Toxicity study about a medicinal plant *Casearia sylvestris*: A contribution to the Brazilian Unified Health System (SUS)

A.Z. Ameni a, O.A. Latorre a, L.M.B. Torres b, S.L. Górniak a,*

a Faculty of Veterinary Medicine and Animal Sciences, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87. CEP: 05508-270 - Cidade Universitária, São Paulo, SP, Brazil

b Botanic Institute of São Paulo Av. Miguel Estéfano, 3687, Água Funda, CEP: 04301-012, São Paulo, São Paulo, Brazil

**A R T I C L E  I N F O**

Article history:
Received 18 February 2015
Received in revised form
31 July 2015
Accepted 21 August 2015
Available online 3 September 2015

Keywords:
Casearia sylvestris
Toxicity evaluation
Acute toxicity
Repeated dose toxicity
Medicinal plants

**A B S T R A C T**

**Ethnopharmacological relevance:** *Casearia sylvestris S.w (Salicaceae)* is catalogued by the Brazilian Unified Health System as a plant of interest for the Brazilian population with the purpose of treating inflammatory disorders, such as pain and gastrointestinal disorders based on the folk use and some literature about efficacy; however, no toxicological studies concerned the safety of extract fluid of this plant have been reported.

**Aim of the study:** The present study was carried out to evaluate the acute and subchronic toxicity of the hydroethanolic extract fluid (FE) obtained from leaves of *C. sylvestris* in Wistar rats.

**Materials and methods:** In the acute toxicity test three female Wistar rats were treated with a single dose of FE (2000 mg/kg) administered by oral gavage and observed for 14 days in order to identify signs of toxicity or death. In subchronic toxicity study animals received, by daily gavage three doses 60, 120 and 240 mg/kg of the FE of the plant for 28 and 90 days. The animals were observed daily for clinical signs and mortality. Body weight and food consumption were measured weekly and at the end of treatment were analysed hematological, biochemical and histopathological parameters. Also was analysed the cellularity of bone marrow and spleen. Moreover, phytochemical analysis by HPLC–PDA–ESI+ /MS and CG/MS/EI was carried out to qualify the constituents of the extract.

**Results:** The results of acute study indicated that the LD50 is higher than 2000 mg/kg and at 28 and 90 day oral toxicity showed that there were no toxic effects detected in any of the parameters evaluated: body weight and relative organ weight, general behavioral changes, haematological and biochemical parameters and histopathological examination. The analysis by HPLC–PDA–ESI+ /MS and CG/MS/EI identified the flavonoids rutin, quercetin and luteolin and also chlorogenic on the extract.

**Conclusion:** Based on this study the hydroethanolic fluid extract of *C. sylvestris* could be safe even when used over a long period for therapeutic uses proposed by the Brazilian Unified Health System.

© 2015 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

In Brazil the use of medicinal plants has an important role in primary health care, mainly in the communities without easily access to medicines. Since 2006, the Brazilian government defined national policies on the use of herbal medicines in The National Policy of Integrative and Complementary Practices (PNPIC) and The National Policy of Medicinal Plants and Herbal Medicines (PNPMF). These documents encourage research into and the development of herbal medicines while prioritizing the protection of biodiversity and promoting greater access to safe and effective treatments (Carvalho et al., 2011). Furthermore, these documents have led to the inclusion of the use of medicinal plants into the public health system. Currently, 47 medicinal plants are approved to be prescribed in the Brazilian Unified Health System (SUS) based on ethnopharmacology data (ANVISA, 2011). However, while there is a widespread use of herbal medicine, little has been done to establish its safety (Carvalho et al., 2009). In this manner, although in Brazil, the National Health Surveillance Agency (ANVISA) is developing standards for the regulation for the pharmaceutical companies submit data on efficacy and safety of herbal medicines, presently, information and reliable data are scarce and often contradictory. Thus, it should be considerate that there is a lack of minimum information necessary for the correct use of medicinal plants and herbal medicine, which makes an easy target for self-medication without liability and also it is necessary to develop protocols of studies and better evaluate the safety of natural products and herbal medicines, thereby providing a...
rational use of them (Alexandre et al., 2005).

