

Full Paper

## Antioxidant Capacity and *In Vitro* Prevention of Dental Plaque Formation by Extracts and Condensed Tannins of *Paullinia cupana*

Elza Yamaguti-Sasaki <sup>1,‡</sup>, Lia Akina Ito <sup>2</sup>, Vanessa Cristina Dias Canteli <sup>2</sup>, Tânia Mara Antonelli Ushirobira <sup>3</sup>, Tânia Ueda-Nakamura <sup>1</sup>, Benedito Prado Dias Filho <sup>1</sup>, Celso Vataru Nakamura <sup>1</sup> and João Carlos Palazzo de Mello <sup>1,2,\*</sup>

<sup>1</sup> Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Estadual de Maringá, Av. Colombo, 5790, BR-87020-900, Maringá, PR, Brazil

<sup>2</sup> Departamento de Farmácia e Farmacologia, Universidade Estadual de Maringá, Av. Colombo, 5790, BR-87020-900, Maringá, PR, Brazil

<sup>3</sup> CCAS, Pontifícia Universidade Católica, Av. Tiradentes, 963, BR-87013-260, Maringá, PR, Brazil

<sup>‡</sup> Part of the M.Sc. Thesis.

\* Author to whom correspondence should be addressed; E-mail: mello@uem.br;

Fax: (+55)-44-3261-4999;

Received: 28 May 2007; in revised form: 1 August 2007 / Accepted: 1 August 2007 / Published: 20 August 2007

---

**Abstract:** Chemical evaluation of the semi-purified fraction from the seeds of *guaraná*, *Paullinia cupana* H.B.K. var. *sorbilis* (Mart.) Ducke, yielded the following compounds: caffeine, catechin, epicatechin, *ent*-epicatechin, and procyanidins B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, B<sub>4</sub>, A<sub>2</sub>, and C<sub>1</sub>. Measurement of the antioxidant activity by reduction of the DPPH radical confirmed the anti-radical properties of the aqueous (AqE) and crude (EBPC) extracts and semi-purified (EPA and EPB) fractions. The EPA fraction showed radical-scavenging activity (RSA) and protected DPPH from discoloration at 5.23±0.08 (RSD%=1.49) µg/mL, and for the phosphomolybdenum complex showed a higher Relative Antioxidant Capacity (RAC) at 0.75±0.01 (1.75). The EPA fraction had a total polyphenolics content of 65.80%±0.62 (RSD%=0.93). The plant drug showed 5.47% ± 0.19 (RSD%=3.51) and 6.19% ± 0.08 (RSD%=1.29) for total polyphenolics and methylxanthines, respectively. *In vitro* assessment of the antibacterial potential of the *Paullinia cupana* extracts against

*Streptococcus mutans* showed that these could be used in the prevention of bacterial dental plaque.

**Keywords:** *Paullinia cupana*, methylxanthines, condensed tannins, antioxidant capacity, prevention of the formation of dental plaque.

---

## Introduction

*Paullinia cupana* H.B.K. var. *sorbilis* (Mart.) Ducke is a plant of the family Sapindaceae, native to the central Amazon basin and known as *guaraná*. Its seeds are used traditionally by the indigenous people as a stimulant drink, for festivals and hunting. They are used mainly as stimulants and contain high amounts of methylxanthines and tannins. Crude and semipurified extracts of *guaraná* seeds show an antidepressant action, including after a long period of treatment, differently from caffeine [1, 2]. The tannin content can be influenced by the location where the plants were grown and the manner of drying the seeds [3, 4]. One important characteristic of tannins is their antioxidant potential [3]. It has been shown that *guaraná* inhibits the lipid peroxidation process, an effect apparently associated with the high tannin content of the seeds [6, 7]. In addition, enzymes such as glucosyltransferase, produced by *Streptococcus mutans* and *S. sobrinus*, are inhibited *in vitro* by tannins, preventing the formation of dental plaque and inflammations of the mouth and throat [8, 9]. Aqueous extracts produced from *guaraná* seeds at 5 and 7.5%, applied via a mouthwash, were efficient in inhibiting the formation of bacterial dental plaque *in vivo* [10].

This article describes monthly monitoring of the tannin content of *guaraná* seeds, and the isolation of condensed tannins from this species, in addition to others isolated in a previous study [11]. *In vitro* biological assays were performed to determine the antioxidant capacity and analyze in preliminary fashion the antibacterial potential against *Streptococcus mutans* of the different extracts (crude-EBPC and aqueous-AqE), and semi-purified fractions (EPA and EPB).

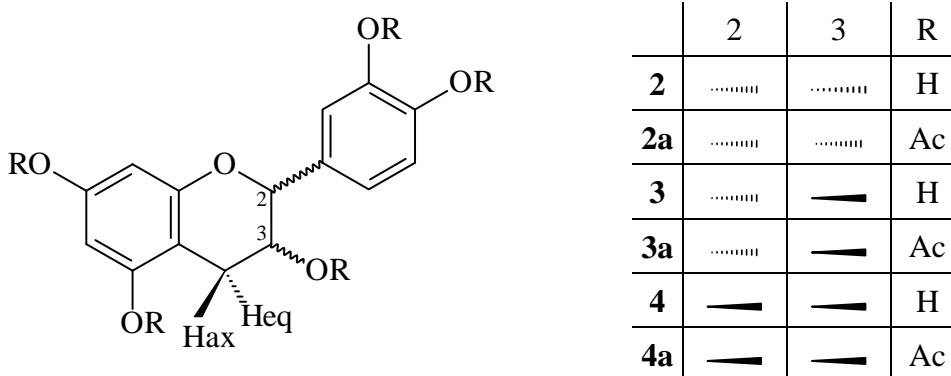
## Results and Discussion

The assessment of the quality of the *P. cupana* seeds established the minimum conditions for the plant drug, demonstrating the quality and equivalence in contents of characteristic chemical substances (methylxanthines and tannins). The total content of tannins was  $5.47\% \pm 0.19$  (Relative Standard Deviation - RSD%=3.51), and of methylxanthines  $6.19\% \pm 0.08$  (RSD%=1.29). Monthly assessment of the total tannin content of *guaraná* seeds stored whole and ground immediately prior to analysis, and of seeds stored in ground form in cardboard barrels demonstrated that the method of storage did not statistically change the tannin content, which remained stable over a 24-month period (data not shown).

