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Original Article

Bioactive Constituents of Brazilian Red Propolis

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In a new propolis type, red Brazilian propolis, 14 compounds were identified (six of them new for propolis), among them simple phenolics, triterepenoids, isoflavonoids, prenylated benzophenones and a naphthoquinone epoxide (isolated for the first time from a natural source). Three of the major components demonstrated significant antimicrobial activity, and two (obtained as inseparable mixture) possessed radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH).

Keywords: antibacterial activity – chemical constituents – propolis – radical scavenging activity

Introduction

Propolis (bee glue) has a long history of being used as a remedy, dating back to the times of ancient Greece and Rome. Nowadays, it is still used for the treatment of various diseases, and in products like 'health foods', 'biocosmetics', etc., because of its versatile biological activities (1). Tropical propolis samples, and especially Brazilian ones, have shown significant differences in their chemical composition to propolis from temperate zone (2,3). For this reason, Brazilian bee glue has recently become a subject of increasing interest for scientists (4–7). It was found that propolis from different regions of Brazil display different chemical composition, depending on the local flora at the site of collection. Park *et al*. (8) have specified 12 types of Brazilian propolis according to its geographical origin, chemical composition and source plant. The most popular and well studied Brazilian propolis is the so-called green or Alecrim propolis, which originates from *Baccharis dracunculifolia* (Asteraceae) (9–12). Till now, no chemical data have been published on red propolis from Brazil. In Brazil, red propolis is collected in the North regions. Red colored propolis is reported to be typical for Cuba, where its plant source was identified as *Clusia nemorosa* (Clusiaceae) (13), and for Venezuela, where bees collect it from *Clusia scrobiculata* (14). In this study, we report our results on antibacterial and antioxidant activity of chemical constituents of red Brazilian propolis.

Materials and Methods

Nuclear magnetic resonance (NMR) spectra were measured on a Bruker AVANCE 250 MNR spectrometer; mass-spectra were measured on a Hewlett Packard 5972 mass spectrometer system.

Propolis. Propolis was collected near Maceio city, Alagoas State, Brazil.

Extraction of propolis. Propolis (61 g) was cut into small pieces and extracted with 70% ethanol (1 : 10, w/v) at room temperature for 24 h. The ethanol extract was concentrated *in vacuo* and extracted successively with petrol ether (40–60°C) three times. The petrol ether extract was evaporated to give 5 g dry residue after evaporation.

Isolation of compounds. The petrol ether extract was subjected to column chromatography on silica gel (300 g) with an *n*-hexane/acetone gradient (1 : 0.05/1 : 0.4) to produce 20 fractions (**I–XX**).

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Fraction **I** (300 mg) was rechromatographed on a silica gel column eluted with *n*-hexane/diethyl ether gradient (1 : 0.01/1 : 1). The first fraction of this column, **I.1** (12 mg), was subjected to gas chromatography-mass spectrometry (GC-MS) analysis with a Hewlett Packard Gas Chromatograph 5890 Series II Plus linked to 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 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, ionization voltage 70 eV. Using computer searches on a NIST98 MS data library, the following compounds were identified in the mixture: *trans*-anethol **1** (13%), methyl eugenol **2** (14%), *trans*-methyl isoeugenol **3** (18%), elemicin **4** (26%) and *trans*-isoelemicin **5** (11%).

The second fraction **I.2** (37 mg), after additional separation by preparative thin layer chromatography (TLC) (silica gel, *n*-hexane/ethyl methyl ketone 1 : 0.06) yielded 20(29)-lupen-3-one **6** (6 mg) and 2,3-epoxy-2-(3-methyl-2-butenyl)-1,4-naphthalenedione **7** (8.6 mg).

Fraction **II** (300 mg) was rechromatographed on a silica gel column eluted with *n*-hexane/diethyl ether gradient (1 : 0.01/1 : 1). After further purification by preparative TLC (silica gel, *n*-hexane/ethyl methyl ketone 1 : 0.06), a mixture of triterpenic alcohols (36 mg) (^{1}H-NMR) was obtained. This mixture was analyzed after silylation, using the above mentioned GC-MS apparatus and the same analysis conditions as with fraction **I.1.** Using computer searches on a NIST98 MS data library, α-amyrin **8**, β-amyrin **9** (identity also confirmed by comparison with an authentic sample), cycloartenol **10** and lupeol **11** were identified.