In this context, our research group performed a toxicity study about *Casearia sylvestris*, which is catalogued by the SUS as a plant of interest for the Brazilian population with the purpose of treating inflammatory disorders, such as pain and gastrointestinal disorders, such as gastritis and ulcers. In addition, it is used as a cicatrizant agent in skin diseases, with its only restriction being against use during pregnancy, according to the “Formulário de Fitoterápicos da Farmacopéia Brasileira” (ANVISA, 2011). Moreover, there are studies showing different uses of it as an antimicrobial (Da Silva et al., 2008), analgesic and anti-inflammatory (Ruppelt et al., 1991), antiviral (Cavalante et al., 2007; Cintra-Françischinelli et al., 2008), cytotoxic effects against tumor cells (Ito-kawa et al., 1990; Morita et al., 1991; Oberlies et al., 2002) and gastric antulcer activities (Basile et al., 1990; Sertié et al., 2000).

Despite the widespread use of *C. sylvestris*, controlled clinical studies have not been conducted and there are paucity of data related to the evaluation of the toxicity of this plant. Therefore, the aim this study was to evaluate the possible toxic effects of the fluid extract of *C. sylvestris* (FE) on preclinical studies, in *Wistar* male rats, as acute oral toxicity and repeated dose 28 and 90 day oral toxicity based on the “Guide to studies of pre-clinical toxicity of herbal medicines” edited by ANVISA (2004).

2. Material and methods

2.1. Plant material

Fresh leaves of *C. sylvestris* Sw (*Salicaceae*) were collected at the University of São Paulo (Parque Esporoe para Todos), São Paulo, SP, Brazil, in February 2009. A voucher herbarium specimen was deposited at the Botanic Institute of São Paulo/SP, under reference number 430190.

The leaves of *C. sylvestris* were dried at room temperature. Next, this material was powdered, and the hydroethanolic fluid extract extract (FE) was obtained by percolation, following Brazilian Official Pharmacopoeia 1st edition instructions. The all material for the study was prepared in one batch lyophilized and kept frozen at −20 °C until use and was resuspended in distilled water at different doses immediately before administration.

2.2. Phytochemicals analysis

2.2.1. HPLC–PDA–ESI+/MS analysis

The spectrometric analysis was performed using a Shimadzu® HPLC system consisting of a LC-10 pump and a SPD-M10A PDA detector coupled to an ESI/MS+ system. All experiments were carried out in positive ion mode with a mass spectrometer Esquire 3000 Ion trap (Bruker Daltonics®, Billerica, MA, USA). PDA signal was acquired scanning wavelengths between 190 ± 800 nm while total ion chromatogram (TIC) mode was employed for MS analysis. Analysis of extracts by analytical HPLC was performed using a Varian Pur- chromatogram (TIC) mode was employed for MS analysis. Analysis was performed using the following program: 5 min isothermal heating (70 °C), a gradient of 5 °C/min to 310 °C, and a final minute of heating at 310 °C. The temperature was then equilibrated before automatic injection of the next sample. The mass spectra were recorded at 2 scans/s with a scanning range of 50–650 m/z. Data from chromatograms and mass spectra were analyzed using the program ChemStation (Agilent®) and compared with the literature (NIST and WILEY 275 L libraries) and data obtained from authentic standards.

2.3. Animals

Eight three Male and female Wistar rats, 60 days years old, bred in the Department of Pathology at the School of Veterinary Medicine and Animal Sciences, University of São Paulo, were used. The rats were randomly assigned to the control and treatment groups, individually housed, maintained under controlled temperature conditions (22–25 °C), relative humidity (50–65%) and lighting (12 h/12 h light/dark cycle). Before the test was conducted, the animals were kept in their cages five days to allow for acclimatization to the laboratory conditions. Drinking water and standard diet (Nuvital-CR1®, Nuvital Nutrientes LTDA) were provided ad libitum. All procedures were performed following the Guide for the Care and Use of Laboratory Animals, NIH publication n°85–23 and were reviewed and approved by the Bioethics Committee of the FMVZ-USP (process n°1738/09).

