Research Article

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Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities

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Abstract

BACKGROUND: Chloroform, ethyl acetate and methanol extracts of a sample of red propolis from the state of Alagoas (northeast Brazil) were analyzed by gas chromatography–mass spectrometry and high-performance liquid chromatography–diode array detection–electrospray ionization-mass spectrometry. Antimicrobial and antioxidant activities were also obtained.

RESULTS: The propolis sample contained low content of narigenin-8-*C*-hexoside, this being the first report of a *C*-glycoside in propolis. The main constituent found was characterized as 3,4,2′,3′-tetrahydroxychalcone. Other important constituents were the chalcone isoliquiritigenin, the isoflavans (3*S*)-vestitol, (3*S*)-7-*O*-methylvestitol, the pterocarpan medicarpin, the phenylpropenes *trans*-anethol, methyl eugenol, elimicin, methoxyeugenol and *cis*-asarone, and the triterpenic alcohols lupeol and α- and β- amyrins. The methanol extract exhibited high antioxidant activities by 2,2-diphenyl-1-picrylhydrazyl and β-carotene/linoleic acid assay methods, and antimicrobial activity toward Gram-positive and Gram-negative bacteria.

CONCLUSION: Structures are suggested for new substances never before seen in any kind of propolis. This is the first report of 3,4,2′,3′-tetrahydroxychalcone and a flavone *C*-glycoside in a propolis sample.

Keywords: red propolis; isoflavonoids; phenylpropene derivatives; chalcones; Leguminosae

INTRODUCTION

Propolis is a resinous hive product produced by honeybees from various plant sources. It is used in the hive to seal holes, smooth out the internal walls, and protect the entrance against intruders.[1] The use of propolis as alternative food and medicine in many countries has increased in the last decades. Many biological activities of propolis have been experimentally observed, such as antioxidant, anti-inflammatory, improvement of heart and circulatory function, antilipidemic, antihyperglycemic, cytotoxic and anticancer.[2]

In general, propolis contains beeswax, resin (often containing volatile and phenolic compounds) and other minor constituents such as sugars, amino acids and pollen. It is worth remembering that the resin is derived from plant material that bees collect from plants and confers the biological activities.[3] Propolis chemical composition is thus dependent on the vegetation around the beehives. In Europe and other temperate zones, the main constituents of propolis are flavonoids and phenolic acid esters (e.g. caffeic acid phenethyl ester (CAPE)), derived from apices of poplar plants (*Populus nigra*).[2] In red propolis from Cuba and Venezuela polyprenylated benzophenones predominate.[4] On the other hand, the red Mexican propolis contains mainly flavanones, isoflavans, and pterocarpans.[5]

In Brazil, the propolis type most commercialized is known as 'green propolis', derived from apices of *Baccharis dracunculifolia* (Asteraceae, alecrim plant), containing predominantly prenylated phenylpropanoids (e.g. artepillin C), chlorogenic and benzoic acids and triterpenoids,[3] obtained in the south and southeast regions. A Brazilian red propolis is obtained in the northeast region (Alagoas state). It has been chemically characterized as containing pterocarpans, isoflavonoids, chalcones, prenylated benzophenones and phenylpropanoids.[6] The ethanol extract of a sample of Brazilian red propolis exhibited cytotoxic activity toward HeLa tumor cells[7] and bactericide activity toward *Staphylococcus aureus*.[8]

Some chemical parameters, such as total phenolic compounds and flavonoids, are valuable data for general characterization of propolis samples.[2] In addition, analysis by high-performance liquid chromatography (HPLC) and gas chromatography (GC), coupled with mass spectrometry (MS), are powerful techniques for identification of constituents of complex mixtures, such as propolis. Electrospray ionization/mass spectrometry (ESI/MS) is a soft ionization technique that allows the generation of ions even from thermally labile non-volatile polar compounds.[9]

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The present work aims to determine total phenolic compounds, total flavonoids and main constituents of chloroform, ethyl acetate and methanol extracts of a sample of Brazilian red propolis. The constituents were characterized through analyses carried out by GC/electron impact mass spectrometry (EIMS), HPLC/ESI/MS and HPLC/ESI/MS/MS and based on comparison with data published in the literature. A further aim was to determine the antioxidant and antimicrobial activities of the methanol extract.

EXPERIMENTAL

Propolis sampling and processing

Propolis produced by *Apis mellifera* was collected in experimental apiaries located in Maceió, state of Alagoas, in northeastern Brazil. For chromatographic analyses, 5.0 g of the powdered sample was extracted in Soxhlet successively with hexane, chloroform, ethyl acetate and methanol, for 3 h. Each extract was concentrated under reduced pressure and the residue dried to constant weight.

For analyses of total polyphenols and flavonoids and determination of antimicrobial and antioxidant activities, 2.5 g of the sample was extracted with methanol in Soxhlet until negative reaction to 5% $FeCl_3$. The methanol extract was maintained in a freezer for 2 h for wax elimination. The cold extract was filtered and diluted to 250 mL to constitute the methanol extract (ME, 10 mg mL^{-1}).

Total polyphenol content

Total polyphenols were determined according to the Folin–Ciocalteu method,[10] using *p*-coumaric acid as reference.

Total flavonoid content

Total flavonoids were determined by the aluminium chloride[10] and dinitrophenylhydrazin[11] methods. Calibration curves were made using quercetin (aluminium chloride method) and pinocembrin (dinitrophenylhydrazin method). Total flavonoids were assumed to be the sum of the values obtained by each method.

GC/EIMS

The hexane, chloroform and ethyl acetate extracts were dissolved in ethyl ether at a concentration of 1000 ppm. Ether solutions (1 µL) were injected into a gas chromatograph (5890 series II plus, Hewlett-Packard, Palo Alto, CA, USA) coupled with a 5989B HP mass spectrometer operating in EI mode at 70 eV. A DB-5HT capillary column (30 m × 0.32 mm internal diameter, 0.25 µm film thickness) was held at 100 °C for 1 min and then heated to 300 °C at 6 °C min^{-1}; final temperature was maintained constant for 2 min. Helium was used as carrier gas in the split mode, flux 1.5 mL min^{-1}, linear velocity 63 cm s^{-1}, total flow 77.3 mL min^{-1}. Injector and detector temperature was 300 °C.

