Chemical Composition and Antibacterial Activity of Brazilian Propolis

V. Bankova[a], R. Christov[a], A. Kujumgiev[b], M. C. Marcucci[c] and S. Popov[a]

[a] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[b] Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[c] Ecological Chemistry Laboratory – Chemical Institute of Universidade Estadual de Campinas, SP, Brazil

Z. Naturforsch. **50c**, 167–172 (1995); received January 10/January 20, 1995

Propolis, Polyphenols, Volatiles, GC/MS, Antibacterial Activity

Four samples of Brazilian propolis were investigated by GC/MS of different fractions. 32 volatile compounds, (10 of them new for propolis), as well as 12 more polar compounds (one of them new for propolis) were identified. Antibacterial activity was found in some propolis fractions.

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, 1979). Now propolis is extensively used in foods and beverages intended to maintain or improve human health.

The chemical composition of propolis appeared to be very complex and more than 160 propolis constituents have been identified so far (Greenaway *et al.,* 1990; Walker and Crane, 1987). Most important constituents of propolis appeared to be phenolics (more than 50% of the weight of propolis) but recently the chemical composition of volatiles was also investigated (Greenaway *et al.,* 1898; Petri *et al.,* 1988; Maciejevicz *et al.,* 1983; Clair and Peyeron, 1981; Bankova *et al.,* 1994).

There are many investigations on the origin of propolis. It is generally accepted that bees collect propolis from resinous tree buds and a tremendous amount of trees has been proposed as sources of propolis (Crane, 1988) but only in few cases chemical analyses have been performed in order to confirm these proposals. Almost always poplar buds appeared to be the source of propolis in temperate zones, especially *Populus nigra* L. (Lavie, 1976; Greenaway *et al.,* 1987; Papay *et al.,* 1986; Bankova *et al.,* 1986). In warmer countries (for example Albania (Bankova *et al.,* 1993) and Egypt (El-Hady and Hegazi, 1994; El-Hady, 1994) other compounds besides poplar components of propolis were identified too. Of special interest is the origin of bee glue in South America, because there are no poplar trees in the tropical regions. Investigations on propolis from Venezuela (Tomas-Barberan *et al.,* 1993) and Brazil (Aga *et al.,* 1993) showed unusual chemical composition: as expected, polyphenols from poplars were entirely absent. Investigated samples showed substantial differences in their chemical composition, depending on the collection site. The main propolis constituents, flavonoids, now are absent or in low concentrations, and substantial amounts of prenylated derivatives of benzophenones and cinnamic acids were found. *Clusia* species have been proved to be a source of propolis in Venezuela (Tomas-Barberan *et al.,* 1993) but their characteristic components have not been found in samples of Brazilian propolis (Aga *et al.,* 143). In order to investigate the origin of South American propolis we must have much more information about its chemical composition, including the minor components. For this reason we performed a detailed investigation with GC/MS on the chemical constituents of four Brazilian propolis samples, collected in regions where different plants predominate.

Experimental

The propolis samples were collected in Brazil, as follows: Br-1 in Sao Paulo State, near Rio Claro City; Br-2 in Parana State, near Prudentopolis;

Reprint requests to Prof. S. Popov.

Br-3 in Ceara State, near Pacajus; Br-4 in Sao Paulo State, near Limera.

Sample preparation

Alcohol extracts. Propolis, cut into pieces, was extracted with 70 % ethanol. The extract was evaporated and the residue dried. About 5 mg of the residue was dissolved in 0.1 ml BSTFA, heated for 20 min at 70°C and analysed by GC/MS.

Volatiles. The propolis samples were grated after cooling and subjected to steam distillation for 4h. The collected distillates were extracted with ethyl ether/*n*-hexane 1:1, the extracts dried over Na_2SO_4 and submitted to GC/MS. The volatile oil content of sample Br-1 was 0.6%, of Br-2 –0.5%, of Br-3 –0.3% and of Br-4 –0.3% from the dry weight of propolis.

Gas chromatography-mass spectrometry

Alcohol extracts. For the GC/MS analysis of the polar compounds a 25 m OV-101 fused silica capillary column was used in a JEOL JGC-20K gas chromatograph directly coupled to a JEOL JMS D-300 mass spectrometer. The samples were introduced *via* an all-glass injector working in the split mode, with helium as a carrier gas, and a temperature program 150–280° at 3°/min.

