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Chemical and Microbiological Study of Extract from Seeds of Guaraná *(Paullinia cupana* var. *sorbilis)*

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SUMMARY. Chemical isolation of a semi-purified extract from the seeds of guaraná, *Paullinia cupana* H.B.K. var. *sorbilis* (Mart.) Ducke, resulted in the identification of caffeine, catechin, epicatechin, and procyanidins B2, B3 and B4. The antibacterial activity of the extracts and isolated substances was evaluated *in vitro* against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa.* Even in concentrations up to 1000 μg/ml, there was no activity against these microorganisms.

RESUMEN. "Estudio Químico y Microbiológico del Extracto de Semilas de Guaraná *(Paullinia cupana* var. *sorbilis)"*. El aislamiento químico de un extracto semipurificado de las semillas de guaraná, *Paullinia cupana* H.B.K. var. *sorbilis* (Mart.) Ducke, resultó en la identificación de cafeína, catequina, epicatequina y procianidinas B2, B3 y B4. La actividad antibacteriana de los extractos y de las substancias se evaluó *in vitro* contra *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* y *Pseudomonas aeruginosa*. En concentraciones hasta 1000 μg/ml ninguna actividad fue demostrada frente a dichos microorganismos.

INTRODUCTION

Guaraná is a plant of the family Sapindaceae, native to the central Amazon basin. Seeds were originally used by the indigenous peoples of this region as a stimulant drink, for festivals and hunting. A possible stimulative effect on the central nervous system is attributed to its seeds, because of the presence of methylxanthines [1].

One of the characteristics of guaraná seeds is the presence of phenolic substances, mainly derivatives of catechin or flavan-3-ols, or condensed tannins, as characterized in some studies through RP-HPLC [2-4]. However, no chemical study involving the isolation and structural identification of monomers and dimers of the condensed tannins, as well as of caffeine in this plant species or its close relatives, has been reported, except for theobromine in *Paullinia pachycarpa* Benth. [5].

Tannins and phenolic compounds are widely recognized as possessing high antibacterial potential [6,7]. This biological activity against microorganisms has been fully established by studies involving several fields of research, such as foods, plants, soil, pharmacology, and human and animal nutrition [7].

This is the first work to combine chemical and microbiological studies of extracts from the seeds of *Paullinia cupana* H.B.K. var. *sorbilis* (Mart.) Ducke.

MATERIAL AND METHODS

Plant material

The seeds of *Paullinia cupana* H.B.K. var. *sorbilis* (Mart.) Ducke were acquired from Mr. J. A. de Sousa, highway MT325, km 8, Alta Floresta, Mato Grosso, Brazil. The seeds were collected from September through November of 2000, combined, dried and toasted in an iron pan, and ground in a hammer mill (Tiger ASN-5). A voucher specimen is deposited at the UEM Department of Biology Herbarium under number HUEM 9.065. The identification of the specimen was confirmed by Prof. Dr. C. M. Sakuragui.

KEY WORDS: Antibacterial activity, Condensed tannins, *Paullinia cupana*.
PALABRAS CLAVE: Actividad antibacteriana, *Paullinia cupana*, Taninos condensados.

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Chemical studies

The NMR spectra were determined in a Varian Mercury 300 Plus at 300 MHz for ^{1}H and 75.45 MHz for ^{13}C, using CD_3OD and $CDCl_3$, and TMS as the internal reference, at the Chemistry Department of the Universidade Estadual de Maringá (UEM). Mass spectra were determined in an ESI-MS Quattro LCZ (Micromass, Manchester, UK) at the Institute of Organic Chemistry of Münster University, Germany. The optical rotation of $[a]_D^{20^\circ}$ was recorded in methanol or acetone on a Perkin-Elmer 241 spectropolarimeter. CD data were recorded in methanol on a Jasco J-720 spectropolarimeter. Identification of the upper and lower units was carried out through hydrolysis with cyanidin, catechin and epicatechin as reference substances according to literature [8].

