Antibacterial Diterpenic Acids from Brazilian Propolis

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Propolis, Diterpenic Acids, Antibacterial Activity, Propolis Plant Sources

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 (Mat-suda, 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., 1995). 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.

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 Et2O (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
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-H2O mixtures, the following compounds were isolated and identified: coniferylaldehyde (5), 25 mg (identified on the basis of MS, ¹H-NMR and UV spectra); isocupressic acid (2) [15-hydroxy-8(17),E-13-labdadien-19-oic acid], 19 mg (¹H-NMR, ¹³C-NMR, MS, IR spectra identical with literature data: Fang et al., 1989; Fang et al., 1991); acetylisocupressic acid (3), 10 mg (¹H-NMR, ¹³C-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 ¹H-NMR, ¹³C-NMR, MS spectra of its Me ester: Fujii and Zinkel, 1984), communic acid (5) [8(17),12,14-labdatrien-19-oic acid], 23 mg (¹H-NMR, ¹³C-NMR, MS identical with published data: Bohmann and Zdero, 1974; Fang et al., 1989), cis/trans 1:2 according to ¹H-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 CH₂Cl₂ 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-hydroxycinnamaldehyde). 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-acetloxy-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 ¹H-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...
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 bioautography (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>
<thead>
<tr>
<th>Substance</th>
<th>Diameter of inhibitory zone [mm]*</th>
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<tr>
<td>Propolis</td>
<td>11.8 ± 0.8</td>
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<tr>
<td>Syringaldehyde (1)</td>
<td>12.7 ± 0.7</td>
</tr>
<tr>
<td>Isocupressic acid (2)</td>
<td>10.5 ± 0</td>
</tr>
<tr>
<td>Acetylisocupressic acid (3)</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>Imbricatolic acid B (4)</td>
<td>9.1 ± 0.7</td>
</tr>
<tr>
<td>Communic acid (5)</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>Methyl isocupressate (6)</td>
<td>5.7 ± 0.6</td>
</tr>
</tbody>
</table>

* 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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