2.4. Acute toxicity study

The single-dose acute oral toxicity test was performed according to OECD Guideline 423 (OECD, 2001).

Three female Wistar rats were treated with a single dose of FE (2000 mg/kg) administered by oral gavage. Next, the rats were closely observed during the first day, hourly, and once daily thereafter for a total of 14 days in order to identify signs of toxicity or death. The body weight and food consumption of each rat were measured every 3 days throughout the observation period. At the end of this period, all survivors were killed to examine macroscopic alterations in their vital organs.

2.5. Subchronic 28 and 90 day toxicity studies

In the present study, the doses used were determinate from the lowest dose (60 mg/kg) with which the extract presented pharmacological activity (antulcer activity) in rats (Basile et al., 1990), and from this dose, applied a factor of 2 and 4 times the therapeutic dose to established the medium and high doses.

For each experiment, forty male Wistar rats were randomly allocated into four groups (n=10) and daily treated, for 28 or 90 days, by oral gavage, the following doses of FE: 0 mg/kg (Co), 60 mg/kg (FE 60), 120 mg/kg (FE 120) and 240 mg/kg (FE 240).

The animals were observed daily for clinical signs and mortality. Body weight and food consumption were measured weekly throughout the study period. At the end of treatment, all rats were anesthetized intraperitoneally (ip) with ketamine (50 mg/kg) and xylazine (5 mg/kg) to collect blood samples by cardiac puncture. Vacutainers containing EDTA and vacutainers that were dry were used to process blood for complete blood count (CBC) and serum biochemistry, respectively. Next, liver, spleen and thymus were harvested and weighed so that relative weight could be calculated.
and histopathology could be evaluated. In addition, one femur from each rat was collected, to verify bone marrow cellularity as well as from as well spleen.

2.6. Statistical analysis
eosin (HE).

2.5.3. Histopathology
Laboratórios Modernos, S.A., Brasil).

2.5. Serum biochemistry

2.5.2. Serum biochemistry
stand for 45 min at room temperature before being centrifuged at 4000 rpm for 10 min. The serum from each sample was recovered and placed at −20 °C until required.

Creatinine, urea, uric acid, chloride, total proteins, albumin, glucose, triglycerides, total cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) were evaluated using an automatic chemistry analyzer CELM SBA-200 (Cia. Equipadora de Laboratórios Modernos, S.A., Brasil).

2.5.3. Histopathology
Histopathological analyses were performed on several organs: liver, spleen, thymus and kidney. They were fixed in 10% neutral formalin and routinely processed for paraffin embedding. Sections (5 μm thick) were prepared and stained with hematoxylin and eosin (HE).

2.6. Statistical analysis

The data were analyzed using GraphPad Prism 4.00® software (GraphPad Software, Inc., San Diego, CA). Homogeneity of variance was tested with Bartlett’s test. If variance was homogeneous, the data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett’s test for multiple comparisons, otherwise, data were analyzed by the Kruskal–Wallis nonparametric ANOVA followed by Dunn’s test. All data were expressed as the mean ± SD, and differences were considered to be statistically significant at p < 0.05.

