Acute and chronic toxicity and antimicrobial activity of the extract of *Stryphnodendron adstringens* (Mart.) Coville

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This study evaluated the antimicrobial activity and acute or chronic toxicity of the extract of *Stryphnodendron adstringens*. The stem bark dry extract was obtained by static maceration with ethanol. Quantification of tannins was performed by the Folin-Denis method, which indicated a total tannin content of 32.7%. The antimicrobial activity of the dry extract of *S. adstringens* was evaluated by agar-based disk diffusion assay with *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) at concentrations of 200, 400 and 600μL/mL. The results indicated that 600μL/mL inhibited microbial growth, i.e. had antimicrobial activity against these species. Acute and chronic toxic effects of *S. adstringens* were evaluated in Wistar rats treated with 200, 400, 600 and 800mg/kg of extract, administrated by gavage. Liver degeneration was observed in the group of rats receiving 800mg/kg in chronic exposure, which may indicate some degree of toxicity at this concentration. However, no systemic toxicity was observed at lower doses. Considering the broad use of *S. adstringens* as a phytotherapeutic agent for various human and animal diseases and the liver toxicity observed at high concentrations, attention should be paid to the possible adverse effect of using the extract from this plant at high concentration.

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INTRODUCTION

Tannins are water-soluble polyphenols that are present in many plants. There is a series of previous studies describing the biological action of tannins including their pharmacological, and toxic activities. The dosage and type of tannins determine these effects (Macáková et al. 2014).

*Stryphnodendron adstringens*, popularly known as barbatimão, is a medicinal plant abundant in central Brazil, which has been traditionally used as a popular herbal medicine (Melo et al. 2007), which is endemic to the savannah, and its bark has a high tannin content (25-37%). Macáková et al. (2014) and Okuda & Ito (2011) described a wide pharmacological spectrum of activity of polyphenols including hydrolysable tannins. However, Ferreira et al. (2009) and Aguilar-Filho (2013) reported intoxication of ruminants with plants that have tannins.

Local populations in the savannah regions of Brazil use infusion or tea of leaves or stem bark from *S. adstringens* as astringent, antimicrobial, homeostatic agent, antidiarrheal, antihypertensive or blood clotting agent. In addition, it is used for treating leucorrhoea, hemorrhoids, hemorrhages, and inflammation. Various studies have suggested the use of infusions and decoctions of this plant in Brazil for the treatment of animal diseases (Confessor et al. 2009, Viú & Viú 2011, Monteiro et al. 2011).

Several in vitro and in vivo studies on the activity of *Strynodendrum* sp. tannins showed that the crude and purified extracts have anti-inflammatory, antiercerogenic, antiprotozoal, antimemolic, antiviral, and antifungal activities (Martins et al. 2003, Silva et al. 2009, Cans & Demo 2011, Moura 2011). Its use in the clinical treatment of bacterial and parasitic diseases and for skin lesions in animals have also been reported (Confessor et al. 2009, Monteiro et al. 2011, Viú & Viú 2011).

Studies on the acute and chronic toxicity of *Strynodendrum* sp. extracts showed variable results depending on the method and extract concentration studied. Some researchers have evaluated the acute toxicity of extract of stem bark from *S. adstringens* orally ingested (Lima et al. 1998, Audi et al. 1999, Rebecca et al. 2002). They demonstrated that LD₅₀ ranged from 400 to 2699mg/kg. An initial preclinical toxicological assay conducted by Almeida et al. (2010) demonstrated that *S. adstringens* leaf extract was most toxic among the tested (leaf hydroalcoholic extracts of *Lippia sidoides, Myrcrodruon urundeuva* and *Caryocar brasiliense*), however, limited studies have been conducted to verify its toxic potential.

The National Policy for Medicinal Plants and Herbal Medicines (PNPMF), and the Brazilian National Program for Medicinal Plants and Phytotherapics published the Brazilian National List of Medicinal Plants of Interest to the SUS (REINSUS) in January 2009. This list contains medicinal plants that exhibit potential for generating products of interest to Brazilian Unified Health System (SUS), including *S. adstringens* (Brasil 2006). This study aimed to evaluate the antimicrobial activity and acute and chronic toxicity of hydroalcoholic extracts of stem bark from *S. adstringens* from the northern region of Minas Gerais, Brazil.