Identification of the constituents was based on comparison of the corresponding mass spectra with data from the libraries Wiley-275 (Hewlett-Packard), Wiley/NBS[12] and standards. Relative amounts (estimative, not quantitative) of constituents were evaluated by the areas under the corresponding peaks.

HPLC/DAD/ESI/MS/MS

Chloroform and methanol extracts were analyzed by HPLC/DAD/ESI/MS and HPLC/DAD/ESI/MS/MS, using a DAD SPD-M10Avp chromatograph (Shimadzu, Kyoto, Japan), equipped with photodiode array detector and coupled to an Esquire 3000 plus mass spectrometer (Bruker Daltonics, Coventry, UK) working with an ESI source, the system controlled by Esquire NT software from Bruker Daltonics. Compounds were detected at 270 nm and the UV spectra were recorded in the range of 250–400 nm. A reverse-phase C18 Zorbax 5B RP-18 (Hewlett-Packard) column (4.6 × 250 mm, 5 µm) was used. The mobile phases comprised eluents A (0.1% aq. HOAc) and B (methanol). A linear gradient 20–90% B (v/v) over 50 min was used. The flow rate was 0.5 mL min^{-1} and the temperature of the column 28 °C. Negative-ion ESI was performed using an ion source voltage of −40 V and a capillary offset voltage of 4500 V. Nebulization was aided by a coaxial nitrogen sheath gas provided at a pressure of 27 psi. The dry gas temperature was set to 130 °C and a dry gas flow of 4 L min^{-1} was used. Desolvation was assisted using a counter-current nitrogen flow set at a flux of 7.0 L min^{-1} and capillary temperature 320 °C. Mass spectra were recorded over the range 50–700 *m/z*. MS/MS data were acquired in the negative ionization mode. Owing to the unavailability of some commercial standards, phenolic compounds were identified by interpretation of their UV absorbance band, and the mass spectra data through cross-comparison of compounds in samples, standards (if possible) and similar compounds previously identified in the published literature. Relative amounts (estimative, not quantitative) of constituents were evaluated by the areas under the corresponding peaks.

Antimicrobial activity

Standard strains of *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* were obtained from the American Type Culture Collection by the Institute of Biomedical Sciences of the University of São Paulo. A strain of *Salmonella typhimurium* was obtained from Institut Pasteur Collection. The microorganisms were maintained at −20 °C in culture with glycerol. Prior to the assays, cultures were activated in trypticase soya broth (TSB) for 24 h at 37 °C. Antimicrobial activity was determined using a macrodilution method,[13] with modifications. Aliquots of ME were transferred to TSB to obtain concentrations ranging from 8 to 2048 µg mL^{-1}. Volumes of 5.0 mL TSB and 100 µL inocula were transferred to eight sterilized tubes. The inocula were standardized at 1.5 10^{-8} cfu mL^{-1} and quantified according to McFarland turbidity standard no. 0.5. Four tubes were used as controls: (i) negative – broth; (ii) negative with ME – broth plus 100 µL ME solution; (iii) positive – broth plus 100 µL inoculum; (iv) positive with methanol – broth plus 100 µL inoculum plus 50 µL methanol. The assays were carried out in duplicate and the tubes were maintained in a stove for 24 h at 37 °C. The minimum inhibitory concentration (MIC) was assumed as the lowest concentration of ME that inhibited growth. The material in tubes corresponding to MIC detection was shown in agar Mueller–Hinton and incubated in the stove for 24 h at 37 °C. The minimum microbicidal concentration (MMC) was assumed as the lowest ME concentration in plaques showing no bacterial growth.

Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method[14] was used with modifications. Solutions used in the test were: (i) methanol solution of DPPH with absorbance 0.7–0.8 at 517 nm (approximately 20 mg mL^{-1}); (ii) 1 mg mL^{-1} ME; (iii) 1 mg mL^{-1} methanol solution of rutin as positive control. The two latter solutions were used as stock for preparation of the following dilutions: 25, 12 and 8 µg mL^{-1}. To 400 µL of the dilutions of ME, 800 µL methanol solution of DPPH was added. A control solution was prepared

with 400 µL methanol instead of ME. After mixing thoroughly, the mixture was allowed to stand in the dark for 20 min and the absorbance measured at 517 nm, using methanol as blank. Radical scavenging activity was expressed as percentage of reduction of DPPH and calculated using the following formula:

$$\% \text{ scavenging} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

The method of linoleic acid oxidation[14] was also carried out with modifications. β-Carotene (1.0 mg) was dissolved in 5.0 mL chloroform. An aliquot of 1.0 mL of this solution was added to 12.5 µL linoleic acid and 100 µL Tween 40. The chloroform was evaporated under nitrogen flow. Distilled water (12.5 mL), previously saturated with oxygen for 30 min, was added and the solution mixed thoroughly. The clear mixture obtained was adjusted to absorb in the range 0.8–0.9 at 470 nm. The assay was carried out using three different concentrations of ME: 1.0, 1.5 and 2.0 mg mL^{-1}. A control was prepared with 120 µL of solvent instead of ME solution, and rutin solutions were used as positive control. Aliquots (1000 µL) of the β-carotene/linoleic acid mixture were mixed with 120 µL ME solution (or rutin solution) and maintained in a water bath at 50 °C. Oxidation was monitored by determination of absorbance at 470 nm over a 30 min period. The antioxidant activity was expressed as percent inhibition (% I) comparing the reduction of sample absorbance ($Abs_s = Abs_{initial} - Abs_{final}$) with the control absorbance ($Abs_c = Abs_{initial} - Abs_{final}$), according to the formula

$$\% I = (Abs_s - Abs_c) \times 100 \times Abs_c^{-1}$$

RESULTS AND DISCUSSION

Total polyphenol and flavonoid contents

The sample analyzed contained 416.31 mg g^{-1} of phenolic compounds. Values reported for red propolis from the same region have been 232 mg g^{-1} [7] and 2.6 mg g^{-1}.[8] Total phenolic substances vary widely among propolis types and even among samples of a similar type. Brazilian propolis samples have been reported to contain total phenolics in the range 88–137 mg g^{-1} [10] and 5–37 mg g^{-1}.[15] The sample analyzed in the present work seems to be exceptionally rich in phenolic compounds. Propolis samples from South American countries (Argentina, Chile, Paraguay, Peru and Uruguay) were reported to contain from 3 to 55 mg g^{-1}.[16] Contents of phenolic compounds reported for European propolis vary from 2 to 111 mg g^{-1}.[17]

