

## Biological Activity and Quality Control of Extract and Stem Bark From *Stryphnodendron adstringens*

Elizabeth Aparecida AUDI<sup>1</sup>, Cleyton Eduardo MENDES DE TOLEDO<sup>1</sup>,  
Fernando SOLERA DOS SANTOS<sup>1</sup>, Patrícia Roberta BELLANDA<sup>1</sup>, Wilson ALVES-DO-PRADO<sup>1</sup>,  
Tânia UEDA-NAKAMURA<sup>2</sup>, Celso Vataru NAKAMURA<sup>2</sup>, Cássia Mônica SAKURAGUI<sup>3</sup>,  
Ciomar Aparecida BERSANI-AMADO<sup>1</sup>, João Carlos PALAZZO DE MELLO<sup>1\*</sup>

<sup>1</sup> *Department of Pharmacy and Pharmacology, University of Maringá - UEM,  
Av. Colombo, 5790; BR-87020-900, Maringá, PR, Brazil*

<sup>2</sup> *Laboratory of Microbiology, Department of Clinical Analyses,  
University of Maringá - UEM, Maringá, PR, Brazil*

<sup>3</sup> *Herbarium, Department of Biology, University of Maringá - UEM, Maringá, PR, Brazil*

**SUMMARY.** The antibacterial and hypotensive activities of an acetone:water and semipurified extracts from the stem bark of *Stryphnodendron adstringens* were evaluated. Both the crude and semipurified extracts showed activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. It wasn't possible to confirm the hypotensive activity. Quality control was determined, using the vegetable drug for two years, by means of pharmacopoeial and chromatographic methods.

**RESUMEN.** "Actividad Biológica y Control de Calidad de Extracto del Tallo de *Stryphnodendron adstringens*". Fueron evaluadas las actividades antibacteriana e hipotensora del extracto acetona:agua y de extractos semipurificados del tallo de *Stryphnodendron adstringens*. El extracto crudo y los extractos semipurificados mostraron actividad contra *Pseudomonas aeruginosa* y *Staphylococcus aureus*. Sin embargo no fue posible confirmar la actividad hipotensora de los extractos de *Stryphnodendron adstringens*. El control de calidad fue determinado con la droga vegetal colectada por dos años, a través de ensayos farmacopeicos y cromatográficos.

### INTRODUCTION

The stem bark of *Stryphnodendron adstringens* (Mart.) Coville, Leguminosae, known as "barbatimão", is used by the native population in the central savanna region of Brazil, as a remedy for several diseases and particularly in cases of skin ulcerations. Lima *et al.*<sup>1</sup> and Audi *et al.*<sup>2</sup> tested fractions of the crude extract in experimental laboratory assays, for anti-inflammatory and anti-ulcer activity, respectively. Toxicological studies were carried out by Rebecca *et al.*<sup>3</sup> with the crude extract of *Stryphnodendron adstringens*. Mello *et al.*<sup>4-6</sup> isolated and identified several flavan-3-ols, prodelphinidins and pro-robinetinidins from an ethyl-acetate extract of the stem bark. The purpose of the present investigation was to determine the existence of other activities, such as hypotensive and antibacterial activity, in addition to developing standards for quality control of the plant materi-

al, in different lyophilized fractions of the stem bark of *S. adstringens*.

### MATERIALS AND METHODS

#### **General experimental procedures**

Analytical HPLC was carried out using a Gilson Model 321, automatic degassing unit type 184 at a temperature of 30 °C on a LiChrospher<sup>®</sup> 100 RP-18 (250 mm x 4 mm; 10 µm) column. The mobile phase (methanol: water = 10:100) was pumped at a flow rate of 1.0 ml per min. The water was acidified with 5% (v/v) HOAc for 30 min. UV detection at 278 and 300 nm was done using a Gilson UV/VIS 156 detector. TLC was performed on pre-coated Silica-gel 60 F<sub>254</sub> (0.25 mm, Merck).