MATERIALS AND METHODS

Obtaining and preparing the *Stryphnodendron adstringens* extract. *S. adstringens* stem bark was harvested at Bela Vista farm, in Botumirim, State of Minas Gerais, Brazil (16° 57.847’ S, 43° 04.308’ W, elevation: 890m). The plant was identified by Rubens Teixeira de Queiroz at the Federal University of Paraíba, Brazil. To obtain the extract, stem bark of *S. adstringens* was dried in a forced air circulation chamber under an average temperature of 50°C, followed by daily weighing until the material reached a constant weight. The dry *S. adstringens* bark was ground using a Willey knife mill in a continuous system. The ground material was subjected to extraction by static soaking in cereal ethanol for eight days (bark:ethanol ratio of 1:2). The mixture was then filtered, and the solvent was evaporated from the extract in an air-circulated oven at 45°C until completely dry. The dried *S. adstringens* extract obtained was stored in an amber vial away from moisture and light.

Phenols and tannins content. Initially, 0.75g of dry extract of *S. adstringens* was dissolved in 150ml of distilled water to obtain a solution 1 which was used to determine total phenolic by the Folin-Denis method using tannic acid (0.1, 0.5, 1.0, 2.5, and 3.75mg/ml) as the standard for the calibration curve (Pansera et al. 2003). The system was maintained at agitation for 30 minutes at 90°C. Next, the system was cooled, the content was transferred to a volumetric flask and the volume was completed to 250ml with distilled water. The flask was maintained at rest for 30 minutes, followed by filtration, and the first 50ml of the filtrate was discarded. The Folin-Denis method comprised addition of the solution 1 which was used to determine total tannic acid by the Folin-Denis method using tannic acid (0.1, 0.5, 1.0, 2.5, and 3.75mg/ml) as the standard for the calibration curve (Pansera et al. 2003). The solution 1 also was used to calculate the content of tannin. This calculation was carried out using a reference solution containing Folin-Denis reagent, pyrogallol and sodium carbonate. The absorbance was measured at wavelength of 715nm. The parameters was used in the expressions below Samples were analyzed using a spectrophotometer 3 minutes after adding the last reagent. The analyses were performed in triplicate, and the tannin content was expressed in percentage (w/w) according to the equation proposed by Dóres & Casali (2007):

\[
A\% = \frac{A_3 \times 10}{c} \\
TT = \frac{FD \times A_1}{(m-p) \times A^{1\%}}
\]

Where: \( A\% \) = specific absorbance of the reference solution, \( A_3 \) = measured absorbance of the reference substance, \( c \) = concentration in mg/ml, TT = total tannins in % (m/m), FD = 50 (dilution factor of the sample), \( A_1 \) = measured absorbance of the total tannins, m = water determination, p = drug mass (g) considering the water determination.

Microbial sensibility test. The microbial sensitivity test of *S. adstringens* extract was performed using the disk-diffusion technique. The minimum-inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were defined according to the National Committee for Clinical Laboratory Standards (CLSI 2012). *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus*
RESULTS AND DISCUSSION

Through the determination of phenolic compounds, it was possible to infer the total tannin content (32.7%) of the of *S. adstringens* bark bark. The result agrees with the Brazilian Pharmacopeia recommends minimum 8.0% of total tannins (Brasil 2010) and was similar to that reported in studies conducted in the same species (20-40%) (Castro et al. 2009, Corrêa et al. 2012). Tannins are the major compounds responsible for pharmacological activity of this particular tree and they exhibit various biological functions. Antimicrobial activity of tannins is well documented; however the mechanisms of this activity, as well as their bioavailability have not been satisfactorily clarified (Macáková et al. 2014).