The content of total flavonoids determined in the sample of Brazilian red propolis was 32.91 mg g^{-1}. Other types of Brazilian propolis have been reported to contain similar contents, for example 43 mg g^{-1} [7] or contents up to 27 mg g^{-1}.[10,15,16] On the other hand, total flavonoid content of European propolis may reach 223 mg g^{-1}.[17] The difference between chemical parameters of Brazilian and European propolis is accounted for by the corresponding plant sources of resins.[18]

Characterization of constituents found by GC/EIMS

The sample from the state of Alagoas displayed a 'ruby' red color, an uncommon feature for propolis. The constituents found in apolar extracts were initially characterized by GC/EIMS analyses. Alkanes such as *n*-tricosane (*m/z* 324, $C_{23}H_{48}$), *n*-pentacosane (*m/z* 352, $C_{25}H_{52}$), *n*-heptacosane (*m/z* 380, $C_{27}H_{56}$), *n*-nonacosane (*m/z* 408, $C_{29}H_{60}$), *n*-hentriacontane (*m/z* 436, $C_{31}H_{64}$) and *n*-tritiacontane (*m/z* 464, $C_{33}H_{68}$) were found in hexane extract.

In chloroform extract analyzed by GC-EIMS phenylpropene derivatives, triterpene alcohols, isoflavonoids and chalcones were detected (Table 1). The following phenylpropene derivatives were identified: *trans*-anethole (*m/z* 148), methyleugenol, (*m/z* 178), elimicin (*m/z* 208) and methoxyeugenol (*m/z* 194). The EI mass spectral data are also shown in Table 1 and for these phenylpropenes the molecular ion and base peak were coincident. Phenylpropene derivatives containing methoxyl groups showed fragments obtained by the loss of a methyl group (15 Da), which is consistent with literature data.[19] Resorcinol (*m/z* 110), *m*-guaiacol (*m/z* 124), anisylacetone (*m/z* 178), *cis*-asarone (*m/z* 208), 5,7,3′,4′-tetramethoxyflavone (*m/z* 342) and farnesol (*m/z* 222) were also found. These compounds were unambiguously identified by comparing their retention times and mass spectra with those of published literature and data from the libraries Wiley-275 (Hewlett-Packard) and Wiley/NBS.

The other group was comprised of triterpene alcohols, which were identified as α- and β-amyrin and lupeol. The most abundant among them was β-amyrin. The main constituents found were isoflavonoids, the pterocarpan medicarpin, and chalcones such as 2′-hydroxy-4′-methoxychalcone (*m/z* 254). Isoflavonoids, chalcones and pterocarpans have already been isolated from plants of the family Leguminosae.[20] The isoflavans, such as vestitol and methylvestitol, are a subclass of the isoflavonoids that possess a chiral center at C3 of the pyran ring, which is created stereoselectively by a specific enzyme; (*S*)-isomers are found in certain woody Leguminosae, such as *Dalbergia* species.[21] (3*S*)-vestitol, (3*S*)-vestitone, (3*S*)-7-*O*-methylvestitol, butein and medicarpin have been isolated from *Dalbergia odorifera*.[20]

The retro-Diels–Alder rearrangement (RDA) have been widely used for structural investigation of flavonoids, isoflavonoids and chalcones under electron impact (EI) and ESI conditions.[20,22] The substance corresponding to the peak with retention time (RT) 24.9 yielded molecular ion *m/z* 272 ($C_{16}H_{16}O_4$) and was characterized as (3*S*)-vestitol (7,2′-dihydroxy-4′-methoxyisoflavan). A base peak at *m/z* 150 and another peak at *m/z* 137 (28%) in the mass spectrum corresponded to the basic fragment ions of the isoflavan ring system and indicated a type of substitution pattern with a methoxyl group at the B ring. The mass fragmentation pattern of the peak at RT 24.1 min, with molecular ion at *m/z* 286 ($C_{17}H_{18}O_4$), was structurally similar to vestitol, exhibiting also a base peak at *m/z* 150 and a fragment at *m/z* 137 (80%). The compound was characterized as (3*S*)-7-*O*-methylvestitol.

The main constituent found in the extract (25.6%) corresponded to the peak at RT 25.1 min; the mass spectrum had a molecular ion at *m/z* 272 (M^+, 60), like vestitol, and a base peak, probably obtained by RDA fragmentation reaction, at *m/z* 137 ($C_7O_3H_5^+$), suggesting that it corresponded to a chalcone. The spectral data allowed no identification of the substance. The pterocarpan medicarpin (peak at RT 22.3 min) showed a molecular ion at *m/z* 270 ($C_{16}O_4H_{14}$) (100%) and fragments at *m/z* 255 (37%) and *m/z* 155 (33%). The isoflavone calycosin (*m/z* 284) and the benzofurans 2-(2′-hydroxy-4′-methoxyphenyl)-3-methyl-6-methoxybenzofuran (*m/z* 284) and 2-(2′,4′-dihydroxyphenyl)-3-methyl-6-methoxybenzofuran (*m/z* 270) were also identified by GC/EIMS. Mass spectral data for these compounds are given in Table 1.

