂/EtOAc 60:35:5, and hexane/acetone 92:8, respectively yielding 49 mg of **8**, 44 mg of **9**, and 10 mg of **11**. Fraction 2.5 was applied to CC on silica gel (68×2 cm) with hexane/EtOAc 7:3 followed by HPLC on silica gel with CH₂Cl₂/EtOAc 9:1 yielding 6.2 mg of **10**. Fraction 2.6 was applied to CC on silica gel (100×2.5 cm) with hexane/CH₂Cl₂/acetone, 5:3:2 yielding crude **12** and crude **13**. HPLC with hexane/EtOAc 6:4 yielded 24 mg of **12** and HPLC with hexane/CH₂Cl₂/acetone 60:35:5 yielded 200 mg of **13**. Fraction 3 was applied to CC on silica gel (80×8 cm) with hexane/EtOAc 6:4 followed by FC on silica gel (25×4 cm) with a CH₂Cl₂/EtOAc gradient (10–20% EtOAc) yielding seven subfractions 3.1–3.7. Fr 3.4 contained additional **2** (12 mg), additional **13** (320 mg), additional **11** (61 mg), a mixture of **10** and **5** and a mixture of **1** and **7**. Compounds **10** and **5** were separated by HPLC with hexane/EtOAc 6:4 yielding additional 22 mg of **10** and 2 mg of **5**. The compounds **1** and **7** were separated by HPLC with CH₂Cl₂/EtOAc 9:1 yielding 160 mg of **1** and 24 mg of **7**. Fraction 3.5 contained a mixture of **17** and **12**, which were separated by FC on silica gel RP-18 (38×2.2 cm) with CH₃CN/H₂O 1:1. Compound **17** (137 mg) was purified by HPLC with hexane/EtOAc 6:4. Additional 20 mg of **12** were purified by FC on silica gel RP-18 (MeOH/H₂O, 1:1) followed by HPLC with hexane/EtOAc 6:4. Fraction 3.6 was applied to FC on silica gel (38×2.2 cm) with hexane/EtOAc 6:4 yielding 110 mg of **4** and crude **14**, which was purified by HPLC on silica gel with hexane/EtOAc 1:1 (136 mg). Fraction 3.7 was applied to FC on silica gel with CH₂Cl₂/EtOAc, 7:3 yielding a mixture of **15** and **16**, which were separated by HPLC with hexane/EtOAc 1:1 yielding 46 mg of **15** and 107 mg of **16**.

Identification

Compound **1** (viscidone). $[\alpha]_D^{25}$: +27.7° (c 0.5, CH₂Cl₂); APCI-MS m/z : 235 [M+H]⁺ corresponding to molecular formula (C₁₃H₁₄O₄); ¹H and ¹³C NMR in CDCl₃ coincided with those reported in (Le-Van and Van Cuong Pham, 1981).

Compound **2** (viscidone-14-acetate). $[\alpha]_D^{25}$: +43.1° (c 1.1, CH₂Cl₂); EI-MS m/z (rel. intens.): 276 [M]⁺ (55) corresponding to molecular formula (C₁₅H₁₆O₅), 259 (7), 216 (100), 201 (59), 176 (75), 161 (80), 115 (40), 91 (53), 77 (41); ¹H NMR in CDCl₃ coincided with those reported in (Bohlmann and Zdero, 1976).

Compound **3** (trementone). $[\alpha]_D^{25}$: -21.3° (c 1.2, CH₂Cl₂); APCI-MS m/z : 203 [M+H]⁺ corresponding to molecular formula (C₁₃H₁₄O₂). ¹H and ¹³C NMR in CDCl₃ coincided with those reported in (Banskota *et al.*, 1998).

Compound **4** (14-hydroxytrementone). $[\alpha]_D^{25}$: -101.1° (c 0.14, CH₂Cl₂); APCI-MS m/z : 219 [M+H]⁺ corresponding to molecular formula (C₁₃H₁₄O₃); ¹H NMR in CDCl₃ coincided with those reported in (García de Quesada *et al.*, 1972).

Compound **5** (14-acetoxy-trementone). $[\alpha]_D^{25}$: -23.4° (c 0.2, CH₂Cl₂); APCI-MS m/z : 261 [M+H]⁺ corresponding to molecular formula (C₁₅H₁₆O₄); ¹H NMR in CDCl₃ coincided with those reported (Bohlmann *et al.*, 1984).

Compound **6** (2,2dimethyl-6-acetyl-2H-chromen). $[\alpha]_D^{25}$: -4.28° (c 0.3, CH₂Cl₂); APCI-MS m/z : 203 [M+H]⁺ corresponding to molecular formula (C₁₃H₁₄O₂); ¹H in CDCl₃ coincided with those reported (Bohlmann and Grenz, 1970).