3. Results and discussion

3.1. Phytochemical analysis

Data analysis by HPLC-PDA-ESI/MS+ system of chromatogram profile of C. sylvestris extract at 254 nm, which showed 18 chromatographic peaks with respective retention time (RT). The total ion currents (TIC) of FA components and the positive ion ESI spectrum of rutin showed an abundant [M+H]+ ion peak at m/z 611 (M=610, C27H30O6), ion peak at m/z 303.0 corresponding to quercetin [M+H]+, the aglycone moiety of rutin and the peak at m/z 465 resulted from loss of rhamnose from rutin similar of standard at Rt=26.1 min. The positive ion ESI spectrum of chlorogenic acid at Rt=16.3 min was identified by mass spectrum data with fragment ions m/z 355 [M+H]⁺ (M=354, C16H18O9) and m/z 163 (100%) [MC9H7O3]. The luteolin glucoside was identified at Rt=33.7 min; m/z=645, 639, 286 [luteolin aglycone M+H⁺, M=286, C15H10O6]. Data analysis by GC/MS/EI after derivatization identified the querzetin at Rt=49.646 min, m/z=575 (%) e 487 and rutin Rt=49.486, m/z=543, 207, 133(90%), 73(100%) which data were similar of authentic standards.

These flavonoids have been reported to act in the gastrointestinal tract showing cytoprotective anti-ulcerogenic efficacy and antispasmodic, antidiarrheal, antioxidant and antiinflammatory properties (Borreli and Izzo, 2000; Di Carlo et al., 1999; Havsteen, 2002; La Casa et al., 2000) suggesting they could be correlate with medicinal activity proposed to this plant. Furthermore, studies about C. sylvestris also showed the presence of phenolic compounds, mainly flavonoids with rutin in constitution, which can be pointed as chemical markers (Silva et al., 2006).

Future studies will be conducted in order to verify if flavonoids found in plant are the main responsible for the gastroprotective e/ or anti-inflammatory effects and to standardization this extract.

3.2. Acute toxicity study

The acute toxicity results, with the limit test single dose of 2000 mg/kg of the C. sylvestris extract, in the present study did not show any signs of toxicity. All rats treated with the FE survived on the 14-day observation period. No adverse clinical signs were observed. Body weight and food consumption measurements did not reveal any difference, and no alterations were observed at necropsy.

Taking into account that the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) assumes that substances with values higher than 2000 mg/kg are classified as having relatively low acute toxicity, we can consider C. sylvestris FE safe. Similarly, the low toxicity of C. sylvestris had already been described by Basile et al. (1990), which showed that the LD 50 was higher than 1840 mg/kg.

3.3. Subchronic 28 and 90 day toxicity studies

C. sylvestris has been prescribed by SUS to treat gastrointestinal disorders; therefore, we established the initial dose for the subchronic studies based on the Basile et al. (1990) study that showed that the antiulcerogenic effective dose is 57.5 mg/kg.

The studies with doses of 60, 120 and 240 mg/kg/day of fluid extract C. sylvestris for 28 and 90 days show that the treated rats presented no signs of behavioral changes or clinical toxic signs, as indicated by the lack of alterations in body weight (P > 0.05) and food consumption (P > 0.05) (Fig. 1), hematological (P > 0.05) (Table 1) relative organ weight (P > 0.05) (Table 2), and histopathological parameters compared to control animals.

On the other hand, some alterations were observed in the biochemical values in rats treated with 120 and 240 mg/kg C. sylvestris extract, treated for 28 days as an increase in total proteins (P < 0.01 post test Dunnet) and albumin (P < 0.05 post test Dunnet’s) and decrease in triglyceride (P < 0.01 post test Dunnet) creatinine (P < 0.05 post test Dunnet’s) and chloride levels (P < 0.01 post test Dunnet) (Table 3). Rats treated with 240 mg/kg C. sylvestris extract for 90 days showed significant reductions in creatinine (P < 0.05 post test Dunnet’s) and total cholesterol (P < 0.01 post test Dunnet) while animals treated with 120 mg/kg showed significant reductions in total cholesterol and increases in chloride levels (Table 4).

The result of decrease in triglyceride levels corroborates with a study conducted by Werle et al. (2009) which showed that the hydroalcoholic extract of C. sylvestris at 500 mg/kg lowers blood triglyceride levels, and can indicate this plant as a candidate to a natural triglyceride reducer. At the same way, the results suggest that this extract could be a reducer of cholesterol and creatinine. However, futures experiments should be conducted to better evaluate this possibility.