Chemical assay of the ethyl acetate fraction yielded compounds **1-10**. Compounds **1**, **2**, and **3** were identified as caffeine, epicatechin, and catechin, respectively, by comparison of spectroscopic data ( $^1\text{H}$ - and  $^{13}\text{C}$  NMR, COSY, mass spectrometry-ESI-MS,  $[\alpha]_D^{20}$ ) with authentic material and literature

values [11]. Compound **4** (Figure 1) was obtained as a pale yellow, amorphous powder. The  $^1\text{H-NMR}$  spectrum of acetate **4a** showed no difference from compound **3a** (epicatechin pentaacetate). The heterocyclic protons displayed an ABMX-system characteristic for the spin pattern of 2,3-*cis*-flavan-3-ols ( $J_{2,3} < 2.0$  Hz) [12, 13]. An optical rotation  $[\alpha]_D^{20}$  of  $+6^\circ$  (methanol;  $c$  0.5) verified the 2*S*,3*S* absolute configuration of **4a**. The ESI mass spectrum showed a prominent peak  $[\text{M}+\text{Na}]^+$  at  $m/z$  523.4 and  $[\text{M}-\text{H}]^-$  at  $m/z$  499.4, indicating the molecular formula  $\text{C}_{25}\text{H}_{24}\text{O}_{11}$ ; therefore, **4** was identified as *ent*-epicatechin. A recent report described the analysis of samples of *guaraná* by capillary electrophoresis with a natural quiral selector (cyclodextrin), and demonstrated the presence of compounds 2*R* catechin and epicatechin and their chiral forms 2*S* (*ent*-catechin and *ent*-epicatechin) [14].

**Figure 1.** Flavan-3-ols identified from seeds of *Paullinia cupana*. Epicatechin (**2**), catechin (**3**), and *ent*-epicatechin (**4**).

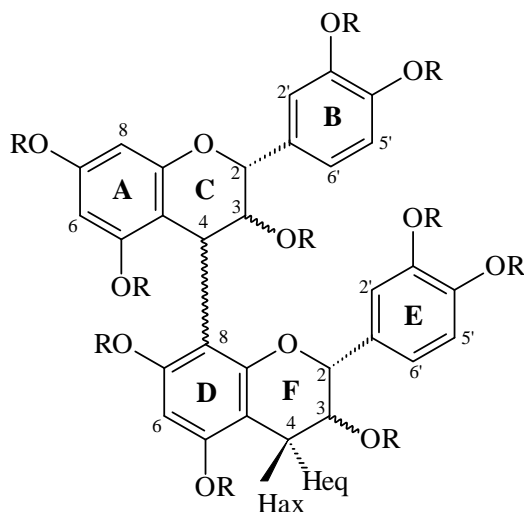


Procyanidins **6**, **7**, and **8** were identified as epicatechin-(4 $\beta$ →8)-epicatechin (procyanidin B<sub>2</sub>), catechin-(4 $\alpha$ →8)-catechin (procyanidin B<sub>3</sub>), and catechin-(4 $\alpha$ →8)-epicatechin (procyanidin B<sub>4</sub>), respectively, by comparison of the physical and spectroscopic data ( $^1\text{H-NMR}$ ,  $^1\text{H-}^1\text{H}$  COSY,  $^{13}\text{C-}$ , HMQC, HMBC, mass spectrometry-ESI-MS,  $[\alpha]_D^{20}$ , CD) of their corresponding peracetates **6a**, **7a**, and **8a** with recently published data [11].

Identification of epicatechin-(4 $\beta$ →8)-catechin (**5**), characterized several times in the literature in the underived phenolic form [15, 16, 17, 18], was effected by comparison of the  $^1\text{H-NMR}$  spectral data of its peracetate **5a** with those of the analogous derivative **6a**. The heterocyclic coupling constant ( $J_{2,3} < 2.0$ ) confirmed a relative 2,3-*cis* configuration of the ‘upper’ constituent unit [12, 13], whereas the 2,3-*trans* stereochemistry of the ‘lower’ flavan unit was indicated by the large coupling constant ( $J_{2,3} = 9.6$  Hz) of the corresponding resonances. The aromatic region ( $\delta$  6.87 and 7.30 ppm) showed two AMX-systems, confirming dihydroxylation on the B- and E-rings. The proton signals of H-6(A), H-8(A) and H-6(D) at  $\delta$  5.99, 6.29 and 6.68 ppm, respectively, as well as the position of the H-2(F) resonance at  $\delta$  4.33 ppm indicated a (4→8)-linked dimeric peracetate proanthocyanidin [19, 20, 21, 22, 23, 24], and this was confirmed by HMBC. The absolute configuration was assigned by optical rotation  $[\alpha]_D^{20} +2^\circ$  (methanol;  $c$  1.0), and in combination with the positive Cotton effect in the 220-240 nm region on the CD spectrum of **5a**, the identity of **5** (Figure 2) was established as epicatechin-

(4 $\beta$ →8)-catechin (procyanidin B<sub>1</sub>). The proposed structure was supported by ESI mass spectrum data, which showed a prominent [M+Na]<sup>+</sup> peak at *m/z* 1021.5 and [M-H]<sup>-</sup> *m/z* 997.5.

**Figure 2.** Dimeric procyanidins isolated from *Paullinia cupana*. Procyanidins B<sub>1</sub> (**5**), B<sub>2</sub> (**6**), B<sub>3</sub> (**7**), and B<sub>4</sub> (**8**).