Comp. **7** $[\alpha]_D^{25}$: $\frac{589 \quad 577 \quad 546 \quad 435 \quad 405 \text{ nm}}{+2.1 \quad +0.3 \quad +1.5 \quad +4.5 \quad +4.1}$ (c 0.3, CHCl₃)

APCI-MS m/z : 221 [M+H]⁺ corresponding to molecular formula (C₁₃H₁₆O₃) (García de Quesada *et al.*, 1972; Zdero *et al.*, 1991).

Compound **8** (coniferyl-9-O-acetate). EI-MS m/z (rel. intens.): 222 [M]⁺ (15) corresponding to molecular formula (C₁₂H₁₄O₄), 179 (15), 151 (13), 137 (33), 119 (16), 91 (22), 77 (16), 43 (100); ¹H NMR in CDCl₃ coincided with those reported in (Zdero *et al.*, 1992).

Compound **9** (ferulic acid ethyl ester). EI-MS m/z (rel. intens.): 222 [M]⁺ (15) corresponding to molecular formula (C₁₂H₁₄O₄), 179 (15), 151 (13), 137 (33), 119 (16), 91 (22), 77 (16), 43 (100); ¹H NMR (CDCl₃) δ: 7.61 (1H, d, *J* = 16.2 Hz, H-7),

7.07 (1H, dd, *J* = 2.1, 8.1 Hz, H-6), 7.03 (1H, d, *J* = 1.8 Hz, H-2), 6.92 (1H, d, *J* = 8.4 Hz, H-5), 6.29 (1H, d, *J* = 15.9 Hz, H-8), 5.95 (1H, br s, OH), 4.26 (2H, q, *J* = 7.2 Hz, O-CH₂-CH₃), 3.92 (3H, s, OCH₃), 1.33 (3H, t, *J* = 7.5 Hz, O-CH₂-CH₃).

Compound **10** (coniferyl aldehyde). The compound was identified by co-chromatography and comparison of its NMR data with those of a standard sample. APCI-MS m/z : 179 [M+H]⁺ corresponding to molecular formula (C₁₀H₁₀O₃); ¹H NMR (CDCl₃) δ: 9.65 (1H, d, *J* = 7.8 Hz, CHO), 7.41 (1H, d, *J* = 15.9 Hz, H-7), 7.13 (1H, dd, *J* = 1.8, 8.4 Hz, H-6), 7.07 (1H, d, *J* = 1.8 Hz, H-2), 6.97 (1H, d, *J* = 8.4 Hz, H-5), 6.06 (1H, s, OH), 6.60 (1H, dd, *J* = 7.8, 15.9 Hz, H-8), 3.95 (3H, s, OCH₃).

Compound **11** (vanillin). The compound was identified by co-chromatography and comparison of its NMR data with those of a standard sample. APCI-MS m/z : 153 [M+H]⁺ corresponding to molecular formula (C₈H₈O₃); ¹H NMR (CDCl₃) δ: 9.83 (1H, s, CHO), 7.43 (1H, dd, *J* = 1.8, 8.5 Hz, H-6), 7.42 (1H, d, *J* = 1.8 Hz, H-2), 7.05 (1H, d, *J* = 9.5 Hz, H-5), 6.26 (1H, br s, OH), 3.97 (3H, s, OCH₃).

Compound **12**. $[\alpha]_D^{25}$: +13.1° (c 1.1, CH₂Cl₂); positive high resolution FAB-MS (mNBA) m/z 398.1374 (calc for C₂₂H₂₂O₇, 398.1365), for ¹H and ¹³C NMR data see (Valcic *et al.*, 1998).

Compound **13**. $[\alpha]_D^{25}$: -2.0° (c 1.2, CH₂Cl₂); negative ESI-MS m/z 441 [M-H]⁻ corresponding to molecular formula (C₂₄H₂₆O₈); for ¹H and ¹³C NMR data see (Valcic *et al.*, 1998).

Compound **14** (9,9'-bisacetyl-olivil).

$[\alpha]_D^{25}$: $\frac{589 \quad 577 \quad 546 \quad 435 \quad 405 \text{ nm}}{+0.3 \quad +0.9 \quad +0.3 \quad +3.5 \quad +6.2}$ (c 1.4, CH₂Cl₂)

positive high resolution FAB-MS (mNBA) m/z 460.1724 (calc for C₂₄H₂₈O₉, 460.1733), for ¹H and ¹³C NMR data see (Chaurasia and Wichtl, 1986).

Compound **15**. $[\alpha]_D^{25}$: +8.8° (c 0.63, CH₂Cl₂); positive high resolution FAB-MS (thioglycerol) m/z 460.1724 (calc for C₂₄H₂₈O₉, 460.1733); for ¹H and ¹³C NMR data see (Valcic *et al.*, 1998).

Compound **16**. $[\alpha]_D^{25}$: -15.6° (c 0.85, CH₂Cl₂); positive high resolution FAB-MS (thioglycerol) m/z 460.1740 (calc for C₂₄H₂₈O₉, 460.1733); for ¹H and ¹³C NMR data see (Valcic *et al.*, 1998).

