Chemical Composition and Biological Activity of Propolis from Brazilian Meliponinae

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Twenty-one propolis samples produced by 12 different Meliponinae species were analyzed by GC-MS. Several chemical types of stingless bees' propolis could be grouped, according to the prevailing type of compounds like: "gallic acid", "diterpenic" and "triterpenic" types. The results confirm that neither the bee species nor the geographical location determine the chemical composition of Meliponinae propolis and the choice of its plant source, respectively. This could be explained by the fact that Meliponinae forage over short distances (maximum 500 m) and thus use as propolis source the first plant exudate they encounter during their flights. The antibacterial, antifungal and cytotoxic activities of the samples were also investigated. Most samples had weak or no activity against *E. coli*, weak action against *Candida albicans*. Some of them showed significant activity against *St. aureus.*, presumably connected to the high concentration of diterpenic acids. Samples rich in diterpenic acids possessed also high cytotoxic activity (*Artemia salina* test).

Introduction

The bees of the subfamily Meliponinae, popularly known as stingless bees, are widespread over the tropical and subtropical areas of the world. Stingless bees have been kept in South America for centuries; meliponiculture existed long before the arrival of Columbus and the common honey bee *Apis mellifera*. Meliponinae are valuable pollinators of many crops, most species produce delectable honey, together with wax and propolis (Kerr, 1987). In Brazil, more than 200 species of Meliponinae are known. Stingless bees' propolis (as alcohol extracts) is used in Brazil as a remedy for healing wounds, gastritis, haemorrhoids etc. (Kerr, 1987). Its chemical composition and biological activity are largely unknown. Only three investigations have been published on the chemistry of this type of propolis, and the results showed that it could or could not be similar to the one of honey bees from the same regions (Tomas-Barberan *et al.*, 1993; Bankova *et al.*, 1998; Bankova *et al.*, 1999). Some samples showed good antibacterial and antifungal activity, comparable to *Apis mellifera* bee glue (Kujumgiev *et al.*, 1999). In the present paper we report the chemical composition of propolis from twelve Brazilian Meliponinae species determined by GC-MS. We also report the results of antibacterial, antifungal and cytotoxicity tests and some comments on the possible composition/activity relationship.

Experimental

Propolis samples were collected at different locations in Brazil. The geographic origin of the samples, bee species and time of collection are listed in Table I.

Propolis extraction and sample preparation

Propolis (1 g of each sample), grated after cooling, was extracted twice (×24h) with 70% EtOH (10 ml) at room temperature. The extract was evaporated to dryness. About 5 mg of the residue were mixed with 40 µl dry pyridine and 60 µl N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA),

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heated at 80 °C for 20 min and analysed by GC-MS.

GC-MS analysis

Analysis was performed with a Hewlett Packard Gas Chromatograph 5890 Series II Plus linked to a Hewlett Packard 5972 mass spectrometer system equipped with a 23 m long, 0.25 mm id, 0.5 μm film thickness HP5-MS capillary column. The temperature was programmed from 100 °C to 310 °C at a rate of 5 °C.min^{-1}. Helium was used as a carrier gas, flow rate 0.7 ml.min^{-1}. Split ratio 1:80, injector temperature 280 °C. The ionization voltage was 70 eV.

Identification of compounds

The identification was accomplished using computer searches on a NIST98 MS Data library. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the basis of its mass-spectral fragmentation. If possible reference compounds were co-chromatographed to confirm GC retention times.

Antibacterial activity

For the investigation of the antibacterial activity the agar cup method was used with *Staphylococcus aureus* 209 and *Escherichia coli* WF+ as test strains. An inhibitory zone with diameter less than 10 mm corresponds to lack of activity (10 mm is the diameter of the cup). 0.1 ml of test solution containing 0.4 mg propolis extract in ethanol was applied to every cup.

Antifungal activity

For the investigation of the antifungal activity the agar cup method was used (Spooner and Sykes, 1972). As a test microorganism, *Candida albicans* 562 was used. The antifungal activity was measured as a diameter of the inhibitory zones. An inhibitory zone with diameter less than 10 mm corresponds to lack of activity (10 mm is the diameter of the agar cup). Control experiments with solvent (ethanol) showed that solvent does not have any activity. 0.1 ml of the test solution containing 0.5 mg propolis extract in ethanol was applied to every cup.