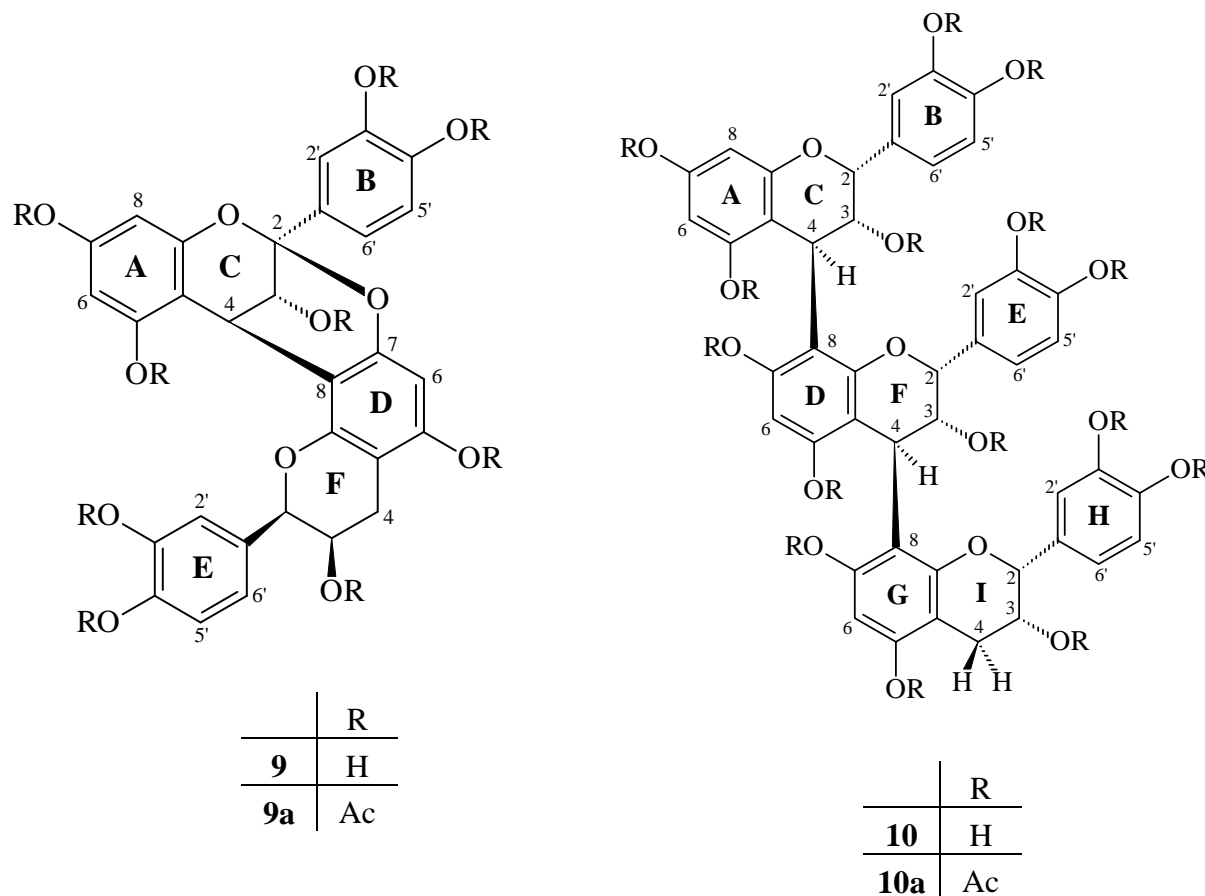
	3(C)	3(F)	4→8	R
<b>5</b>	.....	—	—	H
<b>5a</b>	.....	—	—	Ac
<b>6</b>	.....	.....	—	H
<b>6a</b>	.....	.....	—	Ac
<b>7</b>	—	—	.....	H
<b>7a</b>	—	—	.....	Ac
<b>8</b>	—	.....	.....	H
<b>8a</b>	—	.....	.....	Ac

Compound **9** was obtained as a pale yellow, amorphous powder. The positive and negative mass spectral mode (ESI-MS) of peracetate compound **9** showed a [M+Na]<sup>+</sup> ion at *m/z* 977.4 and a [M-H]<sup>-</sup> at *m/z* 953.5, corresponding to a biflavonoid structure (C<sub>48</sub>H<sub>42</sub>O<sub>21</sub>). These spectra showed 42 and 1 mass units fewer than procyanidin B<sub>2</sub>, respectively, providing initial evidence that it was a doubly linked procyanidin dimer of the A-type. The integration of the <sup>1</sup>H-NMR spectrum showed the presence of 15 protons, all magnetically nonequivalent (excluding the protons of hydroxyl groups), confirming the above observation. A diagnostic feature of the <sup>1</sup>H-NMR spectrum of compound **9a** was the presence of an isolated AB system in the heterocyclic region [ $\delta$  5.05 and 4.60, H-3(C) and H-4(C),  $J_{3,4}=4.2$  Hz, respectively], characteristic of the C ring protons of an A-type proanthocyanidin [25]. This suggested the existence of a double linkage between the C- and D-rings (4 $\beta$ →8;2 $\beta$ →O→7) [25]. Insignificant coupling for  $J_{2,3(F)}$  established that the “lower” flavan-3-ol unit has a 2,3-*cis* relative configuration [19, 23, 24]. The aromatic region protons demonstrated a two-AMX spin system. This is in accordance with dihydroxylated B- and E-rings. Compound **9** was, also in comparison with the literature, thus determined to be epicatechin-(4 $\beta$ →8;2 $\beta$ →O→7)-epicatechin (procyanidin A<sub>2</sub>, Figure 3). In addition, a solid-state <sup>13</sup>C-NMR spectrum analysis of the EPB fraction indicated chemical shifts in the region consistent with C5 and C7 of the phloroglucinol A-ring involved in the double linkage. The chemical shift of the ketal carbon (C2) formed as a result of this additional bond observed at 105.66 ppm provided further support for A-type procyanidin [26].

Structure elucidation and assignment of proton resonances of the trimer epicatechin-(4 $\beta$ →8)-epicatechin-(4 $\beta$ →8)-epicatechin (**10**) were achieved by <sup>1</sup>H-NMR (<sup>1</sup>H-<sup>1</sup>H COSY) of its peracetate **10a**. The three proton resonances of the AMX-type rings at 6.65 to 7.30 (3x 3H) were assigned to the B-, E- and H-rings, by comparison with the spectra of procyanidin C<sub>1</sub> [27]. The signals due to the heterocyclic protons of the flavanyl units were subsequently identified by the long-range couplings in the <sup>1</sup>H-<sup>1</sup>H COSY spectra between the C-2 protons of the flavanyl units and the corresponding three

proton resonances of the B-rings. All heterocyclic protons showed a small coupling constant ( $J_{2,3} < 2$  Hz), confirming a 2,3-*cis* relative configuration. The high-amplitude negative Cotton effects in the 220-300 nm region of the CD spectrum indicated the configuration at both interflavan linkages to be 4*S*. ESI mass spectral ( $[M-H]^-$   $m/z$  865.5 and  $[M+Na]^+$   $m/z$  889.4) analysis and chemical degradation studies suggested that all three units were epicatechins. These results confirm the structure of **10** to be identical to that previously described from *Tilia* sp. (Tiliaceae) [27].

**Figure 3** – Procyanidins A<sub>2</sub> (**9**) and C<sub>1</sub> (**10**) from the seeds of *Paullinia cupana*.



The compounds *ent*-epicatechin (**4**) and the procyanidins B<sub>1</sub> (**5**), A<sub>2</sub> (**9**), and C<sub>1</sub> (**10**) are reported here as natural constituents of *guaraná* (genus *Paullinia*) for the first time.