#### *Plant material*

*Stryphnodendron adstringens* (Mart.) Coville (Leguminosae) was collected at São Jerônimo da

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**PALABRAS CLAVE:** Actividad antibacteriana; Actividad hipotensiva; Barbatimão; Control de calidad; *Stryphnodendron adstringens*.

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

Serra, state of Paraná, Brazil (23°43'7,8" S; 50°45'23,5" W; 926 m altitude; GPS Garmin - Legend version 2.24), from 1996 to 1998, and identified by Dr. Cássia Mônica Sakuragui. A voucher specimen (HUM-3800) is deposited in the Herbarium of the Biology Department (UEM).

#### *Preparation of the extracts*

The plant raw material (stem bark and leaf) was dried at room temperature away from direct light, and ground in a cutter mill. For the biological assay, the air-dried stem bark (2900 g) was extracted in Ultra-Turrax® for 20 min with Me<sub>2</sub>CO-H<sub>2</sub>O (7:3; 29 l). The extract was evaporated *in vacuo* and lyophilised (**F1**; 1142 g). The residue was suspended in H<sub>2</sub>O (11.5 l) and then shaken with EtOAc (33 l) to yield H<sub>2</sub>O (**F2**) and EtOAc (**F3**) layers containing dark-brown solids. The n-BuOH fraction (**F4**) was obtained by the same method, and the remaining H<sub>2</sub>O phase (**F5**) yielded a dark-brown solid. The EtOAc fractions (**F3**), from plants collected in different seasons of the year, was chromatographed using a bidimensional TLC on a) EtOAc-HCO<sub>2</sub>H-H<sub>2</sub>O (18:1:1; v/v) and b) EtOAc-Toluene-HCO<sub>2</sub>H-H<sub>2</sub>O (16:2:1:1; v/v). The reference substances, gallicocatechin (1), epigallocatechin (2), 4'-O-methylgallicocatechin (3) and 4'-O-methylgallicocatechin-(4 $\alpha$ →8)-4'-O-methylgallicocatechin (4), were visualised as brown and blue spots by applying 1% FeCl<sub>3</sub> in EtOH.

#### *Characterisation of the vegetable drug*

The drug raw material (stem bark and leaves collected in spring, winter, autumn and summer) was evaluated by the following tests: a) Loss on drying <sup>7</sup>; b) Saponin test through foam formation assay <sup>8</sup>; c) Determination of extractives <sup>7</sup>; d) Total flavonoids content, calculated as quercetin <sup>7</sup>; e) Total tannins content <sup>9</sup>; f) Determination of total ash <sup>10</sup>; g) Determination of acid-insoluble ash <sup>10</sup>; h) Granulometric analysis by sieving <sup>7</sup> and i) Determination of moisture <sup>10</sup>. To evaluate the dry residue from the stem bark and leaves, a different extractor solvents-mixture was used <sup>7</sup>.

#### *Antibacterial activity assay*

Bacteria strains from the American Type Culture Collection (ATCC; Rockville, MD, USA) were used: *Staphylococcus aureus* (25923), *Escherichia coli* (25922) and *Pseudomonas aeruginosa* (15442). These bacteria were grown in Mueller-Hinton broth (Difco) at 37 °C and main-