Volatiles. A 30 m SPB-1 fused silica capillary column was used in the same apparatus. The samples were introduced *via* an all-glass injector working in the split mode, with helium as a carrier gas, and a temperature program 60–280° at 6°/min.

In both analyses the mass spectrometer was run in the electron impact mode, the ionization potential was 70 ev, ionization current 100 μA and the ion source temperature 100 °C.

The components were identified by comparison with library mass-spectra.

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 microorganism *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. For every extract, the inhibitory zone of 0.4 mg of substance was measured.

Results and Discussion

Propolis samples have been collected from four different locations in Brazil, every one of them characterized by some type of predominating trees or brushes. Sample Br-1 was collected from hives in an *Eucalyptus* reforestation, sample Br-2 in a native forest, sample Br-3 in a cashew plantation and sample Br-4 in an orange plantation (for geographical locations see Experimental). Propolis samples have been powdered and a part of them extracted with ethanol, while other part was subjected to distillation with water vapour. Ethanol extracts were silylated and subjected to GC/MS investigation, the results obtained summarized in Table II. Total mixtures of the volatile constituents were investigated by the same method (Table I). For comparison, in Table I the composition of volatiles from Bulgarian propolis is shown (Bankova *et al.,* 1994). The identification of the propolis constituents was based on the comparison with the mass spectra of authentic samples. In the cases when such spectra have not been available, only the partial structure of the corresponding component was proposed on the basis of the mass spectral fragmentation observed. The quantitation of the components of the mixtures of silylated polar compounds and of volatile compounds was based on the ion currents generated by different compounds (Greenaway *et al.,* 1987; Bankova *et al.,* 1992).

Chemical composition of Brazilian propolis

From the results obtained it is evident that samples Br-1 and Br-2 have almost identical chemical composition, independently on the different collection sites and plant environments. For this reason we included in the tables data for Br-1 only. Samples Br-1 and Br-4 were collected in Sao Paulo state but show some differences in their composition, mainly in the volatile compounds.

The composition of the polar fraction in all investigated samples (Table II) appeared to be unusual for propolis and only few of the peaks were identified. All compounds identified (besides *m*-coumaric acid) have been found earlier in European propolis, originating from poplar buds. However, these compounds are wide-spread in nature and must have some other origin in Brazilian bee glue, since no poplars grow in the tropic

Table I. Composition of propolis volatile components[a,b].

Compound	Br-1	Br-3	Br-4	Bulg[c]
Ketones, aldehydes				
Acetophenone	0.7	–	2.8	
Methoxyacetophenone	–	–	–	3.3
Methoxyacetophenone (isomer)	–	–	–	0.6
4-Phenyl-3-buten-2-on	–	–	–	1.1
Prenyl acetophenone[a]	3.6	–	8.2	–
Diprenyl acetophenone[a]	11.1	–	1.7	–
Methoxy-benzaldehyde	–	–	1.5	–
Alcohols, phenols				
2-Phenylethanol	–	–	0.6	–
3-Phenylpropanol[a]	–	–	3.7	–
α-Methyl benzylalcohol[a]	–	–	1.2	–
Isoeugenol	–	–	–	0.8
Ethylphenol	0.6	–	4.6	–
Esters				
Methyl dihydrocinnamate[a]	–	–	1.2	–
Ethyl dihydrocinnamate[a]	0.3	–	0.7	–
Ethyl phenylacetate[a]	–	–	0.7	–
Benzyl acetate	–	–	–	1.6
Acids				
Nonanoic acid	0.7	–	–	–
Decanoic acid[a]	4.7	–	–	–
Tetradecanoic acid	2.2	–	–	–
Terpenoids				
α-Terpineol	1.5	–	1.6	–
2*Z*,6*E*-Farnesol	17.4	–	6.1	–
β-Caryophyllene	1.9	–	–	1.2
δ-Cadinene	3.3	3.3	0.7	5.3
Ledol[a]	5.7	–	0.1	–
Guajol	–	–	–	2.9
α-Copaene	–	–	–	0.9
β-Selinene	–	–	–	1.2
α-Elemene	–	–	–	2.3
Calamenene	–	–	–	2.2
α-Muurolene	2.4	–	–	2.0
γ-Muurolene	–	–	–	4.7
β-Eudesmol	–	–	–	8.8
Humulene	–	1.0	–	–
Bulnesol	–	–	–	2.3
Sesquiterpene alcohol, $M^+ = 220$	12.9	–	7.1	–
Hydrocarbons				
Xylene	0.9	0.3	–	–
Octadecane	–	2.5	–	–
Nonadecane	–	3.0	0.6	–
Heneicosane	–	3.8	1.3	–
Tricosane	–	5.2	2.3	4.9
Pentacosane	–	3.9	1.8	4.4
Heptacosane	–	–	2.9	2.7
Other compounds				
Dihydrobenzofurane[a]	–	0.5	2.0	–

[a] For the first time in propolis.
[b] The total ion current generated depends on the characteristics of the compounds concerned and is not a true quantitation.
[c] After Bankova *et al.* (1994).