### Biological activity

The results indicate that the antioxidant capacity was directly proportional to the content of polyphenols (Table 1). The semi-purified fraction (EPA) showed the highest content of total polyphenols, reflecting the antioxidant analysis, with a low IC<sub>50</sub> and a higher RAC in relation to the other extracts. Inverse relationships were observed for the semi-purified fraction (EPB). All the samples were significantly different from vitamin C in the relative antioxidant capacity assay (RAC) ( $P < 0.001$ ) and in the IC<sub>50</sub> ( $P < 0.05$  for EPA, and  $P < 0.001$  for all other samples); EPA also showed a significant difference in the EBPC (RAC,  $P < 0.01$ ; IC<sub>50</sub>,  $P < 0.001$ ). The results from the DPPH (IC<sub>50</sub>)

method for the substances epicatechin-(4 $\beta$ →8)-catechin (PB<sub>1</sub>), epigallocatechin, catechin, and gallic acid isolated from this and from another plant species by our research group showed values of 3.29, 8.56, 9.93, and 15.26  $\mu$ M, respectively. These data show that the larger the amount of phenolic hydroxyls, the more the free-radical scavenging. Additionally, the stereochemistry of catechin and gallic acid (2*R*,3*S*) in relation to epigallocatechin (2*R*,3*R*) may be responsible for the decrease in free-radical scavenging activity. This last condition demonstrates that trihydroxylation at the B-ring increases the power of scavenging DPPH radicals. Substances of the cinchonain Ia and Ib type, obtained from *Trichilia catigua* A. Juss., show that the phenylpropanoid unit significantly increases the antioxidant activity of the condensed tannins [28]. Thus, it is possible to establish correlations between the antioxidant capacity in plant extracts and the antioxidant activity of substances isolated from plant drugs containing tannins, and demonstrates the efficiency of these substances in capturing free radicals.

**Table 1.** Assessment of contents of total polyphenols (TP) and antioxidant capacity by the methods of the phosphomolybdenum complex (RAC) and DPPH-radical scavenging (IC<sub>50</sub>).

Sample	TP (%) $\pm$ s.d. (RSD%)	RAC $\pm$ s.d. (RSD%)	IC <sub>50</sub> ( $\mu$ g/ml) $\pm$ s.d. (RSD%)
AqE	36.47 $\pm$ 0.18 (0.51)	0.46 $\pm$ 0.01 (0.96)	9.59 $\pm$ 0.04 (0.41)
EBPC	55.95 $\pm$ 1.12 (2.01)	0.69 $\pm$ 0.03 (4.15)*	6.67 $\pm$ 0.05 (0.82)
EPA	65.80 $\pm$ 0.62 (0.93)	0.75 $\pm$ 0.01 (1.75)*	5.23 $\pm$ 0.08 (1.49)**
EPB	22.1 $\pm$ 0.37 (1.64)	0.36 $\pm$ 0.02 (5.20)	14.97 $\pm$ 0.15 (1.01)
Vitamin C		1.00	4.93 $\pm$ 0.05 (1.07)

RAC = relative antioxidant capacity; IC<sub>50</sub>= amount of antioxidant necessary to decrease the concentration of the DPPH radical by 50%; s.d.= standard deviation; RSD= relative standard deviation. ANOVA, \*P<0.01; \*\*P<0.05 in relation to vitamin C.

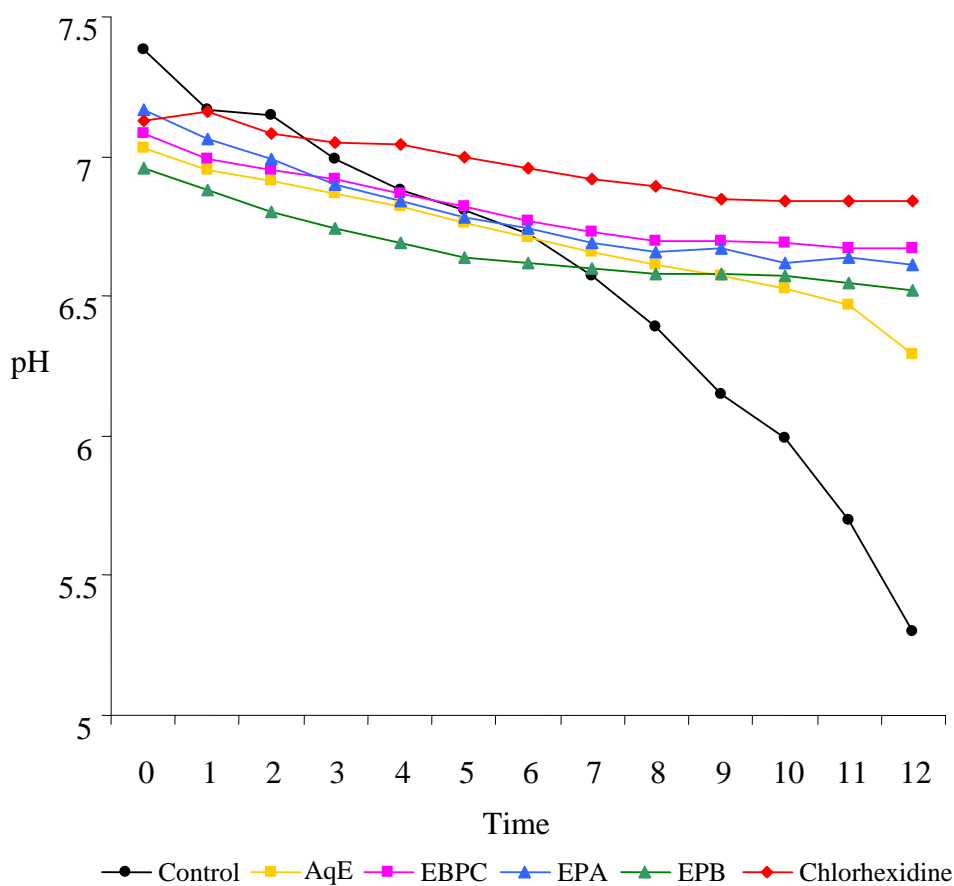
Previous results obtained by *in vivo* evaluation of the antibacterial potential against *Streptococcus mutans* suggested that extracts from *P. cupana* could be used to prevent the formation of dental bacterial plaque [10]. All the extracts evaluated inhibited the adherence of *S. mutans* on a smooth glass surface, and also reduced the production of acids, compared to a solution of chlorhexidine gluconate. In the test of adherence to glass, we used extracts with a tannin concentration of 750  $\mu$ g/mL (Table 2) and a solution of chlorhexidine gluconate at 1.2  $\mu$ g/mL. The EBPC gave the best result; even at a lower concentration, it showed the best action on the adherence of *S. mutans*, with 79.69% inhibition. The AqE extract, and semi-purified fractions (EPA and EPB) showed adherence inhibitions of 62.18%, 52.69%, and 69.95%, respectively. As these fractions did not affect the growth of *S. mutans*, as evaluated through determination of the minimum inhibitory concentration [29], with a MIC>5,000  $\mu$ g/mL, the extracts affected the adherent action of *S. mutans* rather than its growth. All the samples were efficient in reducing the production of acids, with an effect comparable to the chlorhexidine gluconate solution (Figure 4). The decrease of pH was significantly inhibited in the presence of AqE, EBPC, EPA, EPB, and the positive control (chlorhexidine), showing at the onset pH 7.0, 7.1, 7.2, 7.0, and 7.1 and after 12 h of incubation pH 6.3, 6.7, 6.6, 6.5, and 6.8, respectively. On the other hand,

untreated cells (negative control) showed pH 7.3 at the onset, and pH 5.3 after 12 h of incubation. This is significant, because to the extent that the process of acidification of the medium between the bacterial plaque and the outer surface of the tooth is decreased, the possibility of demineralization of this surface, an essential factor in the formation of dental caries, is also decreased.