Compound **17**.

$[\alpha]_D^{25}$: $\frac{589 \quad 577 \quad 546 \quad 435 \quad 405 \text{ nm}}{-0.6 \quad +0.8 \quad -0.8 \quad -0.6 \quad +2.9}$ (c 1.5, CH₂Cl₂)

positive high resolution FAB-MS (thioglycerol) m/z 664.2543 (calc for $C_{36}H_{40}O_{12}$, 664.2519); for 1H and ^{13}C NMR data see (Valcic *et al.*, 1998).

Coniferyl aldehyde and vanillin were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) as 4-hydroxy-3-methoxycinnamaldehyde, 98% and vanillin, 99%, respectively and used for co-chromatography with compounds **10** and **11**.

Antimicrobial bioassays

The crude extract, fractions, and purified compounds were tested for inhibition of *Mycobacterium tuberculosis* H₃₇Rv ATCC 27294 and *M. avium* ATCC 25291 using the BACTEC 460 system (Collins and Franzblau, 1997). Percent inhibition was calculated as $1 - (\text{growth index of test sample} / \text{growth index of control}) \times 100$. The minimum inhibitory concentration is defined as the lowest concentration which inhibited 99% of the inoculum.

In vitro antimicrobial activities against methicillin-sensitive (MSSA) and -resistant (MRSA) *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium* (VREF), *Escherichia coli*, *E. coli imp* (a mutant strain with increased permeability to large molecular weight compounds) and against *Candida albicans* were determined by agar diffusion method. Media used were Difco nutrient agar (pH 6.8) for *S. aureus*, LB (Luria-Bertani) agar for *E. faecium* and *E. coli*, and YM agar for *C. albicans*. Assay plates (12"×12" Sumilon) were prepared by pouring 125 ml volume of agar medium (tempered at 50 °C) inoculated with an overnight broth culture of the test organisms (adjusted to approximately 10^6 cells per ml). Ten μ l volume of antibiotic solution diluted in DMSO were spotted onto agar surface and the plates were incubated at 37 °C for 18 h. The zone of growth inhibition was measured using a hand-held digital caliper.

Results

Morphological analysis

In order to determine the plant origin of propolis, five different samples were taken from the methanol insoluble portion (sediment) and observed under an optical and a scanning electron microscope for the presence of pollen grains and

leaf fragments. For the identification of the plant sources at the species level, pollen grains were observed under the scanning electron microscope and compared with pollen grains obtained directly from the plant species surrounding the beehive area and with those in the permanent slide collection of the Pontificia Universidad Católica de Chile. The pollen grains of every plant species found in the sediment were quantified under the optical microscope to determine their percentage in the sample (Table I).

The analysis of the pollen grains in propolis indicated that they belong to a mixture of native and introduced species. The results showed that the pollen grains that appeared significantly more frequent in the sample ($p > 0.05$, X^2 test) corresponded to the native species *Salix humboldtiana*, *Baccharis linearis* (Fig. 1A), *Buddleja globosa*, and *Peumus boldus* and the introduced species *Eucalyptus globulus* and *Ricinus communis*. Pollen grains which did not appear in significant quantities, but contributed at least 2% to the total amount of pollen grains, corresponded to the native species *Aristotelia chilensis*, *Eupatorium glehnophyllum*, *Colliguaja odorifera*, *Eupatorium salvia*, *Lithrea caustica*, *Hypochaeris radicata*, *Trevoa trinervis* (Fig. 1B), and to the introduced *Citrus limon*.

For the identification of leaf fragments such as stomata, epidermis and plant trichomes, thin slides of propolis were observed under a scanning electron microscope and compared with those of control preparations from plants surrounding the beehives as well as with literature data (Montenegro, 1984).