**Acute toxicity.** After obtaining the microbiological test results, 200, 400, and 600mg/kg concentrations were tested. These doses were also defined by considering previously published data that reported concentrations up to 600mg/kg of *S. adstringens* bark extract administered orally that did not induce animal death (Rebecca et al. 2002). For evaluation of acute systemic toxicity, a single dose of each concentration prepared in sterile saline solution was administered by gavage. The rats were divided into four groups with five rats in each group: group 1 (control with sterile saline solution), group 2 (200mg/kg), group 3 (400mg/kg), and group 4 (600mg/kg). After treatment, all rats received food and water and a 12-h light/dark circadian cycle at 22±2°C. The rats were weighed every other day. At the end of the experimental period (14 days), the surviving rats were sedated with a combination of ketamine (80mg) and xylazine (15mg/kg) administered intraperitoneally and killed. Blood samples (approximately 4mL) were collected by cardiac puncture to determine the hematological (erythrocyte, hemoglobin, hematocrit (PCV), MCV, MCH, MCHC, platelet, and lymphocyte count) and biochemical parameters (ALT, AST, urea and creatinine). Macroscopic analyses were performed, and the liver, heart, and kidneys were weighed to determine their relative weights. Tissue samples were collected for histopathological analyses. These tissues were fixed by immersion in 10% buffered formalin for 24 hours, followed by dehydration in increasing ethanol concentrations, diaphanization in xylene, and paraffin embedding. Five-μm thick sections were cut and stained with hematoxylin and eosin (HE). Lesions were evaluated according to the intensity, and a score was given to each lesion, where 0 = absence of lesions, 1 = mild lesion, 2 = moderate lesion, and 3 = severe lesion. The analyses were performed at the Molecular Pathology Laboratory of the Veterinary School of the UFMG.

**Chronic toxicity.** Rats were kept under the same conditions described for the acute toxicity tests. The experiment was divided into two phases, which had either 15 or 30 days of duration. For each of these phases, two groups of five rats each were formed. One group received sterile saline solution (control) and the other received 800mg/kg of the *S. adstringens* extract, daily, once a day. At the end of each phase the rats were killed and fragments of the kidneys, liver, and heart were collected. Animal weight, hematomatological and biochemical parameters, macroscopic evaluation of the organs and histopathological analyses were performed according to the procedure described for acute toxicity evaluation.

**Statistical analysis.** Data were analyzed using analysis of variance (ANOVA) followed by Bonferroni post test or Dunn’s multiple comparison test. When appropriate, the Student t-test with the Bonferroni adjustment was used for pairwise comparisons. A value of p<0.05 was used to indicate a statistically significant difference. For histological analyses, the Kruskal-Wallis non-parametric test was performed followed by Dunn’s multiple comparison test. All statistical tests were performed using the software Graph Pad Prism (San Diego, CA).
Results of the sensitivity tests using the *S. adstringens* extract disks (600μL/mL) exhibited a significant inhibition zone against *S. aureus*; however, there was no significant difference against *Escherichia coli* (Fig.1). This same concentration inhibited *Staphylococcus aureus* and *E. coli* growth in the test tube. This was confirmed by the absence of microbial growth in Petri dishes, according to the recommendations of NCCLS (2003). Costa et al. (2011) used extract obtained by methodology different from the one adopted in this study to evaluate growth of *E. coli* and *S. aureus* isolated from bovine milk, and Pinho et al. (2012) evaluated the activity of *S. adstringens* extract obtained from leaves in these same microorganisms. These studies showed that concentrations ranging from 25 to 500mg/mL of dried crude *S. adstringens* extract did not inhibit *E. coli* growth (Costa et al. 2011, Pinho et al. 2012). Concentrations higher than or equal to 300mg/mL can inhibit *S. aureus* growth (Pinho et al. 2012). Although it is not possible to make a comparison of our results with the former ones, as they used methodology and sources of different extracts, our results corroborate with those findings and suggest that *S. adstringens* extract also inhibits microbial growth.

The rat weights (Fig.2A) and relative organ weights (Fig.2B) of the groups treated with the different concentrations of *S. adstringens* extract did not statistically differ compared to the control group, in contrast to the study of Rebecca et al. (2002) and Costa et al. (2013) that observed impairment in animal development at the dosages tested.

Hematological parameters were within the reference ranges, whereas no significant changes were observed in the complete blood count analyses and biochemical parameters, indicating that the rats did not develop any clinical abnormalities throughout the experiment.

Biochemical analysis of blood taken from rats treated with *S. adstringens* showed that there were changes only in concentration of aspartate aminotransferase (AST) in rats treated with 200mg/kg of *S. adstringens*, indicating a possible hepatic alteration in this group (Table 1). However, results of the histopathological analyses of the organs collected for the acute toxicity tests (based on histopathology scores) revealed that only the livers of the group treated with 600mg/kg of *S. adstringens* extract exhibited significant microscopic changes compared with the control group.