**Table 2.** Total tannins content, concentration of sample evaluated, and inhibition of bacterial adherence on a smooth surface.

Sample	Total Tannins g (%)±sd (RSD%)	Concentration of the sample (mg/ml)	Adherence Inhibition (%)
AqE	16.16±0.44 (2.75)	4.64	62.18±2.09
EBPC	31.15±1.46 (4.68)	2.41	79.69±5.23
EPA	30.05±0.54 (1.80)	2.50	52.69±17.95
EPB	17.09±0.52 (3.03)	4.39	69.95±2.90
Chlorhexidine			82.88±4.28

**Figure 4** – Effect of the samples in reducing production of acids by *Streptococcus mutans*.



## Experimental Section

### General

The  $^1\text{H-NMR}$  spectra were obtained on a 300 MHz NMR Varian model Mercury plus BB spectrometer, using  $\text{CDCl}_3$  and TMS as references. Solid-state  $^{13}\text{C-NMR}$  spectroscopy was carried out on a Varian NMR spectrometer operating at a frequency of 75 MHz. The sample was packed into a 7 mm diameter silicon nitride rotor, retained with Kel-F end caps, and spun at 5 kHz. Each proton preparation pulse was 4.9  $\mu\text{s}$  at 33.9°, followed by 50 ms of data acquisition and recovery delay of 10 s. Transients from 5120 such sequences were averaged. The mass spectra (MS) were obtained on an ESI-MS Quattro LCZ (Micromass, Manchester, UK). The optical rotation measurements ( $[\alpha]_D^{20}$ ) were performed in methanol on a Perkin-Elmer 241 spectropolarimeter. Column chromatography (CC; Sephadex® LH-20) was carried out using ethanol-water in increasing volumetric proportions. Multi-layer counter-current chromatography (MLCCC): PC ITO Multi-layer Coil Separator-Extractor, Model #1; 800 rpm; eluent system: ethyl acetate-*n*-propanol-water (140:8:80; v/v); upper layer=mobile phase; flow: 1.0 mL/min. The CC and MLCCC were monitored by TLC (aluminum sheets: silica gel 60 F<sub>254</sub>; Merck®; 0.200 mm), under UV<sub>254</sub> light and with a solution of 1%  $\text{FeCl}_3$  in ethanol. DPPH (Fluka) and ammonium molybdate (Merck®) were used as the antioxidant solution.

### Plant Material

Seeds of *Paullinia cupana* H.B.K. var. *sorbilis* (Mart.) Ducke were acquired from Mr. José Augusto de Souza, in the region of Alta Floresta, Mato Grosso, Brazil. A dried specimen was deposited in the Herbarium of the Department of Biology of the State University of Maringá under number HUEM 9.065. It was identified by Prof. Dr. Cássia Mônica Sakuragui. The methylxanthine content was determined according to [30].

### Extraction and isolation of compounds

The seeds were ground in a hammer mill (<180  $\mu\text{m}$ ) (Tiger ASN-5). The aqueous extract (AqE) from the 5% (w/v) *guaraná* seeds used in the biological assays was prepared by Ultra-Turrax® (UTC115KT) processing for 15 min. The AqE was then concentrated and lyophilized.

The crude extract from the *guaraná* seeds (EBPC) (100 g) was prepared by Ultra-Turrax® (UTC-115KT) processing for 15 min, using acetone-water, giving a 15.76% yield. From the EBPC, two semi-purified fractions were obtained: EPB (29.11 g) and EPA (43.93 g) (Patent No. PI 0006638-9, Brazil) [31]. The EPA fraction was used for multi-layer counter-current chromatography (MLCCC) and column chromatography (CC), to isolate the compounds.

Compounds **2** (16.3 mg), **3** (15.1 mg), **4** (4.0 mg), **5** (10.5 mg), **6** (80.1 mg), **8** (4.5 mg), **9** (4.3 mg), and **10** (5.9 mg) were obtained directly by MLCCC [22]. Compounds **1** (40.6 mg) and **7** (10.2 mg) were obtained sequentially by CC in Sephadex® LH-20 [19, 22]. All compounds were obtained from the semi-purified EPA fraction.



*Total polyphenolics and tannins in EBPC, EPA, EPB, and AqE samples*

The total polyphenolics (TP) and tannins (TT) contents in the EBPC, EPA, EPB, and AqE samples were estimated by a colorimetric assay based on procedures described in [32], with slight modifications. Briefly, the EBPC (0.187 mg), EPA (0.082 mg), EPB (0.054 mg) or AqE (0.135 mg) was dissolved in water (250 mL; MS), respectively. An aliquot (5 mL) was diluted in water to 25 mL. A aliquot of the later solution (2 mL) was transferred to a 25-ml vial with Folin-Ciocalteu phenol reagent (1 mL) and water (10 mL), and made up to volume with a solution of 14.06 % sodium carbonate. After 15 min the absorbance was measured at 691 nm (TP). Water was used as the blank. For determination of the nonadsorbent polyphenols (NAP), MS (10 mL) was transferred with of Hide powder (100 mg) and vortexed for 60 min. Next, the solution was filtered, and an aliquot (5 mL) was diluted in water to 25 mL. An aliquot of this solution (2 mL) was transferred to a 25-ml vial with of the Folin-Ciocalteu phenol reagent (1 mL) and water (10 mL), and made up to volume with a 14.06 % sodium carbonate solution. After 15 min the absorbance was measured at 691 nm (NAP). Water was used as the blank. The percentages of total phenolics and tannins were determined (in triplicate) as follows:

$$TP(\%) = \frac{15625.Abs}{1000.m} \qquad NAP(\%) = \frac{15625.Abs}{1000.m} \qquad TT(\%) = TP - NAP$$

where TP = total polyphenolics (%); NAP = non-adsorbent phenolics (%); TT = total tannins (%); Abs = absorbance;  $m$  = mass (g) of EBPC, EPA, EPB, or AqE.

*Relative Antioxidant Capacity (RAC)*

An aliquot of sample solution (0.3 mL) containing a reducing species (in water) was combined in a test tube with reagent solution (3 mL, 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The test tubes were capped and incubated in a water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained reagent solution (1 mL) and the appropriate volume of the same solvent used for the sample, and was incubated under the same conditions as the rest of the samples. For samples of unknown composition, the water-soluble antioxidant capacity was expressed as equivalents of ascorbic acid. The antioxidant capacity of the extracts was expressed in relation to ascorbic acid (0.3 ml of a 2 mM solution) used as the standard, the antioxidant activity of which was considered as 1 (100%) [33].