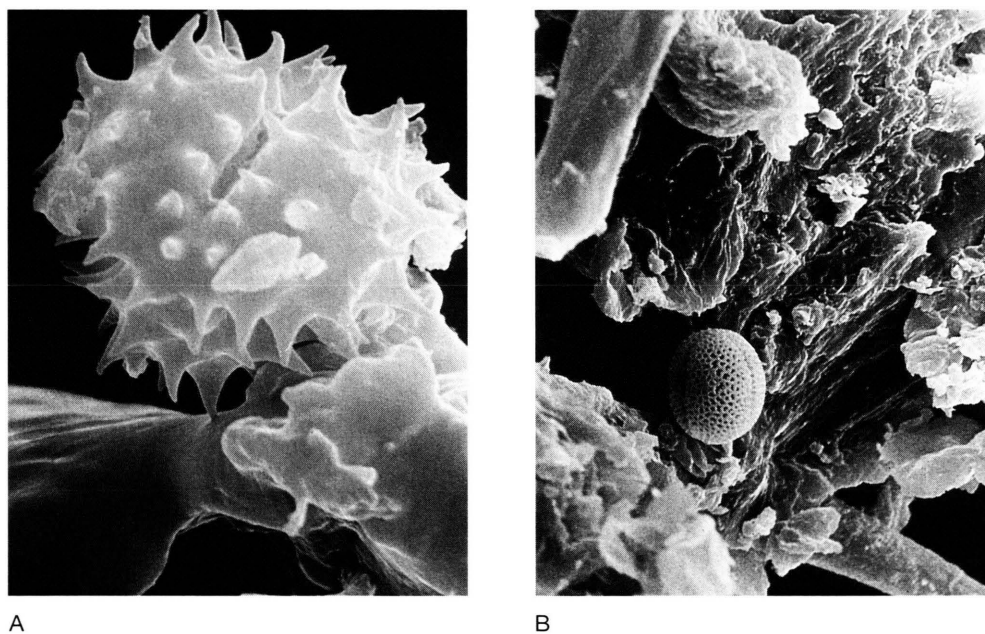
Leaf fragments of *Lithrea caustica*, *Trevoa trinervis*, *Kageneckia oblonga*, and *Baccharis linearis* were easily recognized in the sample by their typical glandular or filamentous trichomes and stomata shapes. From these species, pollen grains were also found in the sample (Table I). All species identified in the sample were present in the area where the beehives are located.

Chemical analysis

The crude methanolic extract of Chilean propolis was partitioned between water and dichloromethane. From the organic phase, 17 phenolic compounds (**1-17**) were isolated (Fig. 2). Com-

Table I. Botanical origin of pollen grains and their percentage distribution in propolis from Central Chile.

Plant species	i = introduced n = native	mean% value
<i>Eucalyptus globulus</i> Labill, Myrtaceae	i	11.56*
<i>Salix humboldtiana</i> Willd, Salicaceae	n	11.49*
<i>Baccharis linearis</i> (R. et P.) Pers., Asteraceae	n	11.02*
<i>Buddleja globosa</i> Hope, Loganiaceae	n	10.69*
<i>Peumus boldus</i> Mol., Monimiaceae	n	9.98*
<i>Ricinus communis</i> L., Euphorbiaceae	i	7.33*
<i>Aristotelia chilensis</i> (Mol.) Stuntz., Elaeocarpaceae	n	3.00
<i>Eupatorium glehnophyllum</i> Less., Euphorbiaceae	n	2.87
<i>Colliguaja odorifera</i> Mol., Euphorbiaceae	n	2.80
<i>Eupatorium salvia</i> Colla., Euphorbiaceae	n	2.80
<i>Lithrea caustica</i> (Mol.) H. et A., Anacardiaceae	n	2.20
<i>Hypochaeris radicata</i> L., Asteraceae	n	2.13
<i>Trevoa trinervis</i> Miers, Rhamnaceae	n	2.00
<i>Citrus limon</i> Burm, Rutaceae	i	2.00
<i>Muehlenbeckia hastulata</i> (J. E.Sm.) Johnst., Polygonaceae	n	1.80
<i>Schinus latifolius</i> (Gill. ex Lindl.) Engler, Anacardiaceae	n	1.73
<i>Ruta graveolens</i> L., Rutaceae	i	1.67
<i>Taraxacum officinalis</i> Weber, Asteraceae	i	1.60
<i>Kageneckia oblonga</i> R. et P., Rosaceae	n	1.60
<i>Talguenea quinquenervia</i> (Gill. et Hook.) Johnst., Rhamnaceae	n	1.60
<i>Brassica</i> sp., Brassicaceae	i	1.53
<i>Fabiana imbricata</i> R. et P., Solanaceae	n	1.53
<i>Maytenus boaria</i> Mol., Celastraceae	n	1.33
unidentified species		1.33
<i>Eschscholzia californica</i> Cham., Papaveraceae	i	1.30
<i>Satureja gilliesii</i> (Graham) Briq., Lamiaceae	n	1.13

* Significant more frequent ($p > 0.05$, X^2 test).Fig. 1. Scanning electron micrographs of pollen grains and leaf fragments found in the Chilean propolis sample. A. Pollen grain from *Baccharis linearis*. B. Pollen grain from *Trevoa trinervis*.

