Antibacterial Diterpenic Acids from Brazilian Propolis

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Four labdane-type diterpenic acids and syringaldehyde were isolated and identified from Brazilian propolis. All the compounds exhibit antibacterial activity. The diterpenes, found for the first time in propolis, are typical for some *Araucaria* species and thus indicate a possible plant source of Brazilian propolis.

Introduction

Propolis (bee glue) is a resinous product that accumulates in bee hives. It possesses versatile biological activities: antibacterial, antiviral, fungicidal, antiulcer, immunostimulating, hypotensive, cytostatic, etc. (Ghisalberti, 1977). Now propolis is extensively used in foods and beverages intended to maintain and improve human health (Matsuda, 1994).

The chemical composition of propolis turned out to be very complex and more than 160 propolis constituents have been identified so far (Marcucci, 1995). The prevailing number of data concerned propolis from the temperate zones and its most important constituents appeared to be phenolics (more than 50% of the weight of propolis). There are many investigations on the origin of propolis and almost always poplar buds appeared to be the source of bee glue in temperate zone, especially *P. nigra* (Greenaway *et al.*, 1987; Papay *et al.*, 1986; Bankova *et al.*, 1986). Of special interest is the origin and respectively the chemical composition and biological activity of propolis in tropical South America, because there are no poplars in this region. Investigations on propolis from tropical Venezuela (Tomas-Barberan *et al.*, 1993) and Brazil (Aga *et al.*, 1993; Bankova *et al.*, 1995) showed that, as expected, polyphenols from poplar buds were entirely absent. Such polyphenols (flavonoids and phenolic acid esters) are known to be responsible for the antibacterial activity of propolis (Ghisalberti, 1978; Kujumgiev *et al.*, 1993). Nevertheless, Brazilian samples showed antibacterial activity, similar to that of "poplar" propolis (Aga *et al.*, 1993; Bankova *et al.*, 1995). In order to find out the active principles of Brazilian propolis and to disclose its plant sources, we tried to characterize the main components of a sample from Parana State.

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Experimental

The propolis was collected in Brazil, in Parana State, near Prudentopolis.

Extraction of propolis

Propolis (20 g) was cut into small pieces and extracted with boiling MeOH (200 ml) for 2 h. The extract was filtered hot, diluted with water (150 ml) and extracted successively with hexane (3 x 350 ml) and Et_2O (350 ml). The ether extract was evaporated *in vacuo* to dryness (7,1 g).

Isolation of the individual compounds

The ether extract was subjected to column chromatography on silica gel (substance-silica gel ratio 1:80, column diameter/column height 1:7), and

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eluted with hexane-acetone gradient to produce several fractions. After repeated column chromatography on silica gel (substance-silica gel ratio 1:100, column diameter/column height 3:50 and 1:30) with hexane-acetone and chloroform-ethyl acetate and LPLC on a Lobar RP-18 column size B with MeOH-H_2O mixtures, the following compounds were isolated and identified: coniferylaldehyde (**5**), 25 mg (identified on the basis of MS, ^{1}H-NMR and UV spectra); isocupressic acid (**2**) [15-hydroxy-8(17),E-13-labdadien-19-oic acid], 19 mg (^{1}H-NMR, ^{13}C-NMR, MS, IR spectra identical with literature data: Fang *et al.*, 1989; Fang *et al.*, 1991); acetylisocupressic acid (**3**), 10 mg (^{1}H-NMR, MS identical with published data: Fujii and Zinkel, 1984). After a mild alkaline hydrolysis (2N KOH in MeOH, room temperature, overnight) the product obtained was identical with **2;** imbricatoloic acid (**4**) [15-hydroxy-8(17)-labden-19-oic acid], 11 mg (identified by comparison with the ^{1}H-NMR, ^{13}C-NMR, MS spectra of its Me ester: Fujii and Zinkel, 1984), communic acid (**5**) [8(17),12,14-labdatrien-19-oic acid], 23 mg (^{1}H-NMR, ^{13}C-NMR, MS identical with published data: Bohlmann and Zdero, 1974; Fang *et al.*, 1989), *cis/trans* 1:2 according to ^{1}H-NMR.

