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CHIRAL ANALYSIS OF MONOTERPENES IN VOLATILE OILS FROM PROPOLIS

EUCLÉSIO SIMIONATTO[1], JANAINA T. FACCO[1], ADEMIR F. MOREL[2], SANDRO R. GIACOMELLI[3], CARLOS E. B. LINARES[3]*

[1]*Universidade Estadual de Mato Grosso do Sul, Programa de Pós-Graduação em Recursos Naturais, Naviraí-MS, 79950-000, Brazil.*
[2]*Departamento de Química, Universidade Federal de Santa Maria, 97105-900, Santa Maria- RS, Brazil.*
[3]*Universidade Regional Integrada, 974000-000, Frederico Wetsphalen -RS, Brazil.*
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ABSTRACT

The essential oils obtained by hydrodistillation from samples of propolis manufactured in three regions of Rio Grande do Sul State at Brazil, were analyzed through CG, CG-MS and chiral phase gas chromatography (CPGC). These analyses display the presence of samples with elevated essential oil purport, when compared with plants. The yields obtained were until 3.8%. The samples exhibited similar composition, with predominance of the monoterpenes α-pinene (57-63%), β-pinene (12.5-30.8%) and limonene (1.5-11.2%). In chiral analysis of these constituents were observed modifications in the enantiomeric excess of isomers of α-pinene and limonene in relation with source location of the sample, already in the chiral analysis of β-pinene were detected only elevated excess of the enantiomer with the (-) configuration. The antimicrobial activity of the crude essential oil was assayed against five bacteria. The best result was obtained against *Staphylococcus aureus*.

Keywords: propolis, essential oil, chiral phase gas chromatography, enantiomeric composition, antimicrobial activity.

INTRODUCTION

Propolis is a resinous material multifunctional, collected by the bees of branches, flowers, pollen, sprouts and exuded of trees and shrubs, being used in the maintenance of the beehives, avoiding to the pathologic action.[1,2]

This product presents several biological properties as antimicrobial, antioxidant, antiviral, antifungal, anti-inflammatory and antitumoral. Today, the propolis due to its several beneficial activities is used as a popular remedy commercially available, in the form of capsules, extracts, liquids for buccal cleaning, tablets for throat and creams. It is also available commercially as purified product in which the wax has been removed from beehives.[1-3] The propolis extracts present a complex composition, result of the geographical variations and botanical origin.[4] So, the geographical location and associated flora can determine the propolis composition.[5] Considering that some factors (e.g.; rainfall and variations in temperature), could affect the chemical composition of propolis, investigations on the chemical composition and biological properties are important not only for academic interest, but also for the chemical and biological standardization of a particular type of propolis.[6]

The studies with propolis in Brazil have been surrendering several scientific works. Due to great production of this material, the Brazil stands out as one of the largest world producers, has been generating interest of several national and international research groups. The high variety of active compounds has been contributing to a great international preference, not only commercially, but also in the scientific area. In the bibliographical revisions was observed that several papers published in the international periodical and many patents also present data on the Brazilian propolis. [7-9] The use of propolis in the world is estimated at about 700-800 tonnes per year.[10] Insufficient official statistics on the volume of propolis produced in Brazil each year, which is exported and what is consumed by the domestic market. The consensus is that Brazil is the second largest producer behind China's and Japan is the main importer of Brazilian propolis.[11,12]

Some works, among many found in the literature on the studies with propolis, mention the importance in the discovery of new molecules (new metabolites) and in the identification of the biological properties of substances obtained from propolis extracts. Velikova, [13] analyzing the samples of Brazilian propolis, collected in the municipal district of Prudentópolis in the State of Paraná, described the isolation of new diterpenes of the class of kaurenes. Kusumoto, [14] described the isolation of new chemical constituents, terpenes and phenolic derived, from the essential oil obtained of propolis samples collected in the south of Minas Gerais, Brazil. A new type of Brazilian propolis rich in prenylated benzophenones was characterized recently from Amazon region.[2]