The main constituents found in ethyl acetate extract were a chalcone with a molecular ion at *m/z* 272, the isoflavans (3*S*)-7-*O*-methylvestitol and (3*S*)-vestitol and the pterocarpan medicarpin. These compounds were also reported for Cuban propolis[23] and Brazilian red propolis.[6,7,19] The presence of phenylpropene

Table 1. Identified constituents by GC/EIMS from the chloroform extract of red propolis from Maceió (northeast Brazil)

Peak	Retention time (min)	Relative amounts	*m/z* (intensity)	Proposed structure
1	3.1	0.5	124 (100), 95 (60), 94 (80), 81 (70)	Methylguaiacol
2	3.7	1.2	148 (100), 147 (50), 133 (30), 117 (40), 105 (40), 91 (30), 77 (40)	trans-anethole
3	3.8	0.5	110 (100), 82	Resorcinol
4	5.2	1.0	178 (100), 163 (40), 147 (50), 135 (30), 107 (55), 103 (53), 91 (55)	Methyleugenol
5	6.6	1.0	178 (100), 163 (55), 147 (20), 107 (70), 103 (40), 91(50), 77 (30)	Anisylacetone
6	7.7	1.0	208 (100), 193 (80), 177 (25), 165 (25), 150 (25), 133 (40), 105 (30), 91 (40)	Elimicin
7	8.4	1.1	194 (100), 179 (25), 163 (20), 151 (25), 131 (35), 119 (50), 91 (70), 77 (50)	Methoxyeugenol
8	9.3	1.2	208 (100), 193 (100), 165 (20), 133 (20)	*cis*-Asarone
9	11.8	0.5	222 (2)	Farnesol
10	18.9	1.6	254 (100), 177 (80), 150 (90)	2′-Hydroxy-4′-methoxychalcone
11	19.3	1.0	284 (100), 269 (90)	Calycosin
12	20.3	1.9	342 (100), 327 (80)	5,7,3′,4′-Tetramethoxyflavone
13	21.6	2.3	284 (100), 269 (50), 148 (40)	2-(2′-Hydroxy-4′-methoxyphenyl)-3-methyl-6-methoxybenzofuran
14	22.3	10.0	270 (100), 255 (37), 155 (33)	Medicarpin
15	22.7	6.9	270 (100), 255 (90)	2-(2′,4′-Dihydroxyphenyl)-3-methyl-6-methoxybenzofuran
16	24.1	11.0	286 (60), 150 (100), 137 (80)	(3*S*) -7-*O*-Methylvestitol
17	24.9	8.0	272 (24), 150 (100), 137 (28), 135 (10), 124 (9)	(3*S*) -Vestitol
18	25.1	25.6	272 (60), 137 (100)	3,4,2′,3′-Tetrahydroxychalcone
19	30.4	5.6	426 (10), 411 (7), 218 (100), 203 (50), 189 (30)	β-Amirin
20	30.7	5.0	426 (4), 411 (7), 218 (100), 203 (10), 189 (17)	α-Amirin
21	30.9	4.6	426 (24), 411 (12), 393 (10), 315 (14), 218 (60), 207 (90), 189 (95), 107 (68)	Lupeol

derivatives and triterpenoids was verified only using CG/MS, while isoflavonoids and chalcones were detected using both CG/MS and HPLC/MS methodologies. Similar to the Brazilian red propolis analyzed in the present work, in Cuban red propolis the main constituents are isoflavonoids, such as (3*S*)-vestitol and (6a*S*,11a*S*)-medicarpin, in addition to the chalcone isoliquiritigenin.[24]

Characterization of constituents found by HPLC/DAD/ESI/MS/MS

Chloroform and methanol extracts were also analyzed by HPLC/ESI/MS/MS. The LC technique was also able to separate each single component in complex mixtures. For MS analysis, the negative ion mode of ESI was selected for its better sensitivity and separation than the positive ion mode. In negative ion mode ESI-MS of the chloroform and methanol extracts of Brazilian red propolis, all the flavonoids yielded prominent $[M - H](^-)$ ions in the first-order mass spectra. The RT, wavelength of maximum absorbance (λ max.), deprotonated molecules and the proposed structure are listed in Tables 2 and 3. The structure of each constituent of this propolis was proposed on the basis of the UV and ESI/MS/MS data. This information was achieved by direct comparison with standards and/or data concerning compounds reported for samples of red propolis.[5–7,19,23]

In the analysis of chloroform extract (Table 2), the peak at RT 37.6 min (30.1%) and at RT 38.7 (10.4%) showed similar deprotonated ions at *m/z* 271.1, a precursor to the ion at *m/z* 272.0, suggesting a chalcone and the isoflavan (3*S*)-vestitol, respectively. The chalcone has a catechol subunit at ring B (positions 2 and 4) and a resorcinol type ring A (hydroxyls at positions 3′ and 4′),with UV bands at 240 nm (band II, ring A, resorcinol absorption) and 370 nm (band I, ring B, catechol absorption). The UV maximum absorption of (3*S*)-vestitol and (3*S*)-7-*O*-methylvestitol was 234 and 280 nm, respectively, which are characteristic of isoflavans.

Isoflavanones, flavanones and chalcones are known to undergo RDA fragmentations, under ESI conditions.[9,22] The substance of the peak at RT 32.1 min was characterized as liquiritigenin, an isoflavanone with UV maximum absorption at 275–310 nm and deprotonated molecular ion at *m/z* 255.0. In the MS/MS analysis the deprotonated ion underwent RDA to yield a product ion at *m/z* 153.0 and a base peak at *m/z* 135.0 $(C_7O_3H_3)^-$, corresponding to the A-ring RDA fragment. The peak at RT 37.1 min was characterized as the chalcone isoliquiritigenin (4,2′,4′-trihydroxychalcone), with UV maximum absorption at 240 nm and 370 nm, which was assigned to the presence of a hydroxyl group at position 4 of ring B.[25] Isoliquiritigenin also exhibited a deprotonated ion at *m/z* 255.0; its MS/MS spectrum, similar to liquiritigenin, produced an ion at *m/z* 153.0 and a base peak at *m/z* 135.0. Although possessing similar fragmentation patterns, the two compounds may be distinguished by their distinct UV spectra.[25]