Methylation of **2**

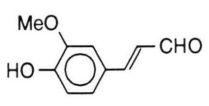
7 mg of isocupressic acid **2** were dissolved in 1 ml 2N NaOH and 1 ml acetone and 4 µl dimethyl sulphate were added. After 1 h at room temperature the reaction mixture was diluted with water, extracted 3 times with CH_2Cl_2 and the combined organic layers were washed with 1N NaOH, 5% HCl and water. After evaporation to dryness, 5.5 mg methyl isocupressate (**6**) were obtained.

Antibacterial tests

For the investigation of the antibacterial activity we used a modification of bioauthography recently developed in our laboratory (Kujumgiev *et al.*, 1993). As a test micro-organism, *Staphylococcus aureus* 209 was used. The antibacterial activity was measured as a diameter of the inhibitory zones in the soft agar layer stained after 72 h incubation at 37°C with methylene blue according to Loeffler (Doetsch, 1981). The inhibitory zones of 0.2 mg of each substance were measured.

Results and Discussion

The ether fraction of the methanol extract of propolis afforded, after repeated column chromatography, five pure compounds. One of them was identified as coniferylaldehyde (**5**) (3-methoxy-4-hydroxycinnamil aldehyde). This compound has been found by Popravko and Sokolov (1980) in Russian propolis originating from *Populus tremula*. The other four compounds were diterpenes, according to their mass spectra. The detailed analysis of their MS and NMR spectra showed that they were diterpenic acids possessing a labdane skeleton, and after comparison with literature data these compounds were identified as isocupressic acid (**2**) [15-hydroxy-8(17),*E*-13-labdadien-19-oic acid], acetylisocupressic acid (**3**) [15-acetyloxy-8(17),*E*-13-labdadien-19-oic acid], imbricatoloic acid (**4**) [15-hydroxy-8(17)-labden-19-oic acid] and communic acid (**5**) [8(17),12,14-labdatrien-19-oic acid] (a mixture of *cis* and *trans* isomers, *cis/trans* 1:2 according to ^{1}H-NMR). These acids are known as components of the oleoresin of some conifers: *Juniperus chinensis* (Fang *et al.*, 1993); *Calocedrus formosana* (Fang *et al.*, 1989); *Pinus ponderosa* (Fujii and Zinkel, 1984); *Araucaria* spp. (Caputo *et al.*, 1974). Their isomers with *ent*-labdane skeletons have been identified in some *Brickellia* spp. (Zdero *et al.*, 1991) and *Baccharis* spp. (Jakupovic *et al.*, 1986). Both *Baccharis* and *Araucaria* spp. are growing in tropical South America, so in order to find out the plant source of the investigated by us propolis sample, it was necessary to establish whether the



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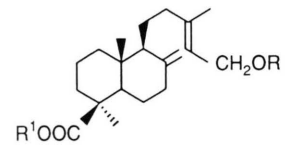
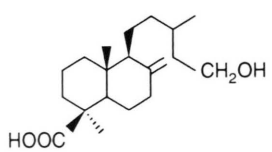


2: R = R^1 = H
3: R = Ac, R^1 = H
6: R = H, R^1 = Me



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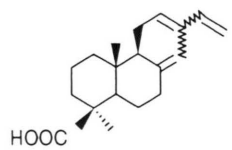

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isolated compounds are labdanes or *ent*-labdanes. In order to solve this problem, we measured the IR spectrum of the methyl ester of **2**, synthesized by us. The appearance of the absorption band of the C-O bond from the $COOCH_3$ group at 1155 cm^{-1} is an indication that the carboxylic group at C-4 is axial and this is the case in the labdane acids (Bohlman and Zdero, 1974). So one of the main sources of the analyzed propolis sample has to be *Araucaria*. *Araucaria heterophylla* was reported to be a source of propolis on Barbados (Crane, 1988), but no chemical evidence was published. The diterpenic acids **2** - **5** are found for the first time in propolis.

The isolated pure compounds were tested for antibacterial activity, using a modification of bioauthography (Kujumgiev *et al.*, 1993). The results obtained are summarized in Table I. Our previous investigations (Bankova *et al.* 1995) showed that Brazilian propolis possessed antibacterial activity and now we identified some of its antibacterial constituents. Our results confirmed once again the well-known fact that no single component of propolis has larger antibacterial activity than the whole extract. Evidently (Table I) both the carboxyl group at C-19 and the hydroxyl group at C-15 are important for the antibacterial action of the isolated labdane acids: **2** and **4** are more active than their derivatives **3** and **6**, as well as than **5**, where an OH group is absent.