Cytotoxicity assay

Artemia salina lethality (Solis *et al.*, 1993) was determined using caffeic acid phenethyl ester (CAPE) as active reference substance. Concentrations of 1000, 100, 10 and 1 ppm were used; 10 *A. salina* per concentration plus control.

Results and Discussion

Propolis samples analyzed were produced by 12 different Meliponinae species. For some species, several samples were analyzed, originating from different locations, to elucidate the influence of the surrounding vegetation on propolis composition. A sample of honey bee propolis from a location near Prudentopolis (Paraná) where many of the Meliponinae samples were collected, was also analyzed (Table I).

The bee glue extracts were silylated to increase volatility and analyzed by GC-MS (see Experimental). The results obtained demonstrated the complex composition of Meliponinae propolis samples and the heterogeneity of their chemical patterns. Over 50 individual compounds have been identified in the samples investigated (part of the GC/MS peaks remained unidentified, because of lack of authentic samples and library spectra of corresponding compounds). For this reason, the chemical composition is presented by means of percentage of the main types of compounds identified, not as percentage of individual compounds (Table II). From the results in Table II it is obvious that there are several types of Meliponinae propolis, according to the prevailing type of compounds. For 5 samples (MAN, UR, MA, MB1, PIS) these are benzoic acid derivatives, the major one being gallic acid. Recently, gallic acid was found to be the major component of the resin of *Eucalyptus cytriodora* (Bankova *et al.*, 1999a), which is probably the plant source of these samples, although they originate from different states (São Paulo and Pernambuco) and from 4 different bee species.

Other distinct types are "diterpenic" (MP, RP, MR, PIR, BO, MIR) and "triterpenic" propolis (MIG, MO1, YG, JMG, JP). Most diterpenes were tentatively identified as diterpenic acids, only kaurenoic acid and dehydroabietic acid have been positively identified. Kaurenoic acid was present in all "diterpenic type" samples. The triterpenes are mainly alcohols of the amyrine type, incl. β-

Table I. Enthomological and geographical origin of propolis samples.

Sample	Bee species		Location	Collection date
	Trivial name	Latin name		(year, month)
MA	Mandacaia	*Melipona quadrifasciata*	Araripina, PE	1997, Nov
ME1	Mandacaia	*Melipona quadrifasciata*	Betim, MG	1997, Mar
ME2	Mandacaia	*Melipona quadrifasciata*	Betim, MG	1997, July
PIS	Mandacaia	*Melipona quadrifasciata*	Pilar do Sul, SP	1998, Dec
RP	Mandacaia	*Melipona quadrifasciata*	Ribeirao Preto, SP	1998, Dec
PIR	Mandacaia	*Melipona quadrifasciata*	Pirina, ES	1998, Dec
MP	Mandacaia	*Melipona quadrifasciata*	Prudentopolis, PR, (other place)	1997, Nov
MIR		*Melipona favora orlinge*	Miranda, MS	1998, Dec
UR	Uruci	*Melipona scutelaris*	Recife, PE	1997, Nov
MAN	Manduri	*Melipona marginata*	Araripina, PE	1997, Nov
MR	Mandaguari	*Scaptotrigona bipunctata*	Prudentopolis, PR	1999, Feb
MB1	Mirim da porta branca	*Plebeia remota*	Prudentopolis, PR	1999, Feb
MB3	Mirim da porta branca	*Plebeia remota*	Prudentopolis, PR, (other place)	1999, Feb
MIG	Mirim guacu	*Plebeia spp.*	Prudentopolis, PR	1999, Feb
MO1	Mirim	*Plebeia droryana*	Betim, MG	1999, Jan
AM	honey bee	*Apis mellifera*	Jaciaba, PR	1999, Feb
YG	Jatai	*Tetragonisca angustula*	Prudentoplis, PR	1999, Feb
JMG	Jatai	*Tetragonisca angustula*	Betim, MG	1999, Jan
NMG		*Nanotrigona testacularis*	Betim, MG	1999, Jan
JP	Iratim	*Lestrimellata spp.*	Prudentoplis, PR	1999, Feb
BO	Bora	*Tetragona clavipes*	Prudentopolis, PR	1999, Feb

Table II. Chemical composition of ethanol extracts of propolis from Brazilian Meliponinae (% of total ion current, GC-MS)*.