The composition of the propolis is intimately linked with the vegetation where the bees inhabit and with the ecological interactions involving bees with local plants.[3] In general its composition varies around vegetable resins (50%), waxes (30%), essential or volatile oils (10%), pollen (5%) and others organic substances (5%).[1] In agreement with the literature, the propolis extract can contain high level of essential oils (up to 10%), which are very superior to the yields of essential oils obtained from extractions of common and classical aromatic plants such as *Eucalyptus globulus*, *Mentha arvensis*, *Thymus vulgaris*, *Foeniculum vulgare* and species of genus *Ocimum*. [15, 16]

In the present work, was investigated the chemical composition of the volatile oils obtained from propolis samples collected in three areas in the Rio Grande do Sul State, Brazil. The collection was made at the municipal districts of São Francisco de Assis, Santiago and Jaguari, region that introduce a strong beekeeping activity. For these investigations was used as main tool analysis gas chromatography enantioselective or chiral gas chromatography. In addition, the antimicrobial activity of essential oil from propolis sample was also examined.

The main application of the enantiomer separation by gas chromatography with enantioselective capillary columns is related with the precise determination of the enantiomeric composition of chiral substances, including natural products, precursors, drugs, pesticides, fungicides, herbicides, pheromones, aromas and fragrances.[17, 18] In essential oils, this analysis possesses a great importance in the control of quality of these oils, where they supply important information about its authenticity detecting the presence of possible synthetic adulterants. [19] So far, no reports are available about the enantiomeric composition of volatile oils from propolis.

MATERIAL AND METHODS

Material

Propolis samples was collected at the municipal districts of Santiago (sample A), São Francisco de Assis (sample B) and Jaguari (sample C), Rio Grande do Sul State, Brazil. Samples were collected between September 2003 to November 2004.

Essential oil extraction

Each sample of propolis (150 g) was subjected to hydrodistillation for 4 h using a modified Clevenger-type apparatus. [20] The essential oils were dried over anhydrous Na_2SO_4, stored in a dark glass bottle and kept at 4°C until analysis.

Chemical analysis

The oils were submitted to GC analysis in a Varian 3800 Gas Chromatograph equipped with two capillary fused silica columns (25 m x 0.25 mm; film thickness 0.2 μm) coated with SE-54 (apolar column) and PEG-20M (polar column). The GC conditions used were: carrier gas H_2 (1 mL/min); injector split/splitless 220°C; FID Detector 280°C; column temperature 50–250°C at 4°C/min. GC/MS analyses were performed on a HP 5973–6890 GC-MSD system, equipped with a HP-5 cross linked capillary column (30 m x 0.25 mm; film thickness 0.2 μm). The MS scan parameters included electron impact ionization voltage of 70 eV, a mass range of 41–380 $m\ z^{-1}$ and a scan

e-mail autor: eusimionatto@yahoo.com.br

interval of 0.5 s. The temperature of the column and the injector were the same as those from GC.

The chiral monoterpene constituents of propolis oils were identified by peak enrichment in enantioselective capillary GC with two fused capillary columns (Dual System), 25 m x 0.25 mm, film thickness 0.2 µm, coated with heptakis-(6-O-methyl-2,3-di-O-pentyl)-β-cyclodextrin and octakis(3-O-butiryl-2,6-di-O-pentyl)-g-CD (Lipodex- E), each diluted with the polysiloxane OV-1701 (1:1).

Identification of essential oil constituents

The identification of the components of the oils was based on comparison of the retention times and Retention Indices on both columns and mass spectra with those of NBS/NIST Library [21] and those described by Adams. [22]

Dual system of capillary columns

A Varian 3800 Gas Chromatograph equipped with injector split/splitless and two FID detectors were used in all applications. The dual system consisted of two chiral columns connected to the same injector. Each column was connected to a different detector (D1 and D2). D1 was connected to a column with heptakis-(6-O-methyl-2,3-di-O-pentyl)-β-cyclodextrin and D2 to a column with octakis(3-O-butiryl-2,6-di-O-pentyl)-g-CD (Lipodex-E) stationary phase. Temperature program: 40–180 °C at 2.0 °C/min; split/splitless injector (220 °C); injection mode: split, 1:10 ratio; injection volume: 1.0 mL; inlet pressure: 7 psi; carrier gas: H_2. Data were collected by the StarChromatography software (Varian).