Table II. Composition of alcohol extracts of propolis[a,b].

Compound	Br-1	Br-3	Br-4
Benzoic acid	1.7	–	1.1
Hydrochinon	1.1	–	0.8
Dihydrocinnamic acid	14.4	–	5.4
Methoxybenzoic acid	1.2	–	–
Hydroxybenzoic acid	1.5	–	0.5
Hydroxybenzoic acid (isomer)	–	–	0.5
m-Coumaric acid[a]	–	2.4	2.9
Palmitic acid	2.0	3.0	2.8
Ethyl caffeate	–	–	0.6
Caffeic acid	2.7	–	3.3
p-Coumaric acid	9.4	–	–
Oleic acid	–	2.4	–

[a] For the first time in propolis.
[b] The total ion current generated depends on the characteristics of the compounds concerned and is not a true quantitation.

regions of South America. Some of these compounds (for example hydroquinone, hydroxybenzoic acids and especially dihydrocinnamic acid) appeared in much higher concentration in Brazilian propolis than in material from temperate zone.

Flavonoids are the main constituents of propolis from the temperate zone. In most of the South American propolis samples investigated till now, flavonoids have not been found. In some samples from Venezuela, only traces of highly methylated 6-oxygenated flavones were identified (Tomas-Barberan, 1993). In sample Br-4 we found trace amounts of two dihydroxydimethoxy flavones and in Br-1 dihydroxydimethoxy flavanone, with both hydroxyl groups in ring A. Their identification requires larger amounts of propolis.

In Br-1 and Br-4 we found trace amounts of three compounds, tentatively identified as dihydroxydimethoxy antraquinone (two isomers) and one dihydroxymethoxy antraquinone. Antraquinones have not been found earlier in propolis but they are constituents of the exudates from some tropical plants and their antibacterial and fungicidal activity resembles that of propolis.

It is evident from Table II that the composition of the polar fraction in Br-1 and Br-4 is similar, while in Br-3 besides oleic and palmitic acids, originating probably from bees wax, we identified only the unusual *m*-coumaric acid.

We found also a similarity between the composition of volatiles from Br-1 and Br-4, the latter containing more components (Table I). Besides hydrocarbons, in Br-3 we found only three sesquiterpenoids.

There are significant differences in the composition of the volatile compounds in Bulgarian and Brazilian propolis. According to Petri *et al.* (1988), propolis from the temperate zone could be separated into two types, the first one being characterized by the presence of substantial amounts of β-eudesmol, while in the other one the main volatile constituent appeared to be benzyl benzoate. None of these compounds has been identified till now in propolis from South America, so it belongs to some other type. However, the final characterization of this type needs much more data on propolis volatiles from different locations in South America.

Derivatives of acetophenone are characteristic for different propolis samples but while Bulgarian samples contained only methoxy- and hydroxyacetophenones, in Brazilian samples we found mono- and diprenylated acetophenones, which appeared to be between the main volatile constituents of Br-1 and Br-4. The elucidation of the location of prenyl substituents needs additional amounts of these compounds. In general, prenylated aromatic compounds are common in tropical plants. In Venezuelan propolis prenylated benzophenones have been found (Tomas-Barberan *et al.*, 1993), while in Brazilian propolis C- and O-prenylated cinnamic acids are between the main constituents (Aga *et al.*, 1994).

While in propolis from the temperate zone esters of cinnamic acids predominate, in Brazilian propolis we found dihydrocinnamic acid and its esters in significant concentrations. The alcohol components of these esters in poplar type propolis are C_2-C_5 alcohols (Greenaway *et al.*, 1990, Bankova *et al.*, 1992), while in Brazilian propolis we found low concentrations of methyl and ethyl esters only.