Comp.	MA	ME1	ME2	PIS	RP	PIR	MP	MIR	UR	MAN	MR	MB1	MB3	MIG	MO1	AM	YG	JMG	NMG	JP	BO
B.a.	22.4	3.4	5.5	20.5	0.3	0.2	6.8	–	28.6	30.2	2.9	20.0	1.4	0.1	–	0.9	0.2	1.5	–	–	0.6
D.h.c.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.9	–	–	–	–	–
Cinn	4.2	1.5	1.6	–	–	–	2.8	–	2.7	7.5	–	1.1	0.9	0.2	–	2.9	–	–	–	–	0.9
Caff. l.c.	–	4.2	7.6	–	–	–	–	–	–	–	–	–	–	–	8.4	–	3.3	2.7	–	–	–
Caff	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.4
PCA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.4	–	–	–	–	–
Diter	–	–	–	–	20.6	46.4	38.3	21.2	–	–	27.0	0.2	1.1	6.9	–	5.6	–	–	18.2	3.4	24.2
Triter	–	2.3	8.9	2.5	11.2	–	–	4.5	–	–	3.7	7.5	0.3	33.5	21.8	10.0	33.3	15.5	13.5	21.3	16.5
Sug	13.3	14.9	0.5	3.8	–	–	–	–	26.1	1.5	1.2	0.8	16.1	4.8	0.1	1.1	1.5	7.6	–	–	1.4

* The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.
B.a., benzoic acids.
D.h.c., dihydrocinnamic acids.
Cinn, cinnamic acids.
Caff. l.c., long chain caffeates.
Caff, prenyl caffeates, benzyl and phenethyl caffeates.
PCA, C-prenylated coumaric acids.
Diter, diterpenic acids.
Triter, triterpenic alcohols.
Sug, sugars.

amyrine itself. In some of these samples (MIG, MO1, YG), significant amount of lanosterol was found. NMG obviously is a combined "diterpenic-triterpenic" type. The rest of the samples represent some "mixed" types, presumably due to the use of several plant sources by bees.

The sample AM is *Apis mellifera* propolis. It is the only one containing C-prenylated *p*-coumaric acids and their cyclic derivatives. These compounds have often been identified in Brazilian propolis and originate from *Baccharis* spp., mainly *B. dracunculifolia* (Bankova *et al.*, 1999a).

Surprisingly, they were totally absent from Meliponinae propolis. Another unexpected finding is the identification of "poplar phenolics" (prenyl caffeates, phenylethyl caffeate, the flavonoid 3-O-acetyl pinobanksin) characteristic for European and North American honey bee propolis (Greenaway *et al.*, 1990) in the sample BO. A possible explanation is the presence of a single introduced poplar tree somewhere near the nest, because generally poplars are not growing in Paraná state.

The results confirm that neither the bee species nor the geographical location determine the chemical composition of Meliponinae propolis and the choice of its plant source, respectively. For example, 6 of the samples (MR, MB1, BO, YG, MIG, JP) were collected at a single location, an apiary in Prudentopolis. However, MB1 belongs to the gallic acid type, MR and BO – to the diterpenic, and YG, MIG, JP – to the triterpenic type. On the other hand, six samples were collected from a single species (*Melipona quadrifasciata anthidioides)* and from different locations (even from different states). Two of them are of the gallic acid type (MA and PIS), three – of the diterpenic type. Triterpenes are present in all samples but are not the main compound group. Samples ME1 and ME2 are from the same bee species and location, the only difference was due to the season. Some qualitative seasonal variations were observed, similar to those in honey bee propolis (Bankova *et al.*, 1998b). In these samples, long-chain caffeic acid esters were identified (C_{14}, C_{16}). These caffeates were recently found in honey bee propolis from Egypt (Christov *et al.*, 1998), their plant source is still unknown.