Fraction **VIII** (241 mg) was rechromatographed on a silica gel column, mobile phase *n*-hexane/acetone gradient (1 : 0.05/1 : 0.8). Additional purification by preparative TLC (silica gel, toluene/acetone 1 : 0.2) yielded isosativan **12** (40.8 mg).

Fraction **X** (454 mg) was rechromatographed on a silica gel column with mobile phase *n*-hexane/acetone gradient (1 : 0.05/1 : 1). Further purification by preparative TLC (*n*-hexane/ethyl methyl ketone 1 : 0.1) led to the isolation of 11.8 mg medicarpin **13**.

Fraction **XIII** (620 mg) was rechromatographed on a silica gel column with mobile phase *n*-hexane/acetone gradient (*n*-hexane/acetone 1 : 0.01/1 : 1) to yield 20.5 mg of an inseparable mixture of guttiferone E **14** and xanthochymol **15**.

20(29)-Lupen-3-one **6** was identified based on comparison of its EIMS, ^{1}H- and ^{13}C-NMR spectra and optical rotation with literature data (15).

2,3-Epoxy-2-(3-methyl-2-butenyl)-1,4-naphthalenedione **7**, colorless oil, $[\alpha]_D$ 0 (c 0.2, acetone). MS (EI, 70 eV), *m/z* (rel. int. %): 242, M^+. (28), 227 $(M-15)^+$ (85), 213 $(M-29)^+$(100), 196 (64), 171 (81), 105 (35%), 89 (36), 69 (30). HRMS (EI) *m/z*: 242.09553 (Calc. for $C_{15}H_{14}O_3$: 242.09430). For ^{1}H-and ^{13}C-NMR, see Table 1.

Table 1. NMR data of **7** in $CDCl_3$, δ in ppm (J in Hz)

C	δ_H^a	δ_C^b	HMBC (H→C)
1	–	192.0	
2	–	63.5	
3	3.85 s	59.0	C-2, C-4, C-4a
4	–	191.8	
4a	–	131.9	
5	8.03 m	127.4	C-4, C-4a, C-7
6	7.73–7.76 m	134.5	
7	7.73–7.76 m	134.3	
8	7.96 m	126.8	C-1, C-6, C-8a
8a	–	132.4	
1′	2.69 dd (15.4; 8) 3.05 dd (15.4; 7)	26.1	C-2, C-3, C-2′, C-3′
2′	5.09 m	115.4	C-1
3′	–	137.3	
4′	1.73 s	25.8	C-2′. C-3′, C-5′
5′	1.69 s	18.0	C-2′. C-3′, C-4′

[a] ^{1}H-NMR, 250 MHz.
[b] ^{13}C-NMR, 62.9 MHz.
HMBC, heteronuclear multiple bond correlation.

Isosativan **12**, colorless crystals. UV, EIMS, ^{1}H- and ^{13}C-NMR spectra identical with literature data (16), $[\alpha]_D$ 0 (c 0.27, chloroform).

(6aS,11aS)-Medicarpin **13**. UV, EIMS, ^{1}H- and ^{13}C-NMR spectra identical with literature data (17), $[\alpha]_D$ +184 (c 0.51, acetone).

Guttiferone E **14** and xanthochymol **15**. The components of this inseparable mixture were identified by comparison of the spectral data for the same mixture published by Gustafson *et al*. (18) ^{1}H- and ^{13}C-NMR, MS (Fig. 1).

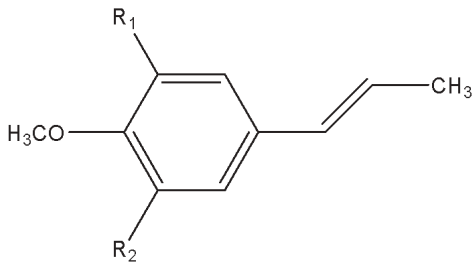
Antimicrobial tests. For the investigation of the antibacterial and antifungal activity, the agar cup method (19) was used with test strains *Staphylococcus aureus* 209 (obtained from the Bulgarian Type Culture Collection, Institute for State Control of Drugs, Sofia), *Escherichia coli* WF+ (obtained from the Collection of ZIMET, Central Institute of Microbiology and Experimental Therapy, Jena, Germany) and *Candida albicans* 562 (obtained from the Bulgarian Type Culture Collection, Institute for State Control of Drugs, Sofia). An inhibitory zone with a diameter <10 mm corresponds to lack of activity (10 mm is the diameter of the cup). The test solution (0.1 ml) containing 0.4 mg of each substance in ethanol was applied to every cup (concentration of the test solution 4 mg ml^{-1}). Control experiments with solvents showed that solvents do not have any activity.