The main compound found in both extracts, a chalcone at RT 37.6 min, showed a UV band at 370 nm (band I, ring B, catechol absorption), again attributed to the presence of OH in position 4 of ring B. In the MS/MS spectrum in negative ion mode, the A-ring RDA fragment was observed at *m/z* 135.0 (70%) $(C_7O_3H_3)^-$, as occurred with isoliquiritigenin. In addition, other fragments were observed: at *m/z* 253.0 (10%) $[M - H - H_2O]^-$, corresponding to the loss of 18 Da, since loss of water by *ortho*-positioned hydroxyl groups is facilitated;[17] at *m/z* 146.9 (40%) $(C_8O_3H_3)^-$ (A ring fragment); and base peak at *m/z* 109.0 $(C_6O_2H_5)^-$ (Fig. 1). The MS/MS of butein

Table 2. Identified constituents by HPLC/DAD//ESI/MS from the chloroform extract of red propolis from Maceió (northeast Brazil)

Peak	Retention time (min)	λ max. (nm)	$[M-H]^-$	Proposed structure
1	5.2	ND	181.0	homovanillic acid
2	31.7	ND	315.3	(3*S*)-Violanone
3	32.1	280, 310	255.1	Liquiritigenin
4	34	280	301.2	Alnustinol
5	34.2	280, 340	285.1	(6a*R*,11a*R*)-3,4-Dihydroxy-9-methoxypterocarpan
6	34.9	280, 340	301.1	(3*S*)-Mucronulatol
7	35.6	290	285.3	(3*S*)-Vestitone
8	35.8	290	283.0	Calycosin
9	36.1	280	283.0	Biochanin A
10	36.3	ND	301.0	(3*S*)-Ferreirin
11	37.1	240, 370	255.0	Isoliquiritigenin
12	37.6	240, 370	271.0	3,4,2′,3′-Tetrahydroxychalcone
13	38.1	285, 360	269.1	(2*S*)-7-Hydroxy-6-methoxyflavanone
14	38.7	280	271.0	(3*S*)-Vestitol
15	39	280, 320	267.2	Formononetin
16	39.2	280	268.9	Medicarpin
17	41.5	ND	283.1	2-(2′-hydroxy-4′-methoxyphenyl)-3-methyl-6-methoxybenzofuran
18	42.2	ND	539.0	Volkensiflavone
19	43.7	280	285.0	(3*S*)-7-*O*-Methylvestitol
20	45.9	285	553.0	Gliricidin

ND, not determined.

Table 3. Constituents identified by HPLC/DAD//ESI/MS in the methanolic extract of red propolis from Maceió (northeast Brazil)

Peak	Retention time (min)	λ max. (nm)	$[M-H]^-$	Proposed structure
1	5	328, 330	341	Caffeic acid-4-O-hexoside
2	17.2	280	433	Naringenin-*C*-hexoside
3	32.1	280, 320	255	Liquiritigenin
4	37.1	240, 372	255	Isoliquiritigenin
5	37.6	250, 370	271	3,4,2′,3′-Tetrahydroxychalcone
6	38.7	280	271	(3*S*)-Vestitol
7	42.2	ND	539	Volkensiflavone
8	43.7	280	285	(3*S*)-7-*O*-Methylvestitol
9	45.9	285	553	Gliricidin

ND, not determined.

(3,4,2′,4′-tetrahydroxychalcone) shows fragments at *m/z* 253.0 $[M-H-H_2O]^-$, *m/z* 153.0 and a base peak at *m/z* 135.0, the latter two assigned to the fragmentation of the A-ring.[17] Our MS/MS data regarding the major chalcone from both extracts suggest that it possesses two dihydroxyl groups in *ortho* positions on both rings A and B. Thus the peak at RT 37.6 min was tentatively identified as 3,4,2′,3′-tetrahydroxychalcone.

The peak at RT 39.0 min exhibited a deprotonated ion at *m/z* 267.0 and was characterized as the isoflavone formononetin. The peak at RT 39.2 min (medicarpin) showed UV maximum absorption at 280 nm and deprotonated molecular ion at *m/z* 269.0. Compounds with higher polarity emerge first from the C18 reversed-phase column used in our analyses, whereby the chalcone eluted first, then (3*S*)-vestitol, medicarpin and 3(*S*)-7-*O*-methylvestitol.

Other constituents detected by HPLC/ESI/MS were homovanillic acid (*m/z* 181.0), (3*S*)-violanone (*m/z* 315.3), alnustinol (*m/z* 301.2), (6a*R*,11a*R*)-3,4-dihydroxy-9-methoxypterocarpan (*m/z* 285.1),

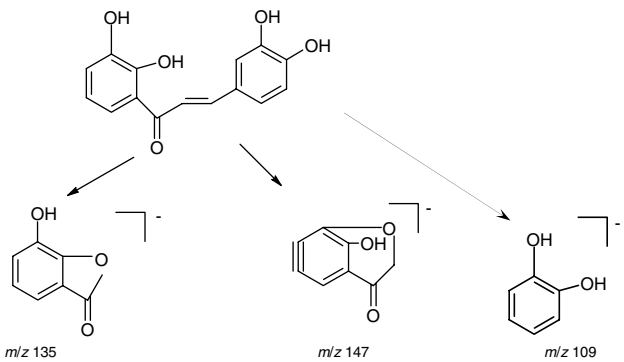

Figure 1. Fragmentation of 3,4,2′,3′-tetrahydroxychalcone in negative ion mode.

(3*S*)-vestitone (*m/z* 285.3), (2*S*)-7-hydroxy-6-methoxyflavanone (*m/z* 269.1), (3*S*)-mucronulatol (*m/z* 301.1),(3*S*)-ferreirin (*m/z* 301.0), biochanin A (*m/z* 283.0), calycosin (*m/z* 283.0) and 2-(2′-hydroxy-4′-methoxyphenyl)-3-methyl-6-methoxybenzofuran (*m/z* 283.1).

Among constituents obtained in low content, one with a peak at 45.9 min showed deprotonated ion at *m/z* 553.0 and is suggested to be the isoflavonoid dimer gliciridin; another with a peak at 42.2 min and deprotonated ion at *m/z* 539.0 is suggested to be volkensiflavone, formed by moieties of naringenin and apigenin (271 + 269 = 540).