Extraction and isolation

The crude extract from the guaraná seeds (EBPC) (100 g) was prepared by ultra-turrax® (UTC-115KT) for 15 min. From the EBPC, two semi-purified fractions were obtained: FAQ (29.11 g) and EPA (43.93 g) (Patent registered under #PI 0006638-9). Fifteen g of EPA were chromatographed on a Sephadex® LH-20 column, using EtOH:H_2O (1:1) [8,9] as the eluent system, to obtain 24 fractions (F#1 - F#24).

The F#2 fraction yielded compound 1 in crystal form during the process of concentration of the organic solvent (2.47 g). Fractions F#5 to F#8 were combined by chromatographic similarity under UV light at 254 nm, and were then developed with a 1% $FeCl_3$ solution in ethanol. This fraction was again submitted to CC on Sephadex® LH-20 using the eluent system EtOH:H_2O (9:1; 8:2; 7:3; 6:4 and 1:1; vv; 150 ml each). From this fraction, compounds **2** (353 mg) and **3** (112.9 mg) were isolated and identified. The F#9 fraction was submitted to chromatography in the same conditions as previously described, and 3 subfractions were obtained. These subfractions were acetylated (anhydride acetic: pyridine; 1.2:1; ambient temperature/24 h) and purified by preparative TLC (silica gel plates PF_{254}, Merck®) using toluene:acetone (7:3; v/v) as the eluent [10], yielding compounds **4** (5.7 mg), **5** (6.4 mg) and **6** (5.1 mg).

Caffeine (**1**). $C_8H_{10}O_2N_4$. MS/ESI *m/z* (rel. int.%): 195 (20), 138 (100), 110 (40), 83 (20), 69 (28), 42 (28) $[M+H]^+$. NMR data (^{1}H and ^{13}C) were compared with the literature [11] (Table 1 and Fig. 1).

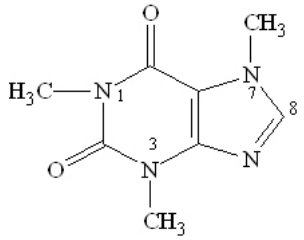

Figure 1. Caffeine.

Epicatechin (**2**). $C_{15}H_{14}O_6$. MS/ESI *m/z* (rel. int. %): 313 (100), 301 (11) $[M+Na]^+$; 289 (92), 255 (11), 97 (40), 89 (100) $[M-H]^+$. ^{1}H NMR [300 MHz, CD_3OD, δ (ppm), multiplicity and *J* (Hz)]: δ 2.72 [*dd,* H-4ax (C), *J*=16.8 and 2.7], 2.86 [*dd,* H-4eq (C), *J*=16.8 and 4.5], 4.80 [*s,* H-2 (C)], 4.16 [*m,* H-3 (C), *J*=4.5 and 2.7], 5.90 [*d,* H-6 (A), *J*=2.4], 5.93 [*d,* H-8 (A), *J*=2.4], 6.75 [*d,* H-5' (B), *J*=8.1], 6.79 [*dd,* H-6' (B), *J*=8.1 and 1.8], 6.97[*d,* H-2' (B), *J*=1.8 Hz]. $[a]_D^{20^\circ}$ -46 ° (methanol; *c* 0.001). CD (*c* 0.2; methanol): $[\theta]_{239}$ 1800 and $[\theta]_{280}$ -1500 (Fig. 2).