Bacterial strains

The essential oils were assayed against five bacterial species: *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 25619, *Klebsiella pneumoniae* ATCC 1003, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25792. Bacterial strains are stored on Muller Hington agar in the URI - FW Mycology Laboratory. The strains were subcultured on to Muller Hington agar tubes prior the assays.

Agar diffusion – screening method

Propolis essential oil was tested for antimicrobial activity using agar diffusion method on solid media. Muller Hington agar plates were used for the test. Sterile paper disc was placed on the agar plates and its surface was spread with 0.1 mL of a logarithmic phase bacteria at a density adjusted by Mc Farland standard scale. After 10 µL of the essential oil was applied to the disc. Agar plates were incubated for 24 h at 37°C. The results were recorded by measuring the zones of growth inhibition around the discs. Tetraciclin and cloranphenicol discs (30µg) were used to a control. After 24 h of incubation, the zones of growth inhibition by the essential oil were measured against the pathogenic microorganisms tested. [23, 24]

RESULTS AND DISCUSSION

The comparative study among volatile oils allowed evidencing the high percentage of monoterpenes, dominated by chiral hydrocarbons α-pinene, β-pinene and limonene that represent together more than 85% of the composition of the oils (Table 1). This study also demonstrated the considerable level of oils in these propolis samples, being obtained medium yields of 3,0%, the highest reached 3,8%. The oil composition and concentrations in both samples presented to be rather similar, not presenting quantitative variations of the main compounds, during the whole study period (one year).

The oil of the sample A (originating from Santiago, yield of 2.5%) presented high amount of α-pinene 62%, proceeded by β-pinene 29.2%. In the sample B (originating from of San Francisco de Assis, yield of 2.7%) the accumulation of β-pineno was of 57% and β-pineno 30.8%. In the sample C (originating from Jaguari, yield of 3.8%) α-pinene was found with 63% and β-pinene with 12.5%. The monoterpene limonene, was found in two samples (A and C) with amounts that varied of 1.5-11.2%. Other components are listed in the Table 1, including some sesquiterpenes present in the samples.

Table 1. Percentage composition of the essential oils of propolis.

Compound[a]	%in samples			Kovats Indices		Identification[e]
	A	B	C	Apolar[b]	Polar[c]	
α-Pinene	62.0	57.0	63.0	931	1007	RI, GC-MS, Co
β-Pinene	29.2	30.8	12.5	939	1016	RI, GC-MS, Co
Limonene	1.5	-	11.2	953	1060	RI, GC-MS, Co
myrcene	0.2	tr	-	976	1065	RI, GC-MS, Co
1,8-cineole	0.2	-	tr	1033	1200	RI, GC-MS, Co
E-caryophyllene	0.5	-	-	1449	1586	RI, GC-MS, Co
E-nerolidol	-	0.7	0.2	1566	2087	RI, GC-MS, Co
sphatulenol	-	1.2	0.5	1579	2164	RI, GC-MS, Co
caryophyllene oxide	-	-	0.6	1580	1986	RI, GC-MS, Co
Total	93.6	89.7	88.0			

[a]Compounds listed in order of elution from a SE-54 column; [b]Kovats Indices determined on apolar SE-54 column (50-250 °C; 4 °C min^{-1}); [c]Kovats Indices determined on polar PEG-20M column (50-250 °C; 4 °C min-1); dI identification: RI, Kovats index by Adams RP[22], GC-MS, gas chromatography-mass spectroscopy; Co, co-injection of authentic material. tr: < 0.1