From the methanol extract (Table 3), a peak at RT 17.2 min showed the deprotonated ion $[M - H]^-$ at *m/z* 433, which was suggested as the product ion mass spectrum of deprotonated naringenin-8-*C*-hexoside. The tandem mass spectrum produced ions of $[M - H - 90]^-$ at *m/z* 342.9 (40%) and $[M - H - 120]^-$ at *m/z* 313.1 (100%), which are consistent with the characteristic ions of a *C*-glycosylflavanone. In fact, it was observed that fragmentation related to a sugar moiety rather than the loss of a whole sugar unit as generally occurs with *O*-glycosides. The absence of the $[(M - H) - 60]^-$ fragment, which is usually generated by the fragmentation of pentose glycosides, in addition to the presence of the ion $[(M - H) - 120]^-$ (base peak) suggests that the sugar substituent is a hexose. Practically only glucose is known as a *C*-glycoside sugar moiety.

The peak at RT 5.0 min showed UV bands at 328.0–330.0 nm and a sharp diagnostic shoulder at 290–300 nm, typical of compounds containing a caffeoyl moiety and deprotonated molecular ion at *m/z* 341.0 $(C_{15}O_9H_{17})^-$. The substance was tentatively characterized as caffeic acid-4-*O*-hexoside, since the tandem mass spectrum exhibited a fragment at *m/z* 179.0 $(C_9O_4H_7)^-$ (deprotonated caffeic acid), corresponding to the loss of a hexose moiety (162 amu), and a fragment at *m/z* 161.0, caused by a subsequent elimination of water from ion *m/z* 179.0.

Antimicrobial activity

The method utilized in this work was the macrodilution in broth, followed by microbial growth in plaque, that allowed evaluation of the antimicrobial activity against both Gram-positive (*Bacillus subtilis, Enterococcus faecalis* and *Streptococcus pyogenes*) and Gram-negative (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhimurium* and *Escherichia coli*) bacteria and the fungi *Candida albicans*.

Methanol extract inhibited the growth of all tested microorganisms. The MIC (256 μg mL^{-1}) and MMC (512 μg mL^{-1}) were observed against *Pseudomonas aeruginosa, Bacillus subtilis* and *Candida albicans* (Table 4). This extract showed the major MMC (1024 μg mL^{-1}) against *Klebsiella pneumoniae*;[26] it is reported that propolis is active against Gram-positive bacteria, showing a limited activity against Gram-negative ones.

In vitro antimicrobial activity of propolis on bacterial strains isolated from human infections showed that the growth of Gram-positive bacteria *Staphylococcus aureus* was inhibited by low propolis concentrations, whereas Gram-negative bacteria were less susceptible.[27]

Propolis antibacterial activity has been attributed to phenolic compounds, especially flavonoids, phenolic acids and their esters.[28] Isoflavonoids are important antimicrobial components of red propolis, especially concerning their activity against *C. albicans*.[19] Pterocarpans possessing a *cis*-6a,11a-dihydro-6H-benzofuro[3,3-c]chromene skeleton constitute the second largest group of natural isoflavonoids, and had been gaining considerable interest due to their wide range of biological effects. Many of them are phytoalexins possessing high antifungal and antibacterial activity, and several of them have been reported to inhibit HIV-1 reverse transcriptase and cytopathic effect on HIV-1 in cell cultures.[29] Chalcones represent an important group of natural compounds with a variety of biological activities, including antibacterial and antifungal.[30]

Table 4. Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) of methanol extracts of red propolis on microorganisms

Microorganism	MIC (μg mL^{-1})	MMC (μg mL^{-1})
Pseudomonas aeruginosa	256	512
Bacillus subtilis	256	512
Candida albicans	256	512
Salmonella typhimurium	512	512
Klebsiella pneumoniae	512	1024
Enterococcus faecalis	512	–
Escherichia coli	512	–
Proteus mirabilis	512	–
Streptococcus pyogenes	512	–

Antioxidant activity

Reactive oxygen species (ROS) are formed continuously in cells as a consequence of both oxidative biochemical reactions and external factors. However, they become harmful when they are produced in excess under certain abnormal conditions such as inflammation, ischemia and in the presence of catalytic iron ions. Under these conditions, endogenous antioxidants may be unable to counter ROS formation. The formation of these species may cause cellular damage by peroxidation of membrane lipids, sulfydryl enzyme inactivation, protein cross-linking and DNA breakdown, which may be involved in the etiology of diverse human diseases, such as coronary heart disease, inflammation, neurodegenerative diseases such as Parkinson's, Alzheimer's and cancer.[31]

The antioxidant activity of ME was evaluated using two methods: DPPH free radical scavenging activity and the β-carotene bleaching test. The DPPH method is representative of the methods employing model radicals in the evaluation of radical scavengers. Such methods have gained high popularity over the last decade because of their rapidity and sensitivity. The β-carotene bleaching test method uses a model lipid substrate (linoleic acid) in an emulsified form, and depends on methods employing only model substrates.[31]

DPPH free radical scavenging method

Antioxidants react with DPPH, a stable free radical, and convert it to 1,1-diphenyl-2-picrylhydrazine. The phenolic compounds intercept the free radical chain oxidation by donating hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate or propagate further oxidation. In phenolic acids and their esters, radical scavenging activity generally depends on the number of phenolic hydroxyl groups.[32] The DPPH method is based on the reduction of alcoholic DPPH solutions at 517 nm and the remaining DPPH measured after a certain time; this difference corresponds inversely to the radical scavenging activity of the antioxidant. As can be seen in Table 5, ME showed reduction of free radical at all concentrations,

Table 5. Antioxidant activity of methanol extract (ME) of Brazilian red propolis sample, compared with rutin solutions (standard), using DPPH free radical scavenging activity