Proton	δ (ppm)	δ Literature* (ppm)	Carbon	δ (ppm)	δ Literature* (ppm)
$\underline{C}H_3$-N_1	3.41 (*s*)	3.37 (*s*)	$\underline{C}H_3$-N1	27.89	27.5
			C-2	151.70	151.3
$\underline{C}H_3$-N_3	3.59 (*s*)	3.55 (*s*)	$\underline{C}H_3$-N_3	29.71	29.3
			C-4	148.68	148.3
			C-5	107.57	107.1
			C-6	155.41	154.9
$\underline{C}H_3$-N_7	4.00 (d, *J*=0.6 Hz)	4.01 (d, *J*=0.7 Hz)	$\underline{C}H_3$-N_7	33.55	33.2
H-8	7.52 (*sl*)	7.58 (q, *J*=0.7 Hz)	C-8	141.35	141.2

Table 1. ^{1}H e ^{13}C NMR data of caffeine (^{1}H NMR = 300 MHz; ^{13}C NMR = 75.45 MHz; $CDCl_3$); *s*: singlet; *d*: dublet; *sl*: singlet broad; *q*: quartet; * Sitkowski *et al.* [11].

Catechin (**3**). $C_{15}H_{14}O_6$. MS/ESI *m/z* (rel. int.%): 313 (100), 301 (21) $[M+Na]^+$; 289 (100), 255 (42) $[M-H]^+$. ^1H NMR [300 MHz, CD_3OD, δ ppm), multiplicity and *J* (Hz)]: δ 2.49 [*dd,* H-4ax (C), *J*=16.2 and 8.1], 2.83 [*dd*, H-4eq (C), *J*=16.2 and 5.1], 4.55 [*d,* H-2 (C), *J*=7.5], 3.96 [*ddd,* H-3 (C), *J*=7.5, 5.1 and 8.1], 5.85 [*d,* H-6 (A), *J*=2.1], 5.92 [*d,* H-8 (A), *J*=2.1], 6.71 [*dd,* H-6' (B), *J*=8.4 and 1.8], 6.76 [*d,* H-5' (B), *J*=8.4], 6.84 [*d,* H-2' (B), *J*=1.8]. $[a]_D^{20°}$ -20.1° (acetone; c 0.04). CD (*c* 0.2; methanol): $[\theta]_{239}$ 850 and $[\theta]_{280}$ -1650 (Fig. 2).

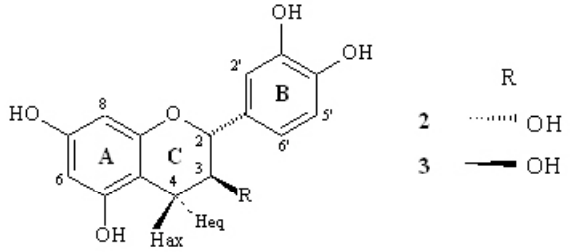

Figure 2. Epicatechin (**2**) and catechin (**3**).

Epicatechin-(4β→8)-epicatechin (procyanidin B_2) (**4**). $C_{50}H_{46}O_{22}$. MS/ESI *m/z* (rel. int. %): 1021 (100), 522 (21) $[M+Na]^+$. ^1H NMR [300 MHz, $CDCl_3$, δ ppm), multiplicity and *J* (Hz)]: δ 2 x 2.88-2.92 [*m,* H-4 (F)], 4.46 [*d,* H-4 (C), *J*=1.8], 4.54 [*s,* H-2 (F)], 5.10 [*m,* H-3 (F)], 5.16 [*m,* H-3 (C)], 5.57 [*s,* H-2 (C)], 5.99 [*d,* H-6 (A), *J*=2.4], 6.24 [*d,* H-8 (A), *J*=2.4], 6.66 [*s,* H-6 (D)], 6.89 [*dd,* H-6' (E), *J*=8.1 and 1.8], 7.01 [*d,* H-2' (E), *J*=1.8], 7.04 [*d,* H-5' (E), *J*=8.1], 7.17 [*d,* H-5'(B), *J*=8.1], 7.29 [*dd,* H-6' (B)], 7.36 [*d,* H-2' (B), *J*=1.8]. CD (*c* 0.1, methanol): $[\theta]_{230}$ 27200 (Fig. 3).