*Determination of antioxidant capacity by the DPPH• (2,2-diphenyl-1-picrylhydrazyl) method*

The antioxidant capacity with the DPPH• radical (1 mM, 0.5 mL) was determined with the sample (4 mL) previously dissolved in methanol in concentrations of 2.0 to 20.0 µg/mL. The solutions were homogenized, and the reaction occurred in ambient temperature for 30 min, protected from light. A solution of DPPH• (0.5 mL) in methanol (4 mL) was used as a control, and another solution of BHT (2

mg) in methanol (4 mL) with DPPH• solution (0.5 mL) was used as the blank (as described in [34], with certain modifications). The absorbance values were measured at 517 nm and converted to a percentage of antioxidant activity or percentage of DPPH• consumed (AA%), using the following formula:

$$AA\% = 100 - [(Abs_{sample} - Abs_{blank}) \cdot 100] / Abs_{control}, \text{ where: } Abs = \text{absorbance}$$

#### Cover slip adherence test

The method of Hamada [35] was used, partially modified. *Streptococcus mutans* ATCC 25175 cultured for 24 h in BHI broth at 37 °C and in microaerophilia (10% CO<sub>2</sub>) were centrifuged for 20 min and resuspended with buffer (3.0 mL) adjusted by the McFarland scale (1.5x10<sup>8</sup> cells/mL). From this bacterial suspension, a sample (1.5 mL) was added to buffer (control, 1.5 mL) or to extract (1.5 mL). This was incubated at 37 °C in microaerophilia for 4 h, centrifuged, and resuspended with BHI + saccharose (3.0 mL). Serial dilutions were made in BHI medium: 10<sup>-1</sup>, 10<sup>-2</sup>. The 10<sup>-2</sup> dilution (500 µL) was placed on a 24-well plate and a cover slip added. The plate was incubated at 37 °C in microaerophilia for 2 h. Next, the suspension was removed from each well, and the cover slip was removed and washed in buffer, and placed on another 24-well plate. 10% sheep-blood agar in BHI (500 µL) was added on the cover slip, and the plate was incubated. The colony-forming units (CFU) were counted after 24 h of incubation at 37 °C in microaerophilia in triplicate. Viable colonies in dishes with 10% sheep-blood agar were also counted.

#### Determination of the minimum inhibitory concentration (MIC) by the plate-dilution method

The minimum inhibitory concentration was determined according to the NCCLS [29], using a sample of *Streptococcus mutans* ATCC 25175 kindly provided by the Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

#### Effect of the extract on the production of acids

*Streptococcus mutans* was seeded (1 mL in 100 mL red phenol broth containing 1% glucose and the extract). This was incubated at 37 °C in microaerophilia, and at regular intervals a sample (4 mL) of the culture was removed and its pH measured in a pH meter [36], with certain modifications.

*Caffeine, epicatechin and catechin* (**1**, **2**, and **3**). The <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 300 MHz) data and the ESI-MS are identical with those recently published in [11].

*ent-Epicatechin* (**4**). C<sub>15</sub>H<sub>14</sub>O<sub>6</sub>; ESI-MS [M+Na]<sup>+</sup> *m/z* 523.4; [M-H]<sup>+</sup> *m/z* 499.4; <sup>1</sup>H-NMR [CDCl<sub>3</sub>, δ (ppm), multiplicity and *J* (Hz)]: δ 2.88 [*dd*, H-4ax (C), *J*=18.0, 2.1], 2.98 [*dd*, H-4eq (C), *J*=17.7, 4.5], 5.12 [*s*, H-2 (C)], 5.39 [*m*, H-3 (C)], 6.57 [*d*, H-6 (A), *J*=2.1], 6.67 [*d*, H-8 (A), *J*=2.4], 7.20 [*d*, H-5' (B), *J*=8.4], 7.27 [*dd*, partially hidden by the CDCl<sub>3</sub>, H-6' (B), *J*=1.8], 7.36 [*d*, H-2' (B), *J*=2.1 Hz]; [α]<sub>D</sub><sup>20</sup> +6° (methanol; *c* 0.5).

*Epicatechin-(4 $\beta$ →8)-catechin* (Procyanidin B<sub>1</sub>) (**5**). C<sub>50</sub>H<sub>46</sub>O<sub>22</sub>; ESI-MS [M+Na<sup>+</sup>]<sup>+</sup> *m/z* 1021.5; [M-H<sup>+</sup>]<sup>-</sup> *m/z* 997.5; <sup>1</sup>H-NMR [CDCl<sub>3</sub>,  $\delta$  (ppm), multiplicity and *J* (Hz)]:  $\delta$  2.56 [*dd*, H-4ax (F), *J*=16.5, 9.0], 3.21 [*dd*, H-4eq (F), *J*=16.5, 6.6], 4.33 [*d*, H-2 (F), *J*=9.6], 4.42 [*d*, H-4 (C), *J*=2.1], 5.05 [*ddd*, H-3 (F), *J*=9.6, 9.3, 6.6], 5.15 [*m*, H-3 (C), *J*=1.8], 5.47 [*s*, H-2 (C)], 5.99 [*d*, H-6 (A), *J*=2.1], 6.29 [*d*, H-8 (A), *J*=2.1], 6.68 [*s*, H-6 (D)], 6.88 [*d*, H-2' (E), *J*=1.8], 6.94 [*dd*, H-6' (E), *J*=8.4, 2.1], 7.09 [*d*, H-5' (E), *J*=8.1], 7.16 [*d*, H-5' (B), *J*=8.7], 7.24 [*dd*, H-6' (B), *J*=8.7, 2.1], 7.29 [*d*, H-2' (B), *J*=1.8]; [ $\alpha$ ]<sub>D</sub><sup>20°</sup> +2° (methanol; *c* 1.0).

*Epicatechin-(4 $\beta$ →8)-epicatechin* (Procyanidin B<sub>2</sub>) (**6**). C<sub>50</sub>H<sub>46</sub>O<sub>22</sub>; ESI-MS [M+Na<sup>+</sup>]<sup>+</sup> *m/z* 1021.5; [M-H<sup>+</sup>]<sup>-</sup> *m/z* 997.5. The spectral data are identical with those recently published [11]; [ $\alpha$ ]<sub>D</sub><sup>20°</sup> -4.5° (methanol; *c* 2.0).

*Catechin-(4 $\alpha$ →8)-catechin* (Procyanidin B<sub>3</sub>) (**7**). C<sub>50</sub>H<sub>46</sub>O<sub>22</sub>; ESI-MS [M+Na<sup>+</sup>]<sup>+</sup> *m/z* 1021.6; [M-H<sup>+</sup>]<sup>-</sup> *m/z* 997.7; The spectral data are identical with those recently published [11]; [ $\alpha$ ]<sub>D</sub><sup>20°</sup> -31.5° (methanol; *c* 1.43).