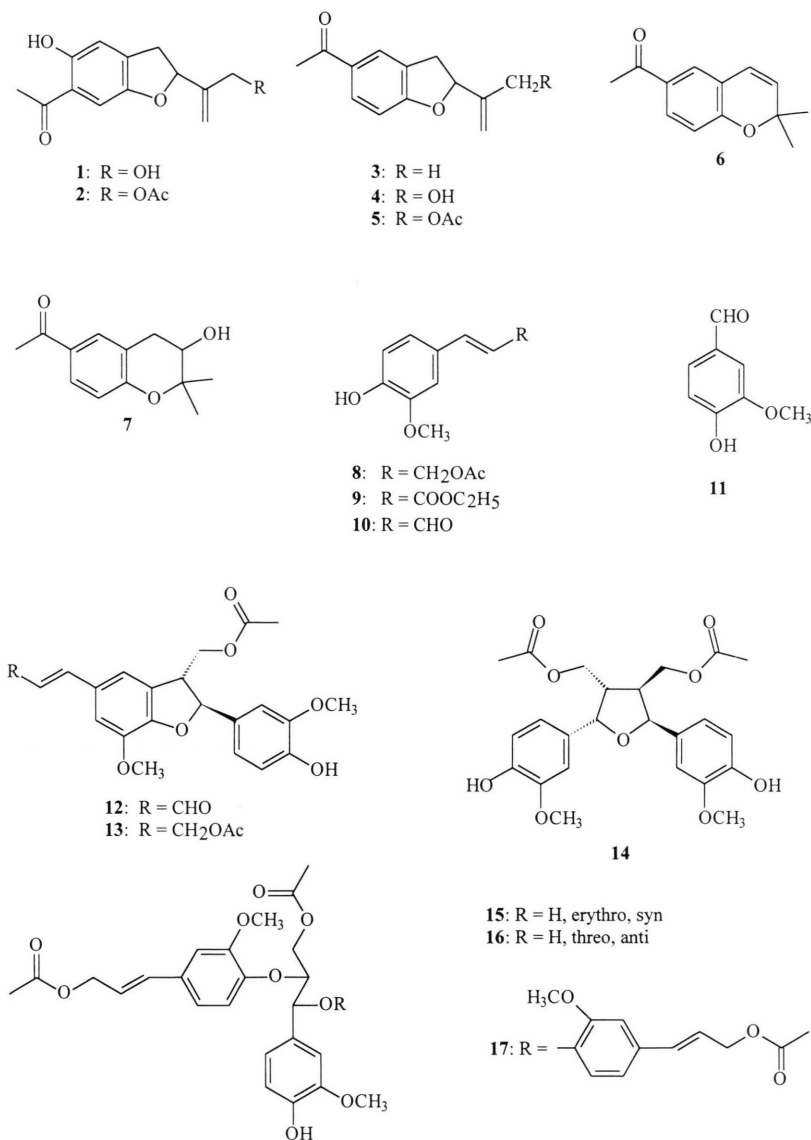


Fig. 2. Compounds **1**–**17** isolated from Chilean propolis.

pound **1** was identified as viscidone (Le-Van and Cuong Pham, 1981) and **2** as its acetate (Bohlmann and Zdero, 1976). Five *p*-hydroxyacetophenones were isolated, trementone (**3**) (Banskota *et al.*, 1998), 14-hydroxytrementone (**4**) (García de Quesada *et al.*, 1972; Le-Van and Van Cuong Pham, 1981) and its acetate (**5**) (Bohlmann *et al.*, 1984) as well as the known 2,2-dimethyl-6-acetyl-2*H*-chromen (**6**) (Bohlmann and Grenz, 1970) and its hydroxylated precursor (**7**) (García de Quesada *et al.*, 1972; Zdero *et al.*, 1991). Compounds **8**–**11**

were identified as coniferyl-9-O-acetate (**8**) (Zdero *et al.*, 1992), ferulic acid ethyl ester (**9**), coniferyl aldehyde (**10**), and vanillin (**11**), respectively. The dihydrobenzofuran lignan aldehyde (**12**) (Valcic *et al.*, 1998) and the related acetate (**13**) (Bankova *et al.*, 1996; Valcic *et al.*, 1998), the 9,9'-bisacetyl-olivil (**14**) (Chaurasia and Wichtl, 1986), two diastereomers of a dimeric coniferyl alcohol (**15**, **16**) (Jakupovic *et al.*, 1987; Valcic *et al.*, 1998), and the trimeric coniferyl acetate (**17**) (Valcic *et al.*, 1998) were also isolated from the Chilean

propolis sample. Compound **12**, **16**, and **17** were isolated from this propolis sample as new natural products that we recently reported in detail (Valcic *et al.*, 1998).

Antimicrobial activity

The crude methanolic extract of Chilean propolis was partitioned between water and dichloromethane. The dried organic phase was tested for antimycobacterial activity against the R₃₇Hv strain of *Mycobacterium tuberculosis* and *M. avium* in the BACTEC 460 system (Collins and Franzblau, 1997) (Table II). At a concentration of 300 µg/ml, a 99% growth inhibition of *M. avium* and a 92% growth inhibition of *M. tuberculosis* were observed. The dichloromethane extract was separated into seven fractions of increasing polarity. Only the two most unpolar fractions (fraction 1 and 2) showed inhibitory effects on the growth of

both *Mycobacterium* species. From fraction 1, a compound responsible for the antimycobacterial activity was found to be **2**. The MIC values of **2** were determined as 64 µg/ml against both *M. avium* and *M. tuberculosis*. Of the six compounds isolated from fraction 2, only **10** and **12** were found to have antimycobacterial activity. The MIC values for **10** and **12** were determined as 64 µg/ml against *M. avium* and 128 µg/ml against *M. tuberculosis*.