Solution ((μg mg^{-1})	Antioxidant activity (%)
Standard	
8.0	60.75
12.8	64.37
25.0	100
Sample	
8.0	30.62
12.5	32.75
25.0	39.12

and chalcones and isoflavonoids may be involved among the active substances. At maximum concentration (25 μg mL^{-1}), the antioxidant activity of ME was 39.12%, while rutin at the same concentration exhibited 98% of the antioxidant activity. Below the minimum concentration (8 μg mL^{-1}), the extract did not exhibit antioxidant activity. 7-*O*-Methylvestitol and medicarpin have been reported to exhibit 4.5% and 0.7% of antioxidant activity, respectively.[19] Isoflavonoids, in general, are known to be biologically active compounds and to possess antioxidant properties, able to reduce cardiovascular diseases.[33] Besides this, flavonoids and various phenolic compounds are the most important pharmacologically active constituents in propolis and have been shown to be capable of scavenging free radicals.[34]

Chalcones containing phenolic and catecholic derivatives may exhibit antioxidant activity as a result of their ability to donate electron (or hydrogen atom) and thereby eliminating reactive oxygen and nitrogen species and decay-free radical propagation reactions.[35] 3,4,2′,3′-Tetrahydroxychalcone found in this red propolis has a structure that appeared with butein, which exhibits a basic structure with two benzene rings that possess two dihydroxyl groups linked by a β-unsaturated carbonyl group and a double bond. The presence of a double bond between the benzene rings can promote antioxidant activity by delocalizing the unpaired electrons of the phenoxyl radicals.[36] Butein contains a catechol moiety and thus is assumed to have at least one hydroxyl group with low dissociation energy capable of donating a hydrogen atom and thus act as a potent antioxidant.[35]

β-Carotene oxidation method

Methanol extracts at concentrations 1.0, 1.5 and 2.0 mg mL^{-1} gave 84.5%, 85.3% and 85.7% antioxidant activity in relation to rutin. Thus the constituents of the extract have higher antioxidant activity by this method than by the DPPH method.

The test made to inhibit β-carotene oxidation in a lipid micelle system is important because membrane lipids are rich in unsaturated fatty acids that are most susceptible to the oxidative process. Lipid oxidation is a free radical chain process leading to the deterioration of lipids and lipid-containing materials. β-Carotene bleaching measured by the decrease in initial absorbance at 470 nm is slowed down in the presence of antioxidants. The method is sensitive due to the strong absorption of β-carotene, but it is slower than the DPPH method. Apolar antioxidants exhibit stronger antioxidative properties in emulsions. On the other hand, polar antioxidants remaining in the aqueous phase are more diluted in organic phase and are less effective in protecting the lipid. However, the differences in solubilities of phenolic compounds in a micellar water–lipid system may influence the results obtained by this test. The main constituents of ME are chalcones and isoflavonoids. Butein has been reported to be an inhibitor of xanthine oxidase, to have inhibitory effects on lipid peroxidation and participate in one side reaction of chain propagation with hydroperoxides.[37]

The methanol extract under investigation showed DPPH radical scavenging action and also β-carotene bleaching. The higher antioxidant activity of ME in the β-carotene method is accounted for by the fact that the principal constituents of the extract are isoflavans and chalcones, probably more concentrated in the organic phase.

CONCLUSIONS

This work is in agreement with previous studies concerning the chemical composition of Brazilian red propolis. However, substances still not detected in propolis were characterized from our propolis sample, such as 3,4,2′,3′-tetrahydroxychalcone and a flavone *C*-glycoside. Furthermore, the results corroborate the suggestion of the contribution of exudates of species of Leguminosae species to produce this type of propolis. However, further studies on the chemical compositions of propolis and plants are needed.

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REFERENCES

1 Watson DG, Peyfoon E, Zheng L, Lu D, Seidel V, Johnston B, *et al*, Application of principal components analysis to H-1-NMR data obtained from propolis samples of different geographical origin. *Phytochem Anal* **17**:323–331 (2006).
2 Bankova V, Recent trends and important developments in propolis research. *Evid Based Complement Alternat Med* **2**:29–32 (2005).
3 Salatino A, Teixeira EW, Negri G and Message D, Origin and chemical variation of Brazilian propolis. *Evid Based Complement Alternat Med* **2**:33–38 (2005).
4 Hernandez IM, Fernandez MC, Cuesta-Rubio O, Piccinelli AL and Rastrelli L, Polyprenylated benzophenone derivatives from Cuban propolis. *J Nat Prod* **68**:931–934 (2005).
5 Lotti C, Fernandez MC, Piccinelli AN, Cuesta-Rubio O, Hernandez IM and Rastrelli L, Chemical constituents of red Mexican propolis. *J Agric Food Chem* **58**:2209–2213 (2010).
6 Awale S, Li F, Onozuka H, Esurni H, Tezuka Y and Kadota S, Constituents of Brazilian red propolis and their preferential cytotoxic activity against human pancreatic PANC-1 cancer cell line in nutrient-deprived condition. *Bioorg Med Chem Lett* **16**:181–189 (2008).
7 Alencar SM, Oldoni TLC, Castro ML, Cabral ISR, Costa-Neto CM, Cury JA, *et al*, Chemical composition and biological activity of a new type of Brazilian propolis: red propolis. *J Ethnopharmacol* **113**:278–283 (2007).
8 Cabral ISR, Oldoni TLC, Prado A, Bezerra RMN and Alencar SM, Composição fenólica, atividade antibacteriana e antioxidante da própolis vermelha brasileira. *Quim Nova* **32**:1523–1527 (2009).
9 Ferreres F, Gil-Izquierdo A, Andrade PB, Valentão P and Tomas-Barberan FA, Characterization of *C*-glycosyl flavones *O*-glycosylated by liquid chromatography–tandem mass spectrometry. *J Chromatogr A* **1161**:214–223 (2007).
10 Woisky RG and Salatino A, Analysis of propolis: some parameters and procedure for chemical quality control. *J Apicul Res* **37**:99–105 (1998).