DPPH free radical scavenging activity. DPPH free radical scavenging activity was measured according to the procedure described in the literature (20). The decrease of the absorption at 516 nm of the DPPH solution after addition of the tested solution was measured. An aliquot (2960 μl) of 0.1 mM ethanolic DPPH solution was mixed with 40 μl of a 3.6 mM solution of the tested substance. The radical scavenging activity was expressed as percentage decrease with respect to control values. Caffeic acid was used as positive control.

Results and Discussion

The petrol ether fraction of the ethanol extract of the investigated propolis sample was subjected to column chromatography on silica gel and several fractions were produced. After further purification by repeated column chromatography and preparative TLC, two complex mixtures, one inseparable mixture of two isomers and four pure compounds were obtained.

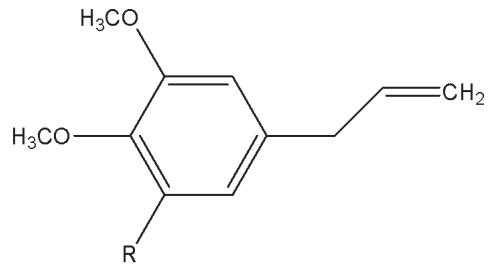
The most unpolar fraction, isolated by repeated column chromatography, was of complex composition and was analyzed by GC-MS. It turned out to be composed of following phenylpropene derivatives: *trans*-anethol **1**, methyl eugenol



1. $R_1 = R_2 = H$

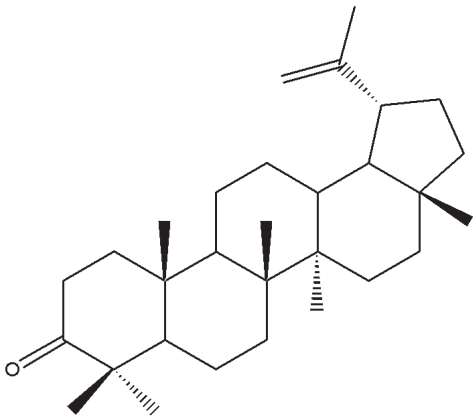
3. $R_1 = OCH_3$, $R_2 = H$

5. $R_1 = R_2 = OCH_3$



2. R = H

4. R = OCH_3



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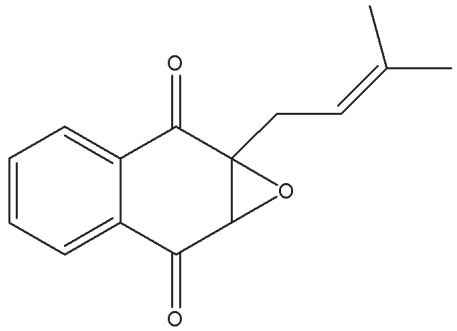

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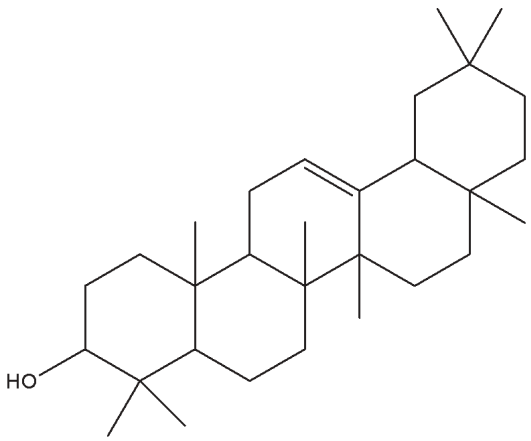

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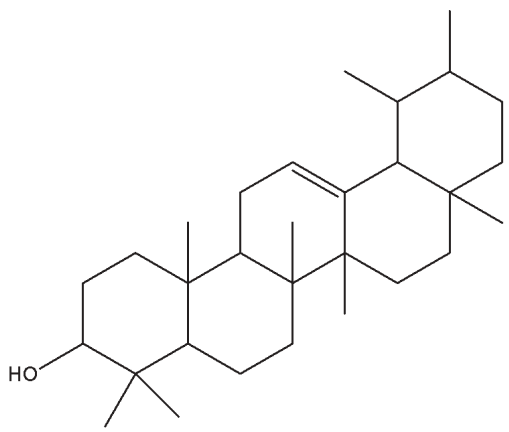