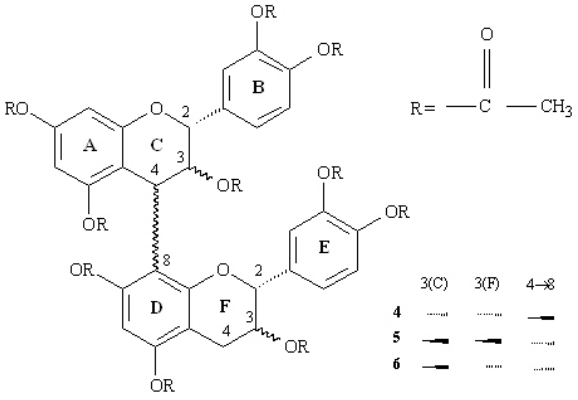

Figure 3. Epicatechin-(4β →8)-epicatechin (**4**), Catechin-(4α→8)-catechin (**5**), and Catechin-(4α →8)-epicatechin (**6**).

Catechin-(4α →8)-catechin (procyanidin B_3) (**5**). $C_{50}H_{46}O_{22}$. MS/ESI *m/z* (rel. int. %): 1021 (100), 522 (29) $[M+Na]^+$. ^1H NMR [300 MHz, $CDCl_3$, δ ppm), multiplicity and *J* (Hz)]: δ 2.66 [*dd,* H-4ax (F), *J*=16.5 and 7.5], 2.93 [*dd,* H-4eq (F), *J*=16.5 and 5.4], 4.48 [*d,* H-4 (C), *J*=9.3], 4.76 [*d,* H-2 (C), *J*=9.9], 4.96 [*d,* H-2 (F), *J*=7.8], 5.02 [*ddd,* H-3 (F), *J*=7.8, 7.5 and 5.4], 5.62 [*pseudo triplet,* H-3 (C)], 6.48 [*d,* H-6 (A), *J*=2.4], 6.51 [*d,* H-8 (A), *J*=2.4], 6.64 [*s,* H-6 (D)], 6.73 [*dd,* H-6' (E), *J*=8.4 and 1.8], 6.93 [*d,* H-2' (E), *J*=1.8], 6.99 [*dd,* H-6'(B), *J*=8.4 and 1.8], 7.02 [*d,* H-2'(B), *J*=1.8], 7.14 [*d,* H-5'(E), *J*=8.4], 7.14 [*d,* H-5'(B), *J*=8.4]. CD (*c* 0.1; methanol): $[\theta]_{230}$ -34000 (Fig. 3).

Catechin-(4α→8)-epicatechin (procyanidin B_4) (**6**). $C_{50}H_{46}O_{22}$. MS/ESI *m/z* (rel. int.%): 1021 (100), 522 (21) $[M+Na]^+$. ^1H NMR [300 MHz, $CDCl_3$, δ ppm), multiplicity and *J* (Hz)]: δ 2.72-2.78 [*m,* H-4ax (F)], 3.02 [*dd,* H-4eq (F), *J*=17.1 and 5.4], 4.53 [*d,* H-4(C), *J*=9.6], 4.82 [*d,* H-2(C), *J*=9.9], 5.01 [*s,* H-2 (F)], 5.21 [*m,* H-3 (F)], 5.71 [*pseudo triplet,* H-3 (C)], 6.53 [*d,* H-6 (A), *J*=2.4], 6.58 [*d,* H-8 (A), *J*=2.4], 6.63 [*s,* H-6 (D)], 6.85-7.30 [6 x H (B/E)]. CD (*c* 0.1; methanol): $[\theta]_{230}$ -5990 (Fig. 3).

Microbiological assay

The antibacterial activity of the EBPC, FAQ, EPA extracts and isolated compounds from *Paullinia cupana* was observed *in vitro* against ATCC strains of *Staphylococcus aureus* (25923), *Bacillus subtilis* (6623), *Escherichia coli* (25922) and *Pseudomonas aeruginosa* (15442).

The Minimum Inhibitory Concentrations (MICs) of all test substances (ST), isolated compounds and reference antibiotics [penicillin, vancomycin and tetracycline (Sigma)] were determined by microdilution techniques in Mueller-Hinton broth [12].