Rebecca et al. (2002) demonstrated that repeated 800mg/kg and 1600mg/kg doses of the extract administered orally caused chronic toxicity in rats, where an LD₅₀ of 2669mg/kg was obtained. Rebecca et al. (2003) evaluated the toxicity of the same methanol extract on mitochondria obtained from liver cells and reported altered energy metabolism. Costa et al. (2013) evaluated acute and chronic toxicity of the same orally ingested fraction and reported no acute toxicity, thereby defining an LD₅₀ of 3015mg/kg. Furthermore, toxicity was not observed at the orally ingested doses of 10, 100, and 200mg/kg during the 90 days of chronic toxicity tests. Oliveira et al. (2013) evaluated the cytotoxicity of aqueous fractions of *S. adstringens* on rat macrophages at 50mg/mL doses and did not observe any decrease in cell viability.

The values obtained in this study were lower than those previously described (Rebecca et al. 2002, Costa et al. 2013), indicating that 400mg/kg of *S. adstringens* extract orally do not result in acute toxicity and can be safe and do not cause acute toxicity. Although this dose has not shown toxicity, was defined the dose of 800mg/kg for chronic toxicity experiments.

The results of the chronic toxicity experiments revealed no changes in body weight during 30 days of the experiment (Fig.4A). No significant changes in the organ weights were observed in treated rats when compared with the control group (Fig.4B). Regarding hematological and biochemical parameters, significant variation was not observed in any of the parameters evaluated (neither at 15 days nor at 30 days) relative to the control group, suggesting that there was no chronic toxicity in the rats. After 30 days of treatment only the MCV (mean corpuscular volume) and MCH (mean corpuscular concentration) had significantly decreased (Table 2). MCH indicates the quantity

Table 1. Biochemical parameters of Wistar rats treated with a single oral dose of the *Stryphnodendron adstringens* bark extract

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>200 mg/mL</th>
<th>400 mg/mL</th>
<th>600 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>55.8±3.8393</td>
<td>64±5.4924</td>
<td>67.4±5.35015</td>
<td>64±9.3005</td>
</tr>
<tr>
<td>AST</td>
<td>197.2±9.0078</td>
<td>148.4±15.917</td>
<td>204.2±23.0703</td>
<td>197.2±21.5021</td>
</tr>
<tr>
<td>Urea</td>
<td>37±3.6055</td>
<td>52±5.573</td>
<td>50.6±4.1545</td>
<td>50.6±5.5973</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.37±0.02502</td>
<td>0.52±0.0483</td>
<td>0.42±0.03219</td>
<td>0.48±0.06592</td>
</tr>
</tbody>
</table>

Biochemical parameters obtained from the serum of rats, treated a single dose of an oral vehicle (Control, n=5) or a single oral dose of *S. adstringens* at doses of 200, 400, and 600mg/kg (n=5). Values represent mean ± standard deviation. Statistical comparisons of the experimental rats with the control group were performed using analysis of variance (ANOVA) and Bonferroni post-tests; n = represents the number of rats in each group. a = represents a significant difference when compared with the control (p<0.001).
of hemoglobin in a sample of blood and MHCH indicates the concentration of hemoglobin; reduction in hemoglobin is associated with anemia, so these results suggested a possible anemia of rats treated with 800mg/mL of S. adstringens, when compared with the control group. Also a decrease in urea concentration after 30 days was observed, which can be the result of a deregulation of renal and hepatic function when rats were treated with 800mg/mL of S. adstringens (Table 2). Similar data were obtained by Costa et al. (2013) who evaluated chronic toxicity with repeated doses for 90 days. They did not report any toxicity following the oral administration of the proanthocyanidin fraction to mice. However, Rebecca et al. (2002) found different results while using 800 or 1,600mg/kg for prolonged periods. The authors reported animal weight loss, increased blood glucose levels, and increased AST levels at the end of the 30-day treatment period.

Chronic toxicity was assessed by histopathological examination, and the results did not indicate any significant microscopic changes in heart and kidneys, neither at 15 days nor at 30 days after administering 800mg/kg of S. adstringens extract. However, in the liver, there was significant microscopic alteration in the group treated with 800mg/kg at 30 days compared to the control group (p<0.05).