Samples Br-1 and Br-4 contained significant amounts of terpenoids, only few of them being found in Bulgarian propolis. Almost all of them are sesquiterpenoids, (hydrocarbons and alcohols), part of them found for the first time in propolis (Table II). Main components appeared to be α-terpineol, 2*Z*,6*E*-farnesol, spatulenol and ledol. Only δ-cadinene was found in all investigated Bulgarian and Brazilian samples and humulene was found in the unusual Br-3 sample.

We can conclude that Brazilian propolis is characterized by very low concentration of flavonoids and esters of phenolic acids. High concentration of dihydrocinnamic acid, the presence of prenylated acetophenones and of some specific terpenoids are characteristic for Brazilian propolis. The results obtained confirm the suggestion that the chemical composition of Brazilian propolis is substantially different from this of propolis in temperate regions because of the different plant sources.

Origin of Brazilian propolis

Many authors refer to bees collecting sticky material on leaf buds of numerous tree species. It was proved unambiguously that in almost all cases bees collect propolis from poplar buds and in some cases from birch buds. There are no poplars in the tropic regions of South America and bees have to find other sources of propolis. Recently, Tomas-Barberan proved that the resin extruded by flowers of *Clusia minor* and *C. major* (Guttiferae) is one of the main sources of propolis in tropical Venezuela. This resin contained the polyprenylated benzophenones found in the majority of Venezuelan propolis samples studied (Tomas-Barberan *et al.*, 1993). We did not indicate even traces of prenylated benzophenones in all Brazilian propolis samples investigated by us and these results exclude *Clusia* sp. as its source. Prenylated cinnamic acid derivatives (Aga *et al.*, 1994) and a new clerodane diterpene (Yoshida *et al.*, 1993), identified in Brazilian propolis, were absent in Venezuelan propolis as well as in our samples. It is important to note that in some South American Asteracean species (*Baccharis* sp. and *Fluorensia* sp.) the same prenylated cinnamic acid derivatives and isomeric clerodane diterpenes were found (Labbe *et al.*, 1986; Zdero *et al.*, 1986; Bohlmann and Jakupovic, 1979). So *Flourensia* and *Baccharis* spp. are probably among the sources of bee glue in some regions of Brazil, but they are not the main plant sources of our samples.

While in temperate regions there are small differences in the chemical composition of propolis from different locations, in South America these differences are very significant. We mentioned the radical differences of the propolis composition of samples from Venezuela and Brazil – two countries with more or less similar climate. Till now there are three investigated propolis samples from Sao Paulo state: Br-1, Br-4 and the publication of H. Aga *et al.* (1994) and their chemical composition appeared to be quite different. This difference is obviously due to the plant sources available in the vicinity of the hives. However, most of the terpenoids identified in Br-1 and Br-4 are widespread and it is impossible to connect them to some definite plant. Sample Br-3 has unusual composition and must have a different source. The identification of some prenylated hydroxyacetophenones in *Fluorensia heterolepis* (Bohlmann and Jakupovic, 1986) suggests that this plant might be one of the sources of propolis in the region of Sao Paulo. In these propolis samples prenylated acetophenones are important constituents.

It is necessary to investigate more samples of South American propolis together with resinous exudates of plants in the vicinity of the hives in order to clear the origin of the South American propolis.

Antibacterial activity of Brazilian propolis

The antibacterial activity of propolis is of great importance for the bee community and independently of its origin propolis always possesses such activity. It is due to a significant part of the propolis constituents, mainly phenols (flavonoids, phenolic acids and their esters) (Ghisalberi, 1979). Antibacterial activity has been found also in the volatile fraction from propolis and could be due to some phenols and/or terpenoids (Petri *et al.*, 1988).

Our investigations on the antibacterial activity of Brazilian propolis were performed by a modification of bioauthography (Kujumgiev *et al.*, 1993) with *Staphylococcus aureus* 209 as an indicator strain. The results obtained are summarized in Table III. From the data obtained it is evident that the main part of the antibacterial activity is concentrated in the polar compounds, mainly phenols. Bioautography showed that the antibacterial activity is due mainly to compounds with chromatographic behaviour identical with that of phenolic acids.

Table III. Antibacterial activity of propolis fractions.