Each ST (2 mg/ml) was aseptically mixed with inoculum prepared in the same medium at a density adjusted by the McFarland scale (10^8 UFC/ml) and diluted 1:10 for the microdilution procedure. The plates (96 wells) were then incubated for 24 h at 37 °C.

The MIC was determined as the lowest concentration of the ST which produced an 80% reduction in visible growth compared with the control. All samples were evaluated in duplicate in separate experiments.

RESULTS AND DISCUSSION

Chemistry

Multiple chromatography on Sephadex® LH-20 resulted in the isolation and identification of compounds **1-3**, and after derivation followed

by the use of prep. TLC in silica gel, compounds **4-6** were identified. Compounds **1**, **2** and **3** were identified as caffeine, epicatechin and catechin, respectively, after analysis of the MS and NMR (^{1}H and ^{13}C), 2D NMR (^{1}H-^{1}H COSY, HETCOR and HMBC) spectra, by comparison with the authentic compounds and the literature [11,13,14].

The ^{1}H NMR spectrum of the caffeine (**1**) showed one singlet, in δ 7.52 ppm corresponding to H-8 proton, and three signals for the three methyl groups. One of these signals appeared as a doublet in δ 4.0 ppm ($J_{\text{H-7-H-8}}$ < 1.0 Hz), corresponding to the methyl group at position 7, observed in the HMBC[11] spectrum. The HMBC spectrum established the correlation at long range (^3J) between C-8 and the methyl-group proton in N7, and these protons were correlated with tetrasubstituted carbon in C-5. It was possible to establish correlations of N1-CH_3 and N_3-CH_3 with the tetrasubstituted carbon C-2 (δ 151.70 ppm). The other two signals of methyl groups appeared as singlets in δ 3.41 ppm and δ 3.59 ppm, corresponding to the proton at positions 1 and 3, respectively. Through the HMQC spectrum, a correlation was established between carbons in N1, N3 and N7 with their proton in δ 27.89 ppm, δ 29.71 ppm and δ 33.55 ppm, respectively, and H-8 with C-8 (δ 141.35 ppm) [11].

The identification of flavan-3-ols was carried out from the MS, NMR (^{1}H), $[\alpha]_D^{20^\circ}$ and CD, which showed similarities with substances of the type catechin/epicatechin [15]. These were differentiated on the basis of the spectroscopic data of aliphatic protons H-3(C) and H-2(C) [16]. Compound **2** showed a coupling constant $J_{\text{H-2-H-3}}$ < 1.0 Hz, demonstrating a relative configuration of type 2,3-*cis*. However, Compound **3** showed a coupling constant of 7.5 Hz, demonstrating a relative configuration of type 2,3-*trans*. Between δ 6.75 and 6.97 ppm the characteristic signals of an AMX-spin system appeared, corresponding to protons H-2', H-5' and H-6', demonstrating dihydroxylation in the B ring. The NMR (^{13}C) spectrum helped in the differentiation of the epimers catechin (**2**) and epicatechin (**3**), from the C-2 displacements. Epicatechin showed a displacement in δ 79.94 ppm, whereas catechin displaced in lower fields, δ 82.93 ppm (Δ 2.99 ppm), in accordance with the literature [8,14,17].

Acetylated compounds **4**, **5** and **5** were identified by interpretation of the MS, ^{1}H NMR and ^{1}H-^{1}H COSY, and CD. The spectra indicated that they are acetates of dimers of proanthocyanidin of type epicatechin-(4β→8)-epicatechin (procyanidin B_2), catechin-(4α→8)-catechin (procyanidin B_3) and catechin-(4α→8)-epicatechin (procyanidin B_4), respectively.