*Catechin-(4 $\alpha$ →8)-epicatechin* (Procyanidin B<sub>4</sub>) (**8**). C<sub>50</sub>H<sub>46</sub>O<sub>22</sub>; ESI-MS [M+Na<sup>+</sup>]<sup>+</sup> *m/z* 1021.5; [M-H<sup>+</sup>]<sup>-</sup> *m/z* 997.6. The spectral data are identical with those recently published [11]; [ $\alpha$ ]<sub>D</sub><sup>20°</sup> -16.8° (methanol; *c* 1.43).

*Epicatechin-(4 $\beta$ →8;2 $\beta$ →O→7)-epicatechin* (Procyanidin A<sub>2</sub>) (**9**). C<sub>48</sub>H<sub>42</sub>O<sub>21</sub>; ESI-MS [M+Na<sup>+</sup>]<sup>+</sup> *m/z* 977.4; [M-H<sup>+</sup>]<sup>-</sup> *m/z* 953.5; <sup>1</sup>H-NMR [CDCl<sub>3</sub>,  $\delta$  (ppm), multiplicity and *J* (Hz)]:  $\delta$  2 x 2.88-2.90 [*m*, H-4 (F)], 4.60 [*d*, H-4 (C), *J*=4.2], 4.85 [*s*, H-2 (F)], 5.05 [*d*, H-3 (C), *J*=4.2], 5.35 [*m*, H-3 (F)], 6.38 [*d*, H-8 (A), *J*=2.1], 6.58 [*s*, H-6 (D)], 6.69 [*d*, H-6 (A), *J*=2.4], 2 x 7.21-7.24 [*m*, H-5' (B and E)], 7.38 [*m*, H-6' (E)], 7.41 [*d*, H-2' (B and E), *J*=2.1], 7.51 [*dd*, H-6' (B), *J*=8.7, 2.1]; [ $\alpha$ ]<sub>D</sub><sup>20°</sup> +5.3° (methanol; *c* 1.5).

*Epicatechin-(4 $\beta$ →8)-epicatechin-(4 $\beta$ →8)-epicatechin* (Procyanidin C<sub>1</sub>) (**10**). C<sub>45</sub>H<sub>38</sub>O<sub>18</sub>; ESI-MS [M+Na<sup>+</sup>]<sup>+</sup> *m/z* 889.4; [M-H<sup>+</sup>]<sup>-</sup> *m/z* 865.5; <sup>1</sup>H-NMR [CDCl<sub>3</sub>,  $\delta$  (ppm), multiplicity and *J* (Hz)]:  $\delta$  2 x 2.93-3.11 [*m*, H-4 (I)], 4.65 [*s*, H-4 (C)], 4.70 [*s*, H-4 (F)], 4.78 [*s*, H-2 (F)], 5.12 [*m*, H-3 (C)], 5.21 [*s*, H-2 (I)], 5.36 [*s*, H-2 (C)], 5.40 [*m*, H-3 (F)], 5.48 [*m*, H-3 (I)], 5.94 [*d*, H-6 (A), *J*=2.4], 6.26 [*d*, H-8 (A), *J*=2.4], 6.65 [*s*, H-6 (D)], 6.71 [*s*, H-6 (G)], 9 x 6.65-7.30 [*m*, H- (B, E, and H)]; [ $\alpha$ ]<sub>D</sub><sup>20°</sup> +5.81° (methanol; *c* 3.1).

## Acknowledgements

We thank CNPq, CAPES, the Programa de Pós-Graduação em Ciências Farmacêuticas, the Institut für Pharmazeutische Biologie und Phytochemie der Universität Münster for MLCCC, and Admir Arantes and Cláudio Roberto Novello for support of this work. The valuable observations of two anonymous reviewers contributed significantly to improvements in the manuscript.

## References

1. Otobone, F.J.; Sanches, A.C.; Nagae, R.L.; Martins, J.V.C.; Obici, S.; Mello, J.C.P.; Audi, E.A. Effect of Crude Extract and its Semi Purified Constituents from Guaraná Seeds [*Paullinia cupana* var. *sorbilis* (Mart.) Ducke] on Cognitive Performance in Morris Water Maze in Rats. *Braz. Arch. Biol. Technol.* **2005**, *48*, 723-728.
2. Otobone, F.J.; Sanches, A.C.; Nagae, R.L.; Martins, J.V.C.; Sela, V.L.; Mello, J.C.P.; Audi, E.A. Effect of lyophilized extracts from guaraná seeds [*Paullinia cupana* var. *sorbilis* (Mart.) Ducke] on behavioral profiles in rats. *Phytother. Res.* **2007**, doi: 10.1002/ptr2089.
3. Antonelli-Ushirobira, T.M.; Yamaguti, E.; Uhemura, L.M.; Mello, J.C.P. Controle de qualidade de amostras de *Paullinia cupana* H.B.K. var. *sorbilis* (Mart.) Ducke. *Acta Farm. Bonaerense* **2004**, *23*, 383-386.
4. Ushirobira, T.M.A.; Yamaguti, E.; Uemura, L.M.; Audi, E.A.; Mello, J.C.P. de. Avaliação físico-química de sementes de guaraná secas por diferentes métodos. *Rev. Bras. Farmacogn.* **2004**, *14*, 15-20.
5. Santos, S.C.; Mello, J.C.P. In *Farmacognosia: da Planta ao Medicamento*; Simões, C.M.O.; Schenkel, E.P.; Gosmann, G.; Mello, J.C.P.; Mentz, L.A.; Petrovick, P.R.; Eds.; UFSC: Florianópolis, **2004**; 5<sup>th</sup> ed., Chapter 24, pp. 517-544.
6. Mattei, R.; Dias, R.F.; Espínola, E.B.; Carlini, E.A.; Barros, S.B.M. Guaraná (*Paullinia cupana*): toxic behavioral effects in laboratory animals and antioxidant activity *in vitro*. *J. Ethnopharmacol.* **1998**, *60*, 111-116.
7. Basile, A.; Ferrara, L.; Del Pezzo, M.; Mele, G.; Sorbo, S.; Bassi, P.; Montesano, D. Antibacterial and antioxidant activities of ethanol extract from *Paullinia cupana* Mart. *J. Ethnopharmacol.* **2005**, *102*, 32-36.
8. Nakahara, K.; Kawabata, S.; Ono, H.; Ogura, K.; Tanaka, T.; Ooshima, T.; Hamada, S. Inhibitory effect of oolong tea polyphenols on glucosyltransferases of mutans streptococci. *Appl. Environ. Microbiol.* **1993**, *59*, 968-973.
9. Scholz, E. Pflanzliche Gerbstoffe: Pharmakologie und Toxikologie. *Deutsch. Apoth. Ztg.* **1994**, *134*, 3167-3179.
10. Barbosa, G.D.A.F.; Mello, J.C.P. Avaliação clínica do extrato de guaraná no controle da placa bacteriana dentária. *Rev. Paulista. Odontol.* **2004**, *4*, 28-30.
11. Antonelli-Ushirobira, T.M.; Yamaguti, E.; Uemura, L.M.; Nakamura, C.V.; Dias Filho, B.P.; Mello, J.C.P. Chemical and microbiological study of extract from seeds of guaraná (*Paullinia cupana* var. *sorbilis*). *Lat. Am. J. Pharm.* **2007**, *26*, 5-9.
12. Weinges, K.; Göritz, K.; Nader, F.; Perner, J. Zur Kenntnis der Proanthocyanidine, XI Konfigurationsermittlung von C<sub>30</sub>H<sub>26</sub>O<sub>12</sub> – Procyanidine und Strukturaufklärung eines neuen Procyanidins. *Liebigs Ann. Chem.* **1968**, *715*, 164-171.
13. Weinges, K.; Bähr, W.; Ebert, W.; Göritz, K.; Marx, H.D. Konstitution, Entstehung und Bedeutung der Flavonoid-Gerbstoffe. *Fortschr. Chem. Org. Naturst.* **1969**, *27*, 158-260.
14. Kofink, M.; Galensa, R. Enantiomerentrennung von Catechinen mittels Kapillarelektrophorese. *Lebensmittelchem.* **2005**, *59*, 111-112.