Through chiral-CG was possible to observe the presence of inversion of the enantiomeric excesses of the main isomer of the monoterpene α-pinene in relationship with the local of the origin propolis (Table 2). The oil of propolis from Jaguari (sample C) was characterized by enantiomeric excess of (-)-α-pinene, while the enantiomeric excess of (+)-α-pinene was encountered in samples of São Francisco de Assis e Santiago (B and A) (Figure 1). With relationship to the amount of β-pinene, was found excesses above 95% of the (-)-β-pinene enantiomer in all the studied samples. Taking in consideration the chiral chemical profile of limonene, the results showed no likeness among the samples. The chiral distribution of limonene was (+)-54% / (-)-46% in Santiago's original sample (A), already in the sample of Jaguari (C), was observed enantiomeric excess of 100% to the configuration (+). The limonene was not detected in the sample originating from of São Francisco de Assis (B).

Conventional chiral-CG is commonly employed for the assessment of essential oil quality, through the determination of the enantiomeric excesses of volatile chiral compounds; one of the most popular chiral selectors is the octakis(3-O-butiryl-2,6-di-O-pentyl)-g-CD (Lipodex- E) stationary phase, the selectivity of which is well known.[7, 8] Using the dual system is possible to accompany the separation effect simultaneously in the two chiral columns. Here, in the case of propolis oils, the dual system allows to observe an increase in the

agility of analyzes. The optical isomers of a-pinene was entirely separated on a column coated with heptakis-(6-O-methyl-2,3-di-O-pentyl)-β-cyclodextrin. The satisfactory separation of isomers of b-pinene and limonene were obtained on a column coated with octakis(3-O-butiryl-2,6-di-O-pentyl)-g-CD (Lipodex-E) stationary phase (Figure 1). The use of dual system applied in the present research allowed the acquisition of the chromatography performance of two columns during the same time, leading to a more accurate investigation of the chiral components. The dual system can be varied, using one achiral and one chiral stationary phases; two achiral stationary phases or two chiral stationary phases.[25, 26]

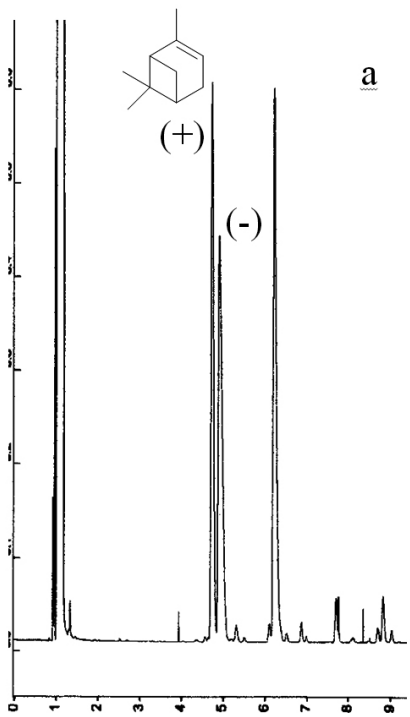
Table 2. Enantiomeric distribuitions (%) of monoterpenes in the propolis essential oils determined by co-injection with authentic material.

Compound	Sample A (*ee*)		Sample B (*ee*)		simple C (*ee*)	
	(+) %[a]	(-) %[a]	(+) %[a]	(-) %[a]	(+) %[a]	(-) %[a]
α-Pinene	40.3	59.7	46.9	56.1	76.2	23.8
β-Pinene	4.1	95.9	2.6	97.4	4.0	96.0
Limonene	54.0	46.0	-	-	100.0	-

[a] Percentage of enantiomeric excess of the chiral monoterpenes analyzed on a dual column system with chiral stationary phase [6-Me-2,3-Pe-β-CD and 3-Bc-2,6-pentyl-g-CD (Lipodex- E)].

According to results, the essential oils obtained from propolis of three regions of southern of Brazil have an expressive concentration of monoterpenes, with a variable enantiomeric composition. Establishing a relation between the monoterpenes and chiral distribution, it is possible to attribute that a-pinene and limonene suffered variations in enantiomeric form in excess, while in the case of β-pinene, the preference is observed by the (-) enantiomeric form in excess (Table 2).