The ^{1}H NMR spectrum of procyanidin B_2 (**4**) demonstrated a coupling constant of 1.8 Hz for the aliphatic protons H-3(C) and H-4(C). The protons H-2(C) and H-3(C) of the upper unit and H-2(F) and H-3(F) of the lower unit showed a coupling constant lower than 1.0 Hz. These data establish a relative configuration of type 2,3-*cis;* 3,4-*trans* for the upper unit and 2,3-*cis* for the lower unit. Comparison of spectral data from the literature [18], added to the MS, and CD, confirmed compound **4** as procyanidin B_2.

Compounds **5** and **6** (procyanidin B_3; procyanidin B_4) showed for the aliphatic protons of the upper unit a coupling constant of $J_{\text{H-2-H-3}}$ = 9.9 Hz, and $J_{\text{H-3-H-4}}$ = 9.3 Hz, demonstrating a relative configuration of type 2,3-*trans;* 3,4-*trans*. These compounds were differentiated based on the aliphatic protons of the lower unit. Compound **5** (procyanidin B_3) showed a coupling constant of $J_{\text{H-2-H-3}}$ = 7.8 Hz, $J_{\text{H-3-H-4ax}}$ = 7.5 Hz and $J_{\text{H-3-H-4eq}}$ = 5.4 Hz, establishing a relative configuration of type 2,3-*trans;* 3,4-*trans*. Compound **6** (procyanidin B_4), however, showed for the aliphatic protons of the lower unit a coupling constant of $J_{\text{H-2-H-3}}$ < 1.0 Hz, determining a relative configuration of type 2,3-*cis*. These data, compared with the literature [15,17,18] and combined with the MS, and CD, confirmed compounds **5** and **6** as procyanidin B_3 and B_4 respectively.

The aromatic protons of compounds **4**, **5** and **6** (between δ 7.36 and 6.73 ppm) were similar to monomeric derivatives catechin/epicatechin, with two AMX-spin systems, characterizing the H-2', H-5' and H-6' protons, indicating the B- and E-rings, demonstrating dihydroxylation in the rings.

The bonds between the upper and lower units of compounds **4**, **5** and **6** were determined as type (4→8), based on previous studies which established the dominance of rotamer signals for this type of bond [19]. Moreover, displacement of the H-2(F) bond was observed between δ 4.37 and 5.01 ppm, similar to data from the literature [20], proving the type (4→8) bond.

Identification of the upper unit resulted in cyanidin. For the lower unit, compound **5** was determined as catechin, and compounds **4** and **6** as epicatechin.

Microbiological study

The evaluations of MICs of ST of *Paullinia cupana* var. *sorbilis* against Gram-positive *(B. subtilis* and *S. aureus)* and Gram-negative *(E. coli* and *P. aeruginosa)* bacteria are shown in Table 2.

Tannin-rich plants possess bactericidal activity [7]. However, this microbiological evaluation demonstrated that ST did not show activity against the microorganisms used, considering values from the literature as parameters [21]. A previous study [6] confirmed the data obtained in this work with the pure compounds catechin and procyanidins B_2, B_3 and B_4, which were active at concentrations above 8.000, 2.000, 2.000 and 1.000 mg/ml, respectively. Therefore, it is not appropriate to continue microbiological tests to establish the minimum bactericidal concentration (MBC).

Extracts and isolated substances	MIC (µg/ml)			
	S. aureus	*B. subtilis*	*E. coli*	*P. aeruginosa*
EBPC	> 1000	> 1000	> 1000	> 1000
FAQ	> 1000	> 1000	> 1000	> 1000
EPA	> 1000	> 1000	> 1000	> 1000
Caffeine	> 100	> 100	> 100	> 100
Epicatechin	> 100	> 100	> 100	> 100
Catechin	> 100	> 100	> 100	> 100
Antibiotics				
Penicillin	0.00975	–	–	–
Tetracycline	–	–	0.78	3.125
Vancomycin	–	0.09	–	–

Table 2. Minimum inhibitory concentrations (MIC) of all test substances (ST) of *Paullinia cupana* var. *sorbilis*.

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