11 Nagy M and Grancai D, Colorimetric determination of flavanones in propolis. *Pharmazie* **51**:100–101 (1996).
12 MacLafferty SW and Stauffer DB, *The Wiley/NBS Registry of Mass Spectral Data*. Wiley, New York (1989).
13 Sutter VL, Barry AL, Wilkins TD and Zabransky RJ, Collaborative evaluation of a proposed reference dilution method of susceptibility testing of anaerobic bacteria. *Antimicrob Agents Chemother* **16**:495–502 (1979).
14 Duarte-Almeida JM, dos Santos RJ, Genovese MI and Lajolo FM, Avaliação da atividade antioxidante utilizando o sistema β-caroteno/ácido linoléico e método de seqüestro de radicais livres por DPPH. *Ciência Tecnol Aliment* **26**:446–452 (2006).
15 Silva JFM, Souza MC, Matta SR, Andrade MR and Vidal FVN, Correlation analysis between phenolic levels of Brazilian propolis extracts and their antimicrobial and antioxidant activities. *Food Chem* **99**:431–435 (2006).
16 Bonvehí JS and Coll FVV, Phenolic composition of propolis from China and South America. *Z Naturfor C-A J Biosci* **49C**:712–718 (1994).
17 Gardana C, Scaglianti M, Pietta P and Simonetti P, Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography–tandem mass spectrometry. *J Pharm Biomed Anal* **45**:390–399 (2007).
18 Tomás-Barberán FA, Garcia-Viguera C, Vit-Olivier P, Ferreres F and Tomás-Lorente F, Phytochemical evidence for the botanical origin of tropical propolis from Venezuela. *Phytochemistry* **34**:191–193 (1993).
19 Trusheva B, Popova M, Bankova V, Simova S, Marcucci MC, Miorin PL, *et al*, Bioactive constituents of Brazilian red propolis. *Evid Based Complement Alternat Med* **3**:249–254 (2006).
20 Volpi N and Bergonzini G, Analysis of flavonoids from propolis by on-line HPLC–electrospray mass spectrometry. *J Pharm Biomed Anal* **42**:354–361 (2006).
21 Padhye S, Ahmad A, Oswal N and Sarkar FH, Emerging role of garcinol, the antioxidant chalcone from *Garcinia indica* Choisy and its synthetic analogs. *J Hematol Oncol* **2**:38 (2009).
22 Liu RX, Ye M, Guo HZ, Bi KS and Guo DA, Liquid chromatography/electrospray ionization mass spectrometry for the characterization of twenty-three flavonoids in the extract of *Dalbergia odorifera*. *Rapid Commun Mass Spectrom* **19**:1557–1565 (2005).
23 Cuesta-Rubio O, Piccinelli AL, Fernandez MC, Marquez Hernandez IM, Rosado A and Rastrelli L, Chemical characterization of Cuban propolis by HPLC-PDA, HPLC-MS, and NMR: the brown, red, and yellow Cuban varieties of propolis. *J Agric Food Chem* **55**:7502–7509 (2007).
24 Fernández MC, Cuesta-Rubio O, Perez AR, Porto RMO, Hernández IM, Piccinelli AL, *et al*, GC-MS determination of isoflavonoids in seven red Cuban propolis samples. *J Agric Food Chem* **56**:9927–9932 (2008).
25 Wang Y, Yang L, He YQ, Wang CH, Welbeck EW, Bligh SWA, *et al*, Characterization of fifty-one flavonoids in a Chinese herbal prescription Longdan Xiegan decoction by high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry and photodiode array detection. *Rapid Commun Mass Spectrom* **22**:1767–1778 (2008).
26 Choi YM, Noh DO, Cho SY, Suh HJ, Kim KM and Kim JM, Antioxidant and antimicrobial activities of propolis from several regions of Korea. *LWT Food Sci Technol* **39**:756–761 (2006).
27 Sforcin JM, Fernandes A, Lopes CAM, Bankova V and Funari SRC, Seasonal effect on Brazilian propolis antibacterial activity. *J Ethnopharmacol* **73**:243–249 (2000).
28 Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R and Popov S, Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. *J Ethnopharmacol* **64**:235–240 (1999).
29 Engler TA, Latessa KO, Iyengar R, Chai WY and Agrios K, Stereoselective syntheses of substituted pterocarpans with anti-HIV activity, and 5-aza-/5-thia-pterocarpan and 2-aryl-2,3-dihydrobenzofuran analogues. *Bioorg Med Chem Lett* **4**:1755–1769 (1996).
30 Nowakowska Z, Kedzia B and Schroeder G, Synthesis, physicochemical properties and antimicrobial evaluation of new (E)-chalcones. *Eur J Med Chem* **43**:707–713 (2008).
31 Ahn MR, Kumazawa S, Usui Y, Nakamura J, Matsuka M, Zhu F, *et al*, Antioxidant activity and constituents of propolis collected in various areas of China. *Food Chem* **101**:1383–1392 (2007).
32 Jayaprakasha GK, Ohnishi-Kameyama M, Ono H, Yoshida M and Jaganmohan Rao L, Phenolic constituents in the fruits of *Cinnamomum zeylanicum* and their antioxidant activity. *J Agric Food Chem* **54**:1672–1679 (2006).
33 Wu T, Bligh SWA, Gu LH, Wang Z-t, Liu He P, Cheng X-m, *et al*, Simultaneous determination of six isoflavonoids in commercial *Radix astragali* by HPLC-UV. *Fitoterapia* **76**:157–165 (2005).
34 Mohammadzadeh S, Sharriatpanahi M, Hamedi M, Amanzadeh Y, Ebrahimi SES and Ostad SN, Antioxidant power of Iranian propolis extract. *Food Chem* **103**:729–733 (2007).
35 Khatib S, Nerya O, Musa R, Shmuel M, Tamir S and Vaya J, Chalcones as potent tyrosinase inhibitors: the importance of a 2,4-substituted resorcinol moiety. *Bioorg Med Chem Lett* **13**:433–441 (2005).
36 Lee SH, Seo GS, Jin XY, Ko G and Sohn DH, Butein blocks tumor necrosis factor alpha-induced interleukin 8 and matrix metalloproteinase 7 production by inhibiting p38 kinase and osteopontin mediated signaling events in HT-29 cells. *Life Sci* **81**:1535–1543 (2007).
37 Farhoosh R, Antioxidant activity and mechanism of action of butein in linoleic acid. *Food Chem* **93**:633–639 (2005).