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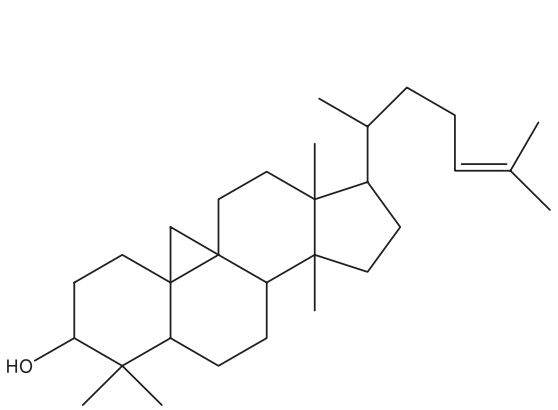
Figure 1. Compounds identified in Brazilian red propolis.



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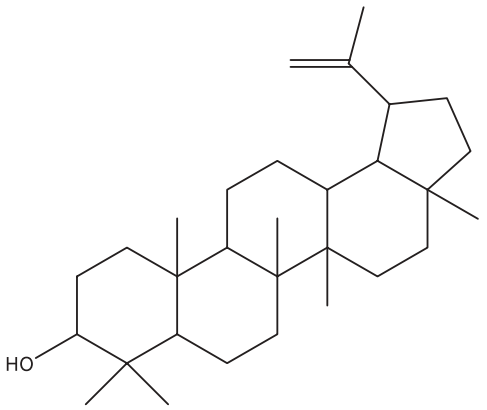

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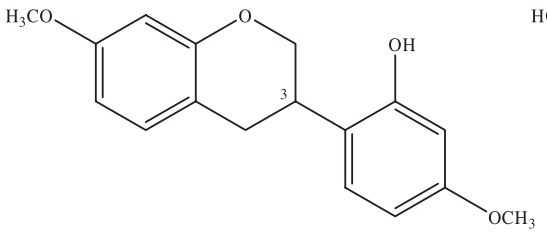

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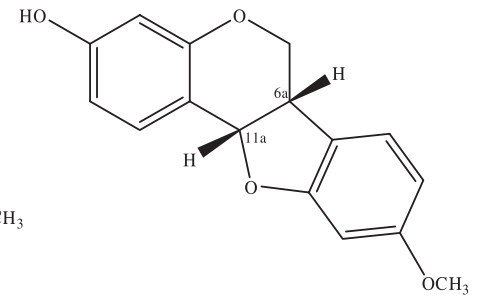

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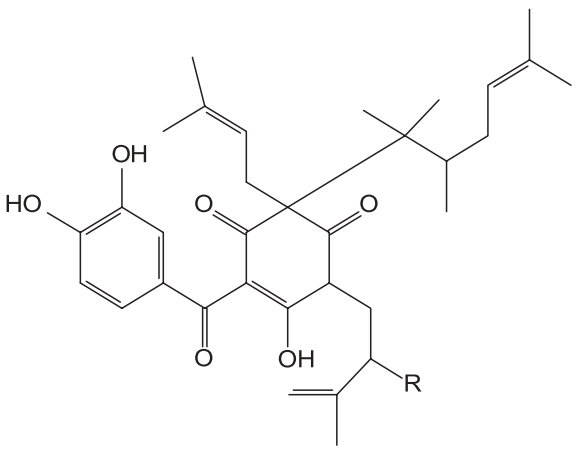

14. R = 3-methyl-2-butenyl **15.** R = 3-methyl-3-butenyl

Figure 1. Continued.

2, *trans*-methyl isoeugenol **3**, elemicin **4** and *trans*-isoelemicin **5**. Elemicin was the most abundant. Of these compounds, methyl eugenol, methyl isoeugenol, elemicin and isoelemicin were found for the first time in propolis. The composition of this fraction also explains the very unusual anis-like odor of this Brazilian red propolis sample.

The second complex mixture (see Materials and Methods) was comprised of triterpenic alcohols, which were identified by means of GC-MS as α-amyrin **8**, β-amyrin **9**, cycloartenol **10** and lupeol **11**. The most abundant among them was β-amyrin. Triterpenic alcohols are typical for Brazilian propolis (2).