Table 2. Hematological and biochemical parameters of Wistar rats treated with oral doses of 800mg/kg of the Stryphnodendron adstringens bark extract for 30 days

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>15 Days</th>
<th>30 Days</th>
<th>Control</th>
<th>15 Days</th>
<th>30 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes (mm$^3$)</td>
<td>7.23 ± 0.03</td>
<td>6.90667 ± 1.59</td>
<td>7.9075 ± 0.45</td>
<td>ALT</td>
<td>79.63 ± 72.6</td>
<td>60.5 ± 5.86</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>15.5 ± 0.10</td>
<td>13.24 ± 2.87</td>
<td>14.88 ± 0.68</td>
<td>AST</td>
<td>165.2 ± 66.51</td>
<td>155.5 ± 66.39</td>
</tr>
<tr>
<td>Packed Cell Volum (%)</td>
<td>44.10 ± 0.7</td>
<td>38.63 ± 8.96</td>
<td>43.15 ± 2.19</td>
<td>Urea</td>
<td>57.6 ± 4.17</td>
<td>44.5 ± 1.89</td>
</tr>
<tr>
<td>VCM (fL)</td>
<td>61.00 ± 1.22</td>
<td>56.01 ± 2.45</td>
<td>54.59 ± 0.81</td>
<td>Creatinine</td>
<td>0.32 ± 0.018</td>
<td>0.52 ± 0.29</td>
</tr>
<tr>
<td>HCM (pg)</td>
<td>21.44 ± 0.23</td>
<td>19.26 ± 1.27</td>
<td>18.83 ± 0.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHCM (g/dL)</td>
<td>35.14 ± 0.32</td>
<td>34.37 ± 0.96</td>
<td>34.49 ± 0.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombocytes (mm$^3$)</td>
<td>613500 ± 95500</td>
<td>277933 ± 220468</td>
<td>449377 ± 304037</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocyte (mm$^3$)</td>
<td>7340 ± 240</td>
<td>10417 ± 3579</td>
<td>5654 ± 6532</td>
<td></td>
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</tr>
</tbody>
</table>

Hematological and biochemical parameters measured in the serum of Wistar rats treated chronically using oral vehicle (Control, n=4) or with daily doses of 120mg/kg of S. adstringens (n=5) for 30 days. The values represent the mean ± standard deviation. Statistical comparisons of the experimental rats with the control group were performed using analysis of variance (ANOVA) and Bonferroni post-test; n = represents the number of rats in each group. The letters a (p<0.04017), b (p<0.005) and c (p<0.0114) represent a significant difference when compared with the control.
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(Fig.5A,B) as demonstrated by histopathology scores. This analysis revealed that the livers of the group treated with of S. adstringens extract exhibited minimal or mild to moderate centrilobular degeneration (hydropic hepatocellular degeneration) (Fig.6).

Rebecca et al. (2002, 2003) observed altered plasma glucose and AST levels combined with a decline in weight of the rats. The effect of the extracts on energy metabolism in the liver was investigated and the results showed that the toxic action may be correlated with interference in the oxidative phosphorylation or electron transport chain or with inhibition ATP-synthase. The authors concluded that such injuries were associated with toxic activity of the tannin present in the extracts when administered at doses above 600mg/kg. Costa et al. (2013) found lesions indicative of toxicity, while analyzing the organs from rats subjected to acute and chronic toxicity evaluation of the proanthocyanidin fraction, where only 4000 and 5000mg/kg doses were used, thereby indicating that the fraction exhibited lower toxicity at the tested concentrations than those used by Rebecca et al. (2002, 2003) and those employed in this study.

Liver lesions have been described in ruminants after intake of mashed beans from Stryphnodendron fissuratum (Ferreira et al. 2009, Aguiar-Filho 2013). Fonseca & Linbradi (2008) reported that possible toxicity of tannins in dye preparations with bark of Stryphnodendron barbatiman occurred in mammalian cells. Regarding the cytotoxicity in other mammalian cells, Oliveira et al. (2013) found mild toxicity of the S. barbatiman Mart. plant extract at 50mg/mL concentration on rat macrophages and significantly reduced the production of TNF-α when compared to the control group.

CONCLUSIONS

According to the sensitivity tests, the Stryphnodendron adstringens extract obtained from northern Minas Gerais exhibited antimicrobial activity at 600μL/mL.

Liver degeneration, although minimal, was observed 30 days after ingestion of the bark extract of S. adstringens in the groups of rats that received 800mg/kg, what may indicate some degree of toxicity.

No systemic toxicity was observed at lower doses.

Considering the broad use of S. adstringens as a phytotherapeutic agent for various human and animal diseases and liver toxicity observed at high concentrations, attention should be paid to the possible adverse effects of using the extract from this tree at high concentrations.

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Anna C. Almeida et al.