Fraction/ sample	Diameter of inhibitory zone [mm]
Volatiles/Br-1	12.1 ± 0.6
Volatiles/B-3	12.8 ± 0.3
Volatiles/Br-4	11.2 ± 0.3
Volatiles/Bulgarian	<4
Alcohol extract/Br-1	7.3± 0.6
Alcohol extract/Br-3	6.7 ± 0.7
Alcohol extract/Br-4	9.2 ± 0.9
Alcohol extract/Bulgarian	9.0 ± 0.5

Antibacterial activity was found also for a mixture of volatile constituents. Many of the identified constituents of volatile compounds possess such activity (phenols, esters, terpenoids etc.). Surprisingly, we did not find analogous antibacterial activity in the volatiles from Bulgarian propolis. This could be explained by the observed significant differences in the chemical composition of Brazilian and Bulgarian propolis, espessially the lower content of phenols.

Recently H. Aga *et al.* (1994) established antibacterial activity of Brazilian propolis. We also found antibacterial activity in all Brazilian propolis samples investigated by us. This activity appeared to be almost identical with the activity of Bulgarian propolis (Table III), inspite of the obtained significant differences in the chemical composition and origin of bee glue from both countries. Evidently, the antibacterial activity of propolis is of great importance for the hive and bees in different countries collect propolis from such plants which can ensure constant antibacterial activity.

Acknowledgements

The authors wish to thank Dr. E. Tsankova for hepful advice on the manuscript. Partial support of this work by the National Foundation for Scientific Research (Contract # X-254) is gratefully aknowledged.

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

Bankova, V.; Christov, R., Popov, S., Pureb, O. and Bocari, G. (1994), Volatile constituents of propolis. Z. Naturforsch. **49 c**, 6–10.

Bankova, V., Dyulgerov, A., Popov, S., Evstatieva, L., Kuleva, L., Pureb, O. and Zamjansan, Z. (1992), Propolis produced in Bulgaria and Mongolia: phenolic compounds and plant origin. Apidologia **23**, 79–85.

Bohlmann, F. and Jakupovic, J. (1979), Neue Sesquiterpene, Triterpene, Flavanone und andere aromatische Verbindungen aus *Fluorensia heterolepis*. Phytochemistry **18**, 1189–1194.

Clair, G. and Peyeron, L. (1981), Contribution a l'etude de huile essentielle de propolis. Riv. Ital. EPPOS **63**, 168–170.

Crane, E. (1988), Beekeeping: Science, Practice and World Recources. Heinemann, London, pp. 367–372.

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

Greenaway, W., May, J., Scaysbrook, T. and Whatley, F. R. (1990), Identification by gas-chromatography-mass spectrometry of 150 compounds in propolis. Z. Naturforsch. **46c**, 111–121.

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

Greenaway, W., Scaysbrook, T. and Whatley, F. (1989), Headspace volatiles from propolis. Flavor Fragrance J. **4**, 173–175.

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

Labbe, C., Rovisora, F., Faini, F., Mahu, M., San-Martin, A. and Castillo, M. (1986), Secondary metabolites from Chilean *Baccharis* species. J. Nat. Prod. **49**, 517–518.

Lavie, P. (1976), Relationship between propolis, poplar buds (*Populus* sp.) and castoreum. Proc. of XXV Int. Beekeep. Congr. (Grenoble 1975), Apimondia Publ., Bucharest, pp. 227–231.

Maciejevicz, W., Scheller, St. and Danievski, M. (1983), GC-MS study of propolis. Analysis of sesquiterpenoids. Acta Pol. Pharm. **40**, 251–253.

Papay, V., Toth, M., Soltes, M., Nagy, E. and Litkei, Gy. (1986), Isolated compounds from Hungarian propolis and *Populi gemma*. Stud. Org. Chem. (Amsterdam) **23**, 233–240.

Petri, G., Lembercovics, E. and Folalvari, M. (1988), Examination of differences between propolis (bee glue) produced from diffeent floral environments. In: Flavors and Fragrances: A World Perspective (B. M. Lawrence, B. D. Mookhergjee and B. J. Williams, eds.). Elsevier, Amsterdam, pp. 439–446,.

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.

Walker, P. and Crane E. (1987), Constituents of propolis. Apidologie **18**; 327–334.

Yoshida, M., Saito, Y. and Metsuno, T. (1993), Diterpenoid from propolis as antitumor agent. Eur. Pat. Appl. EP 523,156 (CA 119: 63032h)

Zdero, Z., Bohlmann, F., King, R. M. and Robinson, H. (1986), Diterpene glycosides and other constituents from Argentinian *Baccharis* species. Phytochemistry **25**, 2841–2855.