15. Foo, L.Y. Polymeric proanthocyanidins of *Photinia glabrescens*, modification of molecular weight and nature of products from hydrogenolysis. *Phytochemistry* **1982**, *21*, 1741-1746.
16. Romeyer, F.M.; Macheix, J.J.; Sapis, J.C. Changes and importance of oligomeric procyanidins during maturation of grape seeds. *Phytochemistry* **1985**, *25*, 219-221.
17. Lu, Y.; Foo, L.Y. The polyphenol constituents of grape pomace. *Food Chem.* **1999**, *65*, 1-8.
18. Khallouki, F.; Haubner, R.; Hull, W.E.; Erben, G.; Spiegelhalder, B.; Bartsch, H.; Owen, R.W. Isolation, purification and identification of ellagic acid derivatives, catechins, and procyanidins from the root bark of *Anisophyllea dichostyla* R. Br. *Food Chem. Toxicol.* **2007**, *45*, 472-485.
19. Petereit, F.; Kolodziej, H.; Nahrstedt, A. Flavan-3-ols and proanthocyanidins from *Cistus incanus*. *Phytochemistry* **1991**, *30*, 981-985.
20. Hemingway, R.W.; Foo, L.Y.; Porter, L.J. Linkage isomerism in trimeric and polymeric 2,3-*cis*-procyanidins. *J. Chem. Soc., Perkin Trans. 1* **1982**, 1209-1216.
21. Kolodziej, H. Tannins of medicinal plants: application of <sup>1</sup>H NMR parameters to the analysis of procyanidins. *Farmaceut. Tijdschr. Belg.* **1989**, *66e*, 44.
22. Mello, J.P.; Petereit, F.; Nahrstedt, A. Flavan-3-ols and prodelfphinidins from *Stryphnodendron adstringens*. *Phytochemistry* **1996**, *41*, 807-813.
23. Kolodziej, H. In *Plant Polyphenols: Synthesis, Properties, Significance*; Hemingway, R.W.; Laks, P.E.; Eds.; Plenum Press: New York, **1992**; pp. 295-320.
24. Hör, M.; Rimpler, H.; Heinrich, M. Inhibition of intestinal chloride secretion by proanthocyanidins from *Guazuma ulmifolia*. *Planta Med.* **1995**, *61*, 208-212.
25. Jacques, D.; Haslam, E.; Bedford, G.R.; Greatbanks, D. Plant proanthocyanidins. Part II. Proanthocyanidin-A2 and its derivatives. *J. Chem. Soc., Perkin Trans. 1* **1974**, 2663-2671.
26. Foo, L.Y.; Lu, Y.; Howell, A.B.; Vorsa, N. The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli* in vitro. *Phytochemistry* **2000**, *54*, 173-181.
27. Ueffing, I. Untersuchung von Procyanidinen in *Tilia spec.*-ein Beitrag zur qualitativen und quantitativen HPLC-Analytik von Flavanolen. PhD. Thesis, Münster, Germany, **1988**.
28. Resende, F.O. *Trichilia catigua*: Avaliação farmacognóstica, fitoquímica e biológica in vitro. **2007**; M.Sc. Thesis, Maringá, Brazil. 164p
29. National Committee For Clinical Laboratory Standards. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; NCCLS Approved Standard M7-A5*; Wayne, PA, **2000**; Vol. 20, n. 2.
30. Andrade, L.; Schenkel, E.P.; Bergold, A.M. Estudo da metodologia de análise de cafeína em sementes de guaraná (*Paullinia cupana*). *Rev. Bras. Farmacogn.* **1999**, *80*, 7-9.
31. Patent No. PI 0006638-9, *Efeito antidepressivo do extrato da droga vegetal guaraná (Paullinia cupana var. sorbilis (Martius) Ducke)*, Brazil, <http://www.inpi.gov.br>. Accessed at 31 July 2007.
32. Glasl, H. Zur Photometrie in der Drogenstandardisierung. 3. Gehaltsbestimmung von Gerbstoffdrogen. *Deutsch. Apoth. Ztg.* **1983**, *123*, 1979-1983.
33. Prieto, P.; Pineda, M.; Aguilar, M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal. Biochem.* **1999**, *269*, 337-341.

34. Amarowicz, R.; Pegg, R.B.; Rahimi-Moghaddam, P.; Barl, B.; Weil, J.A. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem.* **2004**, *84*, 551-562.
35. Hamada, S.; Torii, M.; Kotani, S.; Tsuchitani, Y. Adherence of *Streptococcus sanguis* clinical isolates to smooth surfaces and interaction of the isolates with *Streptococcus mutans* glucosyltransferase. *Infect. Immun.* **1981**, *32*, 364-372.
36. Ooshima, T.; Osaka, Y.; Sasaki, H.; Osawa, K.; Yasuda, H.; Matsumura, M.; Sobue, S.; Matsumoto, M. Caries inhibitory activity of cacao bean husk extract in in-vitro and animal experiments. *Arch. Oral Biol.* **2000**, *45*, 639-645.

*Sample Availability:* Available from the authors.

© 2006 by MDPI (<http://www.mdpi.org>). Reproduction is permitted for noncommercial purposes.