In the same manner, although biochemical analysis of serum collected showed fluctuations of multiple parameters in those
animals treated with *C. sylvestris*, these parameters remained within the normal ranges for Wistar rats (Bihun and Bauck, 2004; Sharp and Regina, 1998). Furthermore, no clinical data were observed to substantiate these results. In addition, one must consider that animals treated with different doses of this plant showed no signs of systemic toxicity, neither was affected the general welfare, growth or normal development.

In the same manner, no alterations were observed in relative organ weight, histology of the thymus and spleen (*P* > 0.05), or in the cellularity of the spleen and bone marrow (*P* > 0.05) (Table 5), suggesting that this extract did not produce immunotoxic effects.

### Table 1

Complete blood count of rats treated daily, for 28 and 90 days, with *C. sylvestris* hydroethanolic extract: 0 (Co), 60 (FE 60), 120 (FE 120) and 240 (FE 240) mg/kg.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Co</th>
<th>FE 60</th>
<th>FE 120</th>
<th>FE 240</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT (%)</td>
<td>41.0±3.1</td>
<td>40.8±2.7</td>
<td>40.5±1.4</td>
<td>41.7±3.0</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>14.4±0.7</td>
<td>14.5±0.6</td>
<td>14.3±0.6</td>
<td>14.9±0.5</td>
</tr>
<tr>
<td>RBC (10⁶/mm³)</td>
<td>8.3±0.6</td>
<td>8.2±0.5</td>
<td>8.3±0.4</td>
<td>8.5±0.5</td>
</tr>
<tr>
<td>WBC (10³/mm³)</td>
<td>5.9±1.0</td>
<td>5.6±1.0</td>
<td>5.6±0.9</td>
<td>5.1±1.0</td>
</tr>
<tr>
<td>PLT (10³/mm³)</td>
<td>6481±56.9</td>
<td>6490±64.6</td>
<td>6379±65.0</td>
<td>6123±77.7</td>
</tr>
<tr>
<td>MCV (fM)</td>
<td>48.8±3.5</td>
<td>50.0±1.7</td>
<td>48.8±1.3</td>
<td>48.8±1.3</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>34.8±1.7</td>
<td>35.6±1.6</td>
<td>35.4±1.4</td>
<td>35.8±1.9</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD (*n* = 10). HCT = Hematocrit, HGB = Hemoglobin, RBC = Red blood cells, WBC = White blood cells, PLT = Platelet, MCV = Mean corpuscular volume, MCH = Mean corpuscular hemoglobin, MCHC = Mean corpuscular hemoglobin concentration.

### Table 2

Relative lymphoid organs weight of rats treated daily for 28 and 90 days with *C. sylvestris* hydroethanolic extract: 0 (Co), 60 (FE 60), 120 (FE 120) and 240 (FE 240) mg/kg (mean ± SD, *n* = 10).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Co</th>
<th>FE 60</th>
<th>FE 120</th>
<th>FE 240</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen (g)</td>
<td>0.21±0.022</td>
<td>0.21±0.028</td>
<td>0.21±0.03</td>
<td>0.22±0.025</td>
</tr>
<tr>
<td>Thymus (g)</td>
<td>0.08±0.021</td>
<td>0.08±0.020</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
</tr>
</tbody>
</table>

In summary, these data suggest that the hydroethanolic extract of *C. sylvestris* could be safe even when used over a long period. Moreover, this study is an important contribution to the rational use of medicinal plants on SUS. Future studies about pharmacokinetics and pharmacodynamic and drug-interactions are suggested for better knowledge of therapeutic safe use.

### 4. Conclusion

In summary, these data suggest that the hydroethanolic extract of *C. sylvestris* could be safe even when used over a long period. Moreover, this study is an important contribution to the rational use of medicinal plants on SUS. Future studies about pharmacokinetics and pharmacodynamic and drug-interactions are suggested for better knowledge of therapeutic safe use.
Data are presented as mean ± SD (n=10). ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; A.T.aminotransferase; ALT, alanine aminotransferase. We are thankful to CAPES (1406395) for the financial support.

References