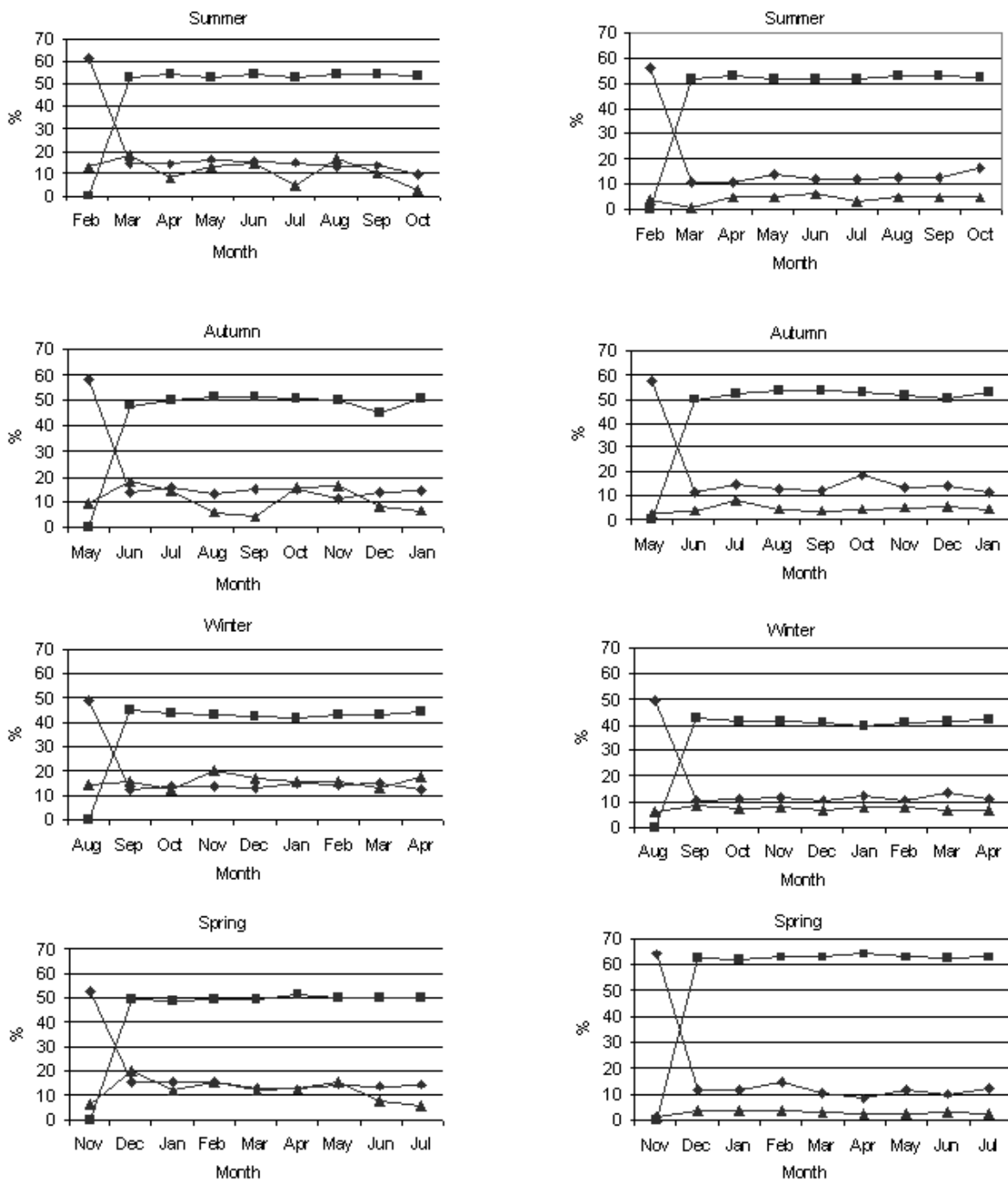
tained in nutrient agar (Difco) at 4 °C. The crude extract (**F1**) and fractions (**F2**, **F3**, **F4**, and **F5**) of *Stryphnodendron adstringens* were tested for antibacterial activity using the diffusion technique on Mueller-Hinton agar <sup>11</sup>.