Table 2. Antimicrobial and antiradical activity of isolated compounds

Sample	Antimicrobial activity inhibitory zone ± SD, mm[a]			DPPH radical scavenging activity
	S. aureus	*E. coli*	*C. albicans*	% inhibition
12	14 ± 0	0	15 ± 1	4.5
13	23 ± 1	14 ± 0	26 ± 0	0.7
14/15	19 ± 1	12 ± 0	0	49
Caffeic acid	–	–	–	85.6

[a]Mean of three measurements.

One of the pure compounds isolated was also of triterpenic nature: the ketone 20(29)-lupen-3-one **6** [identified by comparison of spectral information with literature data (13)], found for the first time in propolis. This compound has recently been found to possess antibiotic activity against bacteria and fungi, and antioxidant activity similar to that of tocopherol (15).

Compound **7** deserves special attention. Its structure was determined as 2,3-epoxy-2-(3-methyl-2-butenyl)-1,4-naphthalenedione on the basis of its MS, infrared, ^{1}H- and ^{13}C-NMR spectra. This is the first isolation of **7** from a natural source. Till now, it was known only as a synthetic product (21,22). The mass- and ^{1}H-NMR spectra of our compound were identical with the literature data (no ^{13}C-NMR data have been published). Compound **7**, obtained synthetically from a natural product, demonstrated antibacterial, antifungal and cytotoxic properties (22).

Two isoflavonoids were isolated and identified: the isoflavan isosativan **12** and the pterocarpan medicarpin **13**, based on comparison of their spectral properties with literature data (including absolute stereochemistry of **13**, confirmed by optical rotation measurements; **12** was racemic). This is the first report of isoflavonoids in propolis other than Cuban. Compounds **12** and **13** were till now found only in Cuban propolis (16,17). This fact suggests that Cuban red propolis and Brazilian red propolis might have a common plant source, but a plant that produces isoflavonoids as components of its exudates is not yet known. The presence of isoflavonoids suggests some plant of the Leguminosae family but further studies are needed for confirmation. Especially **13** is of particular interest: it is an important plant phytoalexin well known for its antimicrobial and especially antifungal activity (23).

Compounds **14** and **15** are double bond isomers which occur as an inseparable mixture, but the structures were deduced by comparison of the spectral data of the mixture with the values for **14** and **15** from the literature (22–25). ^{1}H- and ^{13}C-NMR spectra of the mixture were virtually identical to those of **14** and **15** and all HMBC correlations were fully consistent with these structures. Moreover, Gustafson *et al*. (18) reported the isolation of the same inseparable mixture from *Clusia rosea* leaves as the active anti-HIV principle. These compounds have been detected as traces in red Cuban propolis (13) originating from *C. rosea* floral resins. In Cuban propolis nemorosone, another polyisoprenylated benzophenone, is the most important constituent (13). In our case, however, the mixture of **14** and **15** is among the major components of the extract. So the main plant source is most probably some other *Clusia* species. Moreover, the presence of isoflavonoids in our sample is an indication that another plant source could be involved, as isoflavonoids have never been found in the resins of Clusiaceae plants.

Three of the isolated compounds were tested for their antibacterial and radical scavenging activity against DPPH radicals. The results are represented in Table 2. The results indicated that the isoflavonoids **12** and **13** are important antimicrobial components of red propolis, especially concerning the activity against *C. albicans.* This is not surprising, taking into consideration that pterocarpans are known for their antifungal activity and play a defensive role in many plants due to this activity (26). The mixture of prenylated benzophenones **14/15** demonstrated good activity against *S. aureus*. The mixture showed also significant radical scavenging activity against DPPH, obviously it is one of the most important antioxidant components of the extract.

The identification of new propolis constituents in red Brazilian propolis, most of them having antibacterial, antimycotic and antiradical activities, is a further confirmation of the fact that propolis, independently of its plant source and chemical composition, always possesses antimicrobial and antioxidant activity. This is due to the role that propolis plays in the hive: it is the 'chemical weapon' of bees against pathogen microorganisms and the elements of weather. However, in different propolis types, different chemical constituents are responsible for the valuable activities (27). The results obtained demonstrate once again that propolis remains a fascinating subject for further studies and application to CAM.

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