In another assay, the *in vitro* antimicrobial activity of the propolis samples was determined by the agar-diffusion method against several Gram-positive and -negative bacteria (Table III). The crude dichloromethane extract produced hazy zones of inhibition against methicillin-sensitive (MSSA) and -resistant (MRSA) strains of *Staphylococcus aureus*. Of the 17 isolated compounds tested, only compound **9** showed a similar profile. The compound gave a slight clear inhibition against the MSSA strain of *S. aureus* while yielding only hazy zones against the other test bacteria. Compounds **6**, **9**, and **10** produced clear zones of inhibition against *Candida albicans* and were the only samples that demonstrated any activity against this fungal pathogen.

Discussion

Propolis contains contaminants such as pollen grains and leaf fragments which the bees carry accidentally to the hive during the forage of plant exudates from selected plants, thus, giving a trace as to the botanical source of the bee glue.

Table II. MIC values of propolis crude extract and compounds **2**, **10**, and **12**.

Sample/ compound	MIC ^a [µg/ml] <i>M. tuberculosis</i>	MIC ^a [µg/ml] <i>M. avium</i>
Crude extract	>128	>128
2	64	64
10	64	128
12	64	128
Rifampin ^b	0.125	
Clarithromycin ^b		2

^a Determined in BACTEC 460; see Materials and Methods section for description of MIC determination.

^b For comparison.

Table III. Zones of growth inhibition of propolis crude extract and compounds **6**, **9**, and **10** against six microorganisms.

Sample/ compound	µg/spot	<i>Staphylo-</i> <i>coccus aureus</i> MSSA	<i>Staphylo-</i> <i>coccus aureus</i> MRSA	<i>Entero-</i> <i>coccus faecium</i> VREF	<i>Escherichia</i> <i>coli</i>	<i>Escherichia</i> <i>coli imp</i>	<i>Candida</i> <i>albicans</i>
Crude extract	500	11H	13H				
6	200						6:9H
9	200	6:9H	9H	9H	7VH	6VH	8:13H
10	200						9:11H
Vancomycin ^a	30 µg disc	16	18				
Bacitracin ^a	10 µg disc			18			
Cefoxitin ^a	30 µg disc				18	22	
Nystatin ^a	100 µg disc						16

H = hazy zone of inhibition; VH = very hazy zone of inhibition.

^a Control.

The presence of pollen grains is a valuable indicator of the botanical origin of propolis and is frequently used for its characterization (Ricciardelli d'Albore, 1979). We propose that the presence of leaf fragments can also be considered as an equally important marker since many plant species accumulate their plant exudates in external leaf glands. We have observed that bees foraging for propolis in Chile often target leaves, while those collecting pollen and nectar target only flowers (Ginocchio and Montenegro 1994; Montenegro *et al.* 1997).

In our investigation of the plant origin of propolis from the mediterranean semiarid region near Pichilemu, Chile, we found and identified pollen grains and leaf fragments from numerous species in the material. By quantification of pollen grains, we found that those belonging to six species were significantly more frequent than others. Leaf fragments were found to belong to four species, whose pollen grains were also present in the sample.

Propolis can also be contaminated with pollen grains from plants other than those that contribute to the propolis origin. For example, pollen grains can enter the beehive through atmospheric dust (Ricciardelli d'Albore, 1979). While propolis is spread all over the hive, it is especially deposited at the entrance, thus, creating a wall over which all bees, including those foraging pollen and nectar, pass when entering the hive (Aagaard, 1978). Although the propolis used in this study was collected away from the entrance of the beehive, a careful interpretation of the morphological data is necessary. The plant species whose pollen grains occur in the largest quantities and those species whose leaf fragments were found in addition to their pollen grains may be considered to be the most likely sources of the investigated propolis.

The chemical analysis of propolis could be used as an additional indicator of its botanical origin. However, a direct comparison of the chemical compositions of our propolis sample and its possible plant sources is difficult because of the insufficient knowledge of their chemistry. Also, chemical investigations of plant material are traditionally carried out with the whole plant biomass while the bees utilize only the plant exudates. Interestingly, compounds **1–3**, **5–7** and **13** were previously reported for various *Baccharis* species (Bohlmann *et al.*, 1981, 1984; Jakupovic *et al.*, 1987;

Zdero *et al.*, 1991) but none of the phenolic propolis components **1–17** were found in *B. linearis* (He *et al.*, 1996), a plant species whose leaf fragments and pollen grains were found in the sample of this study. In order to compare the chemical analysis of the Chilean propolis and that of *B. linearis*, detailed GC-MS/ LC-MS investigations of the plant material would be necessary to determine the presence of these phenolic compounds in the exudates of *B. linearis*.