#### *Hypotensive evaluation*

Dogs of both sexes were anaesthetised with sodium thiopental (5 mg/kg), and the femoral artery and vein were catheterised. The **F1**, **F2**, **F3** and **F5** fractions were injected into the femoral vein. The femoral artery was connected to a manometer and the variations in blood pressure were recorded on a Palmer chemograph smoke drum. After the stable blood pressure was determined, the lowest dose of the **F1** fraction which effected a reduction in blood pressure was determined and was used as the first dose. The same dose levels determined for the **F1** were used for the other fractions. Doses were prepared to a volume of 0.05 ml and injected cumulatively into the vein. Administration of the different fractions was followed by 0.05 ml of saline (dead space of catheter = 0.05 ml). The blood pressure after each dose was calculated as a percentage of the control (taken as 100%). Statistical significances were determined by Student's t-test at p<0.05 <sup>12</sup>. The animal experimentation was conducted according World Health Organization and was approved by institutional Committee of Ethical and Experimentation Animals.

#### **RESULTS AND DISCUSSION**

Several factors are responsible for the quality of a vegetable drug. Pharmacopeial data for *Stryphnodendron adstringens* used as a vegetable drug are not yet available. Determination of moisture and loss on drying provides an indication of the water content. These data can be used as one parameter to estimate the efficiency of drying during the operation. In this case the analyses were made from the stem bark and leaves of plants collected in each season during the course of a year. The content of tannins in the stem bark was about twice or more that of the leaves (Fig. 1). The pharmacological data are not yet clear, but the ethyl acetate fraction from *Stryphnodendron adstringens* <sup>1</sup> indicated the importance of the tannins as the responsible class of substances in it. Therefore this fraction serves as an adequate quantitative marker for the vegetable drug and the semi-purified extracts.



**Figure 1.** Loss on drying (■), content of moisture (◆) and content of tannins (▲) from the stem barks (left) and leaves (right) of “barbatimão”.

Total flavonoids content was also determined, as described in the German Pharmacopoeia<sup>13</sup>, which was higher in the leaves than in the stem bark. Nevertheless, this value for flavonoids was much lower than the content of tannins. During autumn, the leaves contained about  $0.84 \pm 0.64\%$ , while the stem bark contained about  $0.031 \pm 0.09\%$ . The concentration

of flavonoids, calculated as quercetin, was highest in this season. There were large differences between leaves and stem bark in total flavonoids and total tannins content. This may suggest a preferential metabolic route for tannins.

The determinations of extractives from the stem bark and leaves of *Stryphnodendron ad-*

Season	Stem Bark ( $\bar{x} \pm SD$ %)	Leaves ( $\bar{x} \pm SD$ %)
Autumn	46.56 $\pm$ 8.07	25.51 $\pm$ 8.38
Winter	46.64 $\pm$ 20.14	35.04 $\pm$ 24.94
Spring	38.03 $\pm$ 7.13	17.16 $\pm$ 4.30
Summer	44.41 $\pm$ 1.51	25.53 $\pm$ 3.90

**Table 1.** Determinations of extractives from the stem bark and leaves in each season of the year (n= 3).

*stringens* are shown in Table 1. These determinations can be considered as a characteristic of the water-extractable substances. It is important for control of the processing of the raw material, and can be used as a quantitative determination.

The correlation between the extractive and the total tannins content for the stem bark and leaves can be observed for the stem bark as well as for the leaves. For this purpose the statistical correlations with the total measure obtained from the stem bark and leaves are realized (linear regression).

Another method, traditional in all pharmacopeias, to characterise raw material is the determination of total ash and acid-insoluble ash. The results for the stem bark were a maximum of 1.6% for total ash, and a maximum of 1% for acid-insoluble ash; the values for leaves were 2.30% and 0.065%, respectively. The stem bark thus contained more organic as well as inorganic substances than did the leaves.

Some saponins have been isolated from *S. coriaceum*<sup>14,15</sup>, but none has yet been isolated from, much less investigated in *S. adstringens*. The foam-formation assay test for saponins gave an index of 125, which indicated that saponins were present in low concentrations in this plant material, if compared with *Pfaffia glomerata* (342) or *Hebanthe paniculata* (500)<sup>16</sup>.