The volatile oil of propolis (sample A) it was evaluated by the diffusion method in agar, with relationship to its properties antimicrobial. In agreement with the results, exposed in the Table 3, it can be observed that the oil possesses a moderate activity, when compared to the standards. The oil presented larger inhibition in the growth of *Staphylococcus aureus*, while the most resistant was *Pseudomonas aeruginosa*. This way, the monoterpenes identified in the oil surely contribute for antimicrobial activity of the propolis, once the properties of α-pinene, β-pinene and limonene are already described in the literature as potential inhibitors of the proliferation of pathogenic microorganisms.[27]



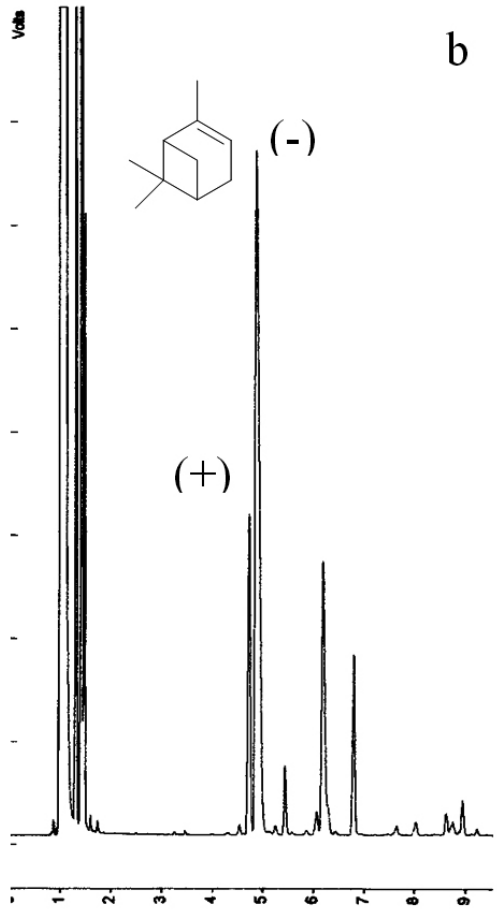


Figure 1. Enantiomer separation of (+/-)-a-pinene: a) sample A. b) sample C. On a 25 m x 0.25 mm CCSF coated with heptakis-(6-O-methyl-2,3-di-O-pentyl)-β-cyclodextrin in OV1701; carrier gas, hydrogen 7 psi.

Table 3. Antibacterial activity of the oil from propolis (Sample A).

Microorganisms	Inhibition of the growth in cm	
	Essential oil (10 µL)	Tetraciclin[a] / Cloranphenicol[b]
Staphylococcus aureus ATCC 6538	1,2	2,7
Pseudomonas aeruginosa ATCC 25619	1,0	3,3
Klebsiella pneumoniae ATCC 1003	1,1	2,6
Bacilo subtilis ATCC 6633	1,1	3,0
Esherichia coli ATCC 25792	1,1	3,0

a: for *Pseudomonas aeruginosa* ATCC 25619
b: for others bacteria

CONCLUSION

Due to its importance, the propolis has been a lot studied, representing an important source of bioactive natural compounds, since they are present in its composition, several compounds with varied biological activities. Furthermore, the propolis extract can be present different quantities of bioactive compounds in accordance with geographical and botanical origin. This way, analyses of the components of the volatile oils from propolis of producing areas can serve as parameters to monitor the origin and quality. Through analyses by enantioselective gas chromatography using dual system, it can be observed the proportion and the chiral distribution of the minority and the main monoterpenic component of the essential oils. Therefore, the qualitative and quantitative enantiomeric composition of α-pinene, β-pinene and limonene,

can be applied as a quality parameter of the oil from this origin (Rio Grande do Sul State, Brazil).

The antimicrobial activity of the oil showed that the monoterpenic composition contributes to the biological activity of crude propolis. The obtained results allow us to infer that α-pinene, β-pinene and limonene, are responsible for to exhibit biological effects of essential oils of propolis, these compounds are already known to play an important inhibitory effect on bacterial.[27-29]

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