European and North American propolis are known to consist of volatile oils and phenolics mostly flavones, flavanones and flavonols (Greenaway *et al.*, 1990; García-Viguera *et al.*, 1993; Bankova *et al.*, 1994). In contrast to the propolis from the Northern Hemisphere, no flavonoids were isolated from our Chilean propolis sample. Interestingly, all 17 compounds we isolated belong to the phenylpropane, benzaldehyde, dihydrobenzofuran, or benzopyran classes. To our knowledge, of the more than one hundred compounds identified in propolis of the Northern Hemisphere, no dihydrobenzofurans, or benzopyrans have been reported (Bankova *et al.*, 1994, 1998; García-Viguera *et al.*, 1992, 1993; Greenaway *et al.* 1987, 1990, 1991; Marcucci, 1995). Also, in propolis from China and Uruguay mostly simple phenylpropanes and flavonoids but no dihydrobenzofurans, or benzopyrans were identified (Serra Bonheví and Ventura Coll, 1994). Several dihydrobenzofurans were reported for Brazilian propolis. Bankova *et al.* (1996) isolated compound **13**, along with the known 4'-O-methyl-kaempferol (kaempferid) from Brazilian propolis, but no indication of the possible plant source was given. In a recent study by Banskota *et al.* (1998) the dihydrobenzofurans **1–3** and **13** were isolated from Brazilian propolis and *Baccharis* spp., *Clusia minor*, *C. major*, and *Araucaria heterophylla* were suggested as possible plant sources based on their chemical compositions. The presence of simple phenylpropanes such as compounds **8–10** in propolis from both hemispheres may be due to their ubiquitous occurrence in the plant kingdom. The occurrence of mostly dihydrobenzofurans, or benzopyrans in our propolis sample might be due to the different plant sources native to Chile when compared with those of other parts of the world.

One of the functions of propolis in the beehive is to protect it against microbial pathogens. This

observation prompted earlier studies in which antimycotic, antiviral, and antibacterial properties of propolis were shown (Marcucci, 1995). In our study, we found moderate antimycobacterial activity against *Mycobacterium avium* and *M. tuberculosis* and we were able to attribute this activity to compounds **2**, **10**, and **12**. The crude propolis extract also showed a moderate growth inhibitory effect on two strains of *Staphylococcus aureus* which could be traced to a single compound (**9**). Compounds **6**, **9**, and **10** also showed moderate inhibitory activity against the fungal pathogen, *Candida albicans*.

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- Aagaard K. L. (1978), Propolis, natural substance – the way to health. In: A remarkable Hive Product: Propolis, Scientific Data and Suggestions Concerning Its Composition, Properties and Possible Use in Therapeutics (Harnaj V., ed.). Apimondia, Bucharest, pp. 124–132.
- Bankova V., Christov R., Popov S., Pureb O. and Bocari G. (1994), Volatile constituents of propolis. *Z. Naturforsch.* **49c**, 6–10.
- Bankova V., Nikolova N. and Marcucci M. (1996), A new lignan from Brazilian propolis. *Z. Naturforsch.* **51b**, 735–737.
- Bankova V. S., Christof R. S. and Tejera A. D. (1998), Lignans and other constituents of propolis from the Canary Islands. *Phytochemistry* **49**, 1411–1415.
- 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.
- Bohlmann F. and Grenz M. (1970), Neue Isopentenylacetophenon-Derivate aus *Helianthella uniflora*. *Chem. Ber.* **103**, 90–96.
- Bohlmann F. and Zdero C. (1976), Weitere Inhaltsstoffe aus *Baccharis conferta* H. B. K. *Chem. Ber.* **109**, 1450–1452.
- Bohlmann F., Kramp W., Grenz M., Robinson H., King R. M. (1981), Diterpenes from *Baccharis* species. *Phytochemistry* **20**, 1907–1913.
- Bohlmann F., Scheidges C., Zdero C., King R. M. and Robinson H. (1984), Ent-labdanes from *Baccharis stenbergiana*. *Phytochemistry* **23**, 1109–1111.
- Brumfitt W., Hamilton-Miller J. T. M. and Franklin I. (1990), Antibiotic activity of natural products: 1. Propolis. *Microbios* **62**, 19–22.
- Chaurasia N. and Wichtl M. (1986), Phenylpropane und Lignane aus der Wurzel von *Urtica dioica* L. *Deut. Apoth. Z.* **126**, 1559–1563.
- Collins L. S. and Franzblau S. G. (1997), Microplate Alamar Blue assay versus BACTEC 460 system for high throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob. Agents Chemother.* **41**, 1004–1009.
- Erdtman G. (1954), An introduction to pollen analysis, Chronica Botanica Company, Waltham, MA.
- García de Quesada T., Rodríguez B. and Valverde S. (1972), The constituents of *Helichrysum stoechas*. *Phytochemistry* **11**, 446–449.
- García-Viguera C., Greenaway W. and Whatley F. R. (1992), Composition of propolis from two different Spanish regions. *Z. Naturforsch.* **47c**, 634–637.
- García-Viguera C., Ferreres F. and Tomás-Barberán F. A. (1993), Study of Canadian propolis by GC/MS and HPLC. *Z. Naturforsch.* **48c**, 731–735.
- Ginocchio R. and Montenegro G. (1994), Effects of insect herbivory on plant architecture. In: Plant-Animal Interactions in Mediterranean Type Ecosystems (Arianoutsou M., Grooves R., eds.). Kluwer Academic Publisher, The Netherlands, Chapter 11, pp. 115–122.
- Gojmerac W. L. (1980), Bees, Beekeeping, Honey, and Pollination, AVI Publishing Company, Inc. Westport, CT, pp. 115–116.
- Greenaway W., Scaysbrook T. and Whatley F. R. (1987), The analysis of bud exudate of *Populus × euroamericana*, and of propolis, by gas chromatography-mass spectrometry. *Proc. R. Soc. Lond. B* **232**, 249–272.
- Greenaway W., Scaysbrook T. and Whatley F. R. (1990), The composition and plant origin of propolis: a report of work at Oxford. *Bee World* **71**, 107–118.
- Greenaway W., May J., Scaysbrook T. and Whatley F. R. (1991), Identification by gas chromatography-mass spectroscopy of 150 compounds in propolis. *Z. Naturforsch.* **46c**, 111–121.