The above mentioned observations do not reveal any system in the plant source choice of Meliponinae. It seems more or less randomly. On the other hand, Brazilian propolis from *Apis mellifera*, although chemically variable, tends to have a constant qualitative composition in a particular region (Marcucci *et al.*, 2000). This could be explained by a different behaviour between stingless bees and honey bees. Possibly because of the greater density of resources in the tropical habitats the Meliponinae forage over shorter distances than the other bees with most species having a maximum distance of 500 m. On the contrary, honey bees often forage over several kilometres. Possibly stingless bees use as propolis source the first plant exudate they encounter during their flights.

Bee glue is the natural defence of bees against microorganisms and for this reason we tested stingless bees' propolis for its antibacterial and antifungal action (Table III). Similarly to honey bee propolis, the Meliponinae samples had weak or no activity against the Gram-negative test strain *E. coli*. Their action against the pathogenic yeast *Candida albicans* was also weak. Most of the samples showed significant activity against *St. aureus*, comparable to the one of *A. mellifera* sample (AM). In most cases high antibacterial activity was related to a high percentage of diterpenic acids (MIR, PIR, BO, MP) or to a combination of gallic acid and diterpenic acids. Antibacterial diterpenes were recently found in Brazilian honey bee propolis (Bankova *et al.*, 1996). The high content of gallic acid alone did not result in high antibacterial activity (UR, MA). In some cases the antibacterial action could be due to unidentified substances.

The cytotoxicity of the samples was also investigated using the *Artemia salina* (brine shrimp) test. The results are presented in Table IV. Several samples showed remarkable cytotoxicity (MP, RP, PIR, NMG). This activity is probably connected to the high content of diterpenic acids in these sam-

Table III. Antibacterial and antifungal activity of propolis samples (extracts with 70% ethanol).

Sample	*S. aureus*	*E. coli*	*C. albicans*
	(diameter of the inhibitory zone ± stand. deviation, mm)[a]		
MA	15 ± 1	0	13 ± 0
ME1	13.7 ± 0.6	0	12 ± 0
ME2	15 ± 1	0	12 ± 0
PIS	18.3 ± 0.6	0	13 ± 1
RP	15 ± 1	11 ± 0	11 ± 1
PIR	17 ± 1	12 ± 0	12.7 ± 0.6
MP	15 ± 1	0	13 ± 0
MIR	18 ± 1	12 ± 0	11 ± 1
UR	13 ± 1	0	12 ± 0
MAN	16 ± 1	0	13 ± 0
MR	16 ± 2	12 ± 0	13 ± 1
MB1	18 ± 1	12 ± 1	13.3 ± 0.6
MB3	15 ± 1	12 ± 0	11 ± 1
MIG	17 ± 1	0	12 ± 2
MO1	12.6 ± 0.6	0	12 ± 1
AM	18 ± 2	11.7 ± 0.5	11 ± 1
YG	10 ± 1	12 ± 0	11 ± 1
JMG	12 ± 0	11 ± 1	13 ± 2
NMG	10 ± 1	12 ± 0	11 ± 1
JP	16.3 ± 0.6	0	12 ± 1
BO	16.7 ± 0.6	13 ± 0	11 ± 1

[a] Mean of three measurements.

Table IV. Cytotoxicity assay of Meliponinae propolis extracts.

Sample	LC_{50} (µg/ml) ± sd[a]
MA	>1000
ME1	12.0 ± 0.2
ME2	197.6 ± 21.0
PIS	29.7 ± 5.7
RP	5.5 ± 0.2
PIR	2.3 ± 0.3
MP	0.3 ± 0.2
MIR	60.2 ± 2.1
UR	not tested
MAN	104.7 ± 9.9
MR	105.3 ± 6.5
MB1	30.0 ± 5.5
MB3	15.0 ± 4.1
MIG	74.1 ± 14.4
MO1	18.2 ± 5.6
AM	11.9 ± 7.1
YG	215.7 ± 35.8
JMG	36.5 ± 5.1
NMG	5.4 ± 1.2
JP	17.6 ± 3.5
BO	41.4 ± 1.6
CAPE (standard)	0.45 ± 0.09

[a] Mean of two measurements.

ples. Recently, several diterpenic acids with significant cytotoxic and antitumor action have been isolated from Brazilian honey bee propolis (Matsuno *et al.*, 1997; Matsuno, 1995). These samples are of interest for future chemical and biological investigations.

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