Granulometric analysis is an important datum for the extractable substances, in conjunction with the extract method. For characterisation of the milled drug, the retention and passage curves were determined, which allowed us to calculate the mean diameter ( $d_{50}$ ), and to infer the level of granulometric homogeneity from the slope of the curve. The milled drug of *S. adstringens* used in this study showed a  $d_{50}$  of 0.440 mm, suggesting that the granulometric classes were distributed homogeneously. Studies to determine the existence of positive or negative influences on the  $d_{50}$  are possible through factorial design-analysis<sup>7,17</sup>.

However, Mello *et al.*<sup>4-6</sup> found several con-

densed tannins, which can be used as marker substances for the chemical, chromatographic and biological control. Like this, a comparative TLC employing a bidimensional solvent system with EtOAc fraction was carried out. The best possibility to identify the extract and the drug material was with the help of the reference substances [gallo catechin (1), epigallo catechin (2), 4'-O-methylgallo catechin (3) and 4'-O-methylgallo catechin-(4 $\alpha$ →8)-4'-O-methylgallo catechin (4)]. Monomeric condensed tannins such as gallo catechin (1) ( $R_{f1}$ = 0.78;  $R_{f2}$ = 0.65) and epigallo catechin (2) ( $R_{f1}$ = 0.70;  $R_{f2}$ = 0.58) are found in many natural sources. Both substances are visualised as a blue color after spraying with  $FeCl_3$ . However, the EtOAc fraction includes a particular natural compound, 4'-O-methylgallo catechin (3) ( $R_{f1}$ = 0.86;  $R_{f2}$ = 0.81), which is visualised as a blue-brown color. This compound has been isolated only from this plant and from *Panda oleosa* (Pandaceae)<sup>4,18</sup>. The dimeric compound, 4'-O-methylgallo catechin-(4 $\alpha$ →8)-4'-O-methylgallo catechin, was not used for TLC analyse. The HPLC method was also employed, and all reference substances at the EtOAc fraction had as retention time ( $t_R$ ; min): (1)= 3.95, (2)= 7.06, (3)= 7.27 and (4)= 6.78. HPLC was also shown to be a possibility for use in quality control of the drug raw material from *Stryphnodendron adstringens*.

The methods employed proved to be suitable for quality control of the drug and of its extract. They are initial steps in attaining complete quality control and in developing the required technological data to produce a phytomedicine. However, additional procedures will be necessary to fully evaluate the drug.

There has been increasing interest in antimicrobial agents from medicinal plants commonly used in folk medicine. Many studies using plant extracts with antibacterial, antiprotozoal, antifungal, insecticidal or antiviral activities<sup>19-23</sup> have been carried out in recent years. In the present study, antibacterial activity of the crude extract and fractions **F2**, **F3**, **F4** and **F5** from *Stryphnodendron adstringens* was demonstrated using diffusion techniques on solid media.

The antibacterial activity of the crude extract from *Stryphnodendron adstringens* against the microorganisms tested is shown in Table 2. *Pseudomonas aeruginosa* and *Staphylococcus aureus* showed inhibition zones ranging from 8 to 11 mm, at a concentration of 50  $\mu$ g. *Escherichia coli* was considered resistant, since no inhibition zone was observed.

Microorganism	Concentrations ( $\mu\text{g}$ )					
	500	250	50	64 <sup>a</sup>	16 <sup>b</sup>	64 <sup>c</sup>
	Inhibition zone (mm)					
<i>Staphylococcus aureus</i>	22	20	11	25	nr	nr
<i>Pseudomonas aeruginosa</i>	15	13	8	nr	15	nr
<i>Escherichia coli</i>	0	0	0	nr	nr	19

**Table 2.** Antibacterial activity of the crude extract of “barbatimão” and reference drugs, as determined by the diffusion technique on solid media (stainless steel cylinders).