- He K., Montenegro G., Hoffmann J. J. and Timmermann B. N. (1996), Diterpenoids from *Baccharis linearis*. *Phytochemistry* **41**, 1123–1127.
- Hoffmann J., A. (1979) Flora Silvestre de Chile, Zona Central. Ediciones Fundacion Claudio Gay, Santiago de Chile.
- Iorish, N. (1978), The use of propolis in medicine. In: A Remarkable Hive Product: Propolis, Scientific Data and Suggestions Concerning Its Composition, Properties and Possible Use in Therapeutics (Harnaj V., ed.). Apimondia, Bucharest, pp. 123–124.
- Iturriaga L., Avila G., Gómez M. and Montenegro G. (1992), Especies vegetales utilizadas como fuente de polen por *Apis mellifera* en la region mediterránea subhúmeda de Chile. *Simiente* **62**, 19–23.
- Jacob F., Jäger E. J., Ohmann E. (1987), Botanik, Gustav Fischer Verlag, Stuttgart, p. 285.
- Jakupovic J., Warning U., Bohlmann F. and King R. M. (1987), New coumaric acid derivatives and other constituents from Argentinian *Baccharis* species. *Rev. Latinoamer. Quim.* **18**, 75–77.
- Krebs K. G., Heusser D. and Wimmer H. (1969), Spray Reagents. In: Thin-Layer Chromatography (Stahl E., ed.). Springer-Verlag, New York, pp. 854–909.
- Lejeune B., Pourrat A. and Dehmouche H. (1988), Propolis utilisation en dermacosmétique. *Parf. Cosmet. Arom.* **82**, 73–77.
- Le-Van N. and Van Cuong Pham T. (1981), An unusual *m*-hydroxyacetophenone and three chromanone derivatives from *Chrysanthamnus viscidiflorus*. *Phytochemistry* **20**, 485–487.
- Marcucci M. C. (1995), Propolis: chemical composition, biological properties and therapeutic activity. *Apidologie* **26**, 83–99.
- Montenegro G. (1984), Anatomía de especies vegetales autóctonas de la Zona Central. Ediciones Universidad Católica, Chile pp.156.
- Montenegro G., Gómez M. and Avila G. (1992), Importancia relativa de especies cuyo polen es utilizado por *Apis mellifera* en el área de la reserva nacional, Los Riles, VII Región de Chile. *Acta Bot Malacitana* **17**, 167–174.
- Montenegro G., Avila G., Rougier D. and Timmermann B. (1997), Pollen loads: Source of carotenoids originating from the communities of central zone of Chile. *Rev. Chil. Hist. Nat.* **70**, 91–99.
- Ricciardelli d'Albore G. (1979), L'origine géographique de la propolis. *Apidologie* **10**, 241–267.
- Serra Bonvehí J. and Ventura Coll F. (1994), Phenolic composition of propolis from China and South America. *Z. Naturforsch.* **49c**, 712–718.
- Valcic S., Montenegro G. and Timmermann B. N. (1998), Lignans from Chilean propolis. *J. Nat. Prod.* **61**, 771–775.
- Varela D., Schuck M. and Montenegro G. (1991), Selectividad de *Apis mellifera* en su recolección de polen en la vegetación de Chile Central (Región Metropolitana). *Ciencia e Investigación Agraria* **18**, 73–78.
- Zdero C., Bohlmann F. and Niemeyer H. M. (1991), An unusual dimeric sesquiterpene and other constituents from Chilean *Baccharis* species. *Phytochemistry* **30**, 1597–1601.
- Zdero C., Bohlmann F. and King R. M. (1992), Clerdane and labdane derivatives from *Olearia teretifolia*. *Phytochemistry* **31**, 1703–1711.