Table I. Antibacterial activity of propolis and its components.

Substance	Diameter of inhibitory zone [mm]*
Propolis	11.8 ± 0.8
Syringaldehyde (**1**)	12.7 ± 0.7
Isocupressic acid (**2**)	10.5 ± 0
Acetylisocupressic acid (**3**)	5.8 ± 0.3
Imbricatoloic acid B (**4**)	9.1 ± 0.7
Communic acid (**5**)	5.7 ± 0.6
Methyl isocupressate (**6**)	5.7 ± 0.6

* Mean value ± standard deviation for three determinations.

A brief review of the results published on the chemistry of tropical propolis shows a remarkable variability of its chemical composition and its plant sources (Tomas-Barberan *et al.*, 1993; Aga *et al.*, 1994; Bankova *et al.*, 1995). It is evident that much more investigations are needed in order to clear the origin of tropical propolis and especially Brazilian propolis and to answer another important question: is it possible to work out some chemical standardization procedure for this valuable hive product in tropical regions?

Acknowledgement

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Aga H., Shibuya T., Sugimoto T., Kutimoto M. and Nakajima Sh. (1994), Isolation and identification of antimicrobial compounds in Brazilian propolis. Biosci. Biotech. Biochem. **58,** 945–946.

Bankova V., Christov R., Kujumgiev A., Marcucci M. C. and Popov S. (1995), Chemical composition and antibacterial activity of Brazilian propolis. Z. Naturforsch. **50c,** 167–172.

Bohlmann F. and Zdero C. (1974), Natürlich vorkommende Terpen-Derivate. XXXIII. Über die Inhaltsstoffe von *Hermas villosa* Thunb. Chem. Ber. **107,** 1416–1419.

Caputo R., Dovinola V. and Mangoni L. (1974), New diterpenes from *Araucaria cunninghami*. Phytochemistry **23,** 475–478.

Doetsch R. N. (1981), Manual Methods for General Bacteriology, p.24, American Society of Microbiology, Washington.

Fang J. M., Hsu K. C. and Cheng Y. S. (1989), Terpenoids from leaves of *Calocedrus formosana*. Phytochemistry **28,** 1173–1175.

Fang J. M., Lang C. I., Chen W. L. and Cheng Y. S. (1991), Diterpenic acids from the leaves of armand pine. Phytochemistry **30,** 2793–2795.

Fang J. M., Sou Y. Ch., Chiu Y. H. and Cheng Y. Sh. (1993), Diterpenes from the bark of *Juniperus chinensis*. Phytochemistry **34,** 1581–1584.

Fujii R. and Zinkel D. F. (1984), Minor components of ponderosa pine oleoresin. Phytochemistry **23,** 875–878.

Ghisalberti E. L. (1979), Propolis: A Review. Bee world **60,** 59–84.

Greenaway W., Scaysbrook T. and Whatley F. R. S. (1987), Analysis of bud exudate of *Populus* x *euramericana*, and of propolis, by gas chromatography-mass spectrometry. Proc. R. Soc. Lond. **B 232,** 249–272.

Jakupovic J., Eid F. and King R. M. (1986), Weitere Inhaltsstoffe von *Baccharis trinervis*. Pharmazie **23,** 875–878.

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

Marcucci M. C. (1995), Propolis: chemical composition, biological properties and therapeutic activity. Apidologie **26,** 83–99.

Matsuda Sh. (1994), Propolis: Health care food. Honeybee Science **25** (4), 145–154.

Popravko S. A. and Sokolov I. V. (1980), [Plant sources of propolis]. Pchelovodstvo (2), 28–29. (in Russian).

Tomas-Barberan F. A., Garcia-Viguera C., Vit-Oliver P., Ferreres F. and Tomas-Lorente F. (1993), Phytochemical evidence for the botanical origin of tropical propolis from Venezuela. Phytochemistry **34,** 191–196.

Zdero C., Bohlmann F. and King R. M. (1991), Guaianolides and labdanes from *Brickellia* species. Phytochemistry **30,** 1591–1595.