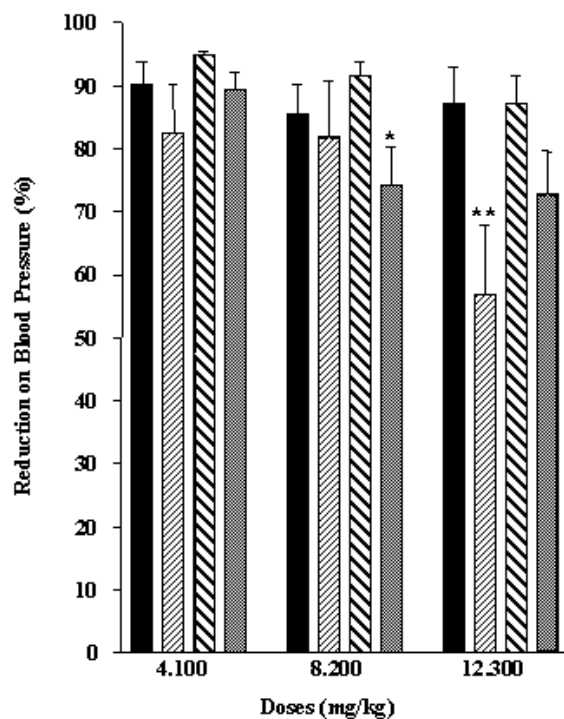
<sup>a</sup> Ceftriaxone; <sup>b</sup> Gentamicin; <sup>c</sup> Tetracyclin; nr: not realized.

Microorganism	Fractions			
	F2	F3	F4	F5
	Inhibition zone (mm)			
<i>Staphylococcus aureus</i>	22	23	22	20
<i>Pseudomonas aeruginosa</i>	15	17	17	15

**Table 3.** Antibacterial activity of different fractions extracted from “barbatimão” at a concentration of 50  $\mu\text{g}$ .

Fractions **F2**, **F3**, **F4** and **F5** were tested against *S. aureus* and *P. aeruginosa* (Table 3). All the fractions showed very similar antibacterial activity. These results are of considerable interest, since this plant may be a new source of antimicrobial compounds that could be used as an alternative chemotherapy against these bacteria.

The blood-pressure study was performed with dogs having normal arterial blood pressure. A dose of 8.2 mg/kg was the lowest dose of **F1** which was effective in reducing blood pressure. Fractions **F2**, **F3** and **F5** had similar effects, but at doses of 4.1, 8.2 and 12.3 mg/kg, respectively. The reduction in blood pressure induced by **F2** (12.3 mg/kg) was similar to **F1** and **F3** (Figure 2). The effects of **F5** were similar to **F3**, but the reduction in blood pressure induced by **F5** (8.2 mg/kg) was greater than **F3** (Figure 2). Thus, all the fractions studied were able to reduce blood pressure in dogs; however, this effect depended both on the doses and the fractions administered. These data suggest that popular use of *Stryphnodendron adstringens* is inadequate for treatment of hypotension. On the other hand, it is possible to verify expressive reduction on blood pressure induced by different fractions. However, caution it is necessary for utilization of such agents on hypertension status, as the mechanism of action was not investigated.



**Figure 2.** Percentage of reduction in blood pressure induced by different fractions of *Stryphnodendron adstringens* (4.1, 8.2 and 12.3 mg/kg) in dogs. On the ordinate, the blood pressure is expressed as a percentage of the control (taken as 100%). The height of the columns represents the mean ( $\pm$  SEM) of 20 experiments.

\* Statistical differences between **F3** and **F5**.

\*\* Statistical differences between **F2**, **F1** and **F3**.

The crude (**F1**) and EtOAc (**F3**) extracts show promise for further investigation. Isolation of the responsible compounds in the **F3** fraction or a subfraction of it, and evaluation of its antimicrobial activity are presently being carried out.

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