Safety Assessment of Esporão de Galo (Celtis iguanaea (Jacq.) Sargent) Crude Extract from Leaves: Acute and Subacute Toxicity Studies in Male Rats

Silva ARH1*, Froeder ALF2, Figueredo KC1, Noda JM1, Borba de Freitas R1, Nunes LT2, Pappis L2, Boligon AA2, Cardoso MS2, Moresco R3, Veiga ML4, Athayde ML2, Bauermann LF8 and Hubacher GW1

1Departamento de Fisiologia e Farmacologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil
2Departamento de Farmácia Industrial, Laboratório de Fisiologia Experimental, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, RS, Brasil
3Departamento de Análises Clínicas e Toxicológicas Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil
4Departamento de Morfologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil
5Departamento de Tecnologia e Ciência dos Alimentos, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

*Corresponding author: Haas da Silva AR, Departamento de Fisiologia e Farmacologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil, Tel: 55-55-3220-9380; E-mail: andreiaregina_silva@yahoo.com.br

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Abstract

Celtis iguanaea is popularly known as “Esporão-de-galo” in Portuguese and its leaves are traditionally used in infusion forms as analgesic, antiasthmatic, digestive and diuretic. The aim of this study was to assess the acute and sub-acute toxicity of the crude extract from the Celtis iguanaea leaves in male rats. The toxicity studies were based on the guidelines of the Organization for Economic Cooperation and Development (OECD-guidelines 423 and 407). In the acute study, a single dose of 2000 mg/kg of C. iguanaea was administered orally. In the sub-acute study, the extract was administered orally to male rats with doses of 100, 200 and 400 mg/kg/day for 28 days. Behavioral changes and biochemical, hematological, and histological analysis were evaluated. The acute administration of C. iguanaea did not cause changes in behavior or mortality. At the sub-acute toxicity study, we observed an increase of glucose and a decrease of the aspartate aminotransferase (ASAT) enzyme. Celtis iguanaea, after acute administration, may be classified as safe (category 5), according to the OECD guide. However, the alterations observed after sub-acute administration with high doses of crude extract from the C. iguanaea leaves suggest that more studies are needed to elucidate the mechanism of action.

Keywords: Toxicity; Esporão-de-galo; Cannabaceae; Crude extract
Introduction

The use of medicinal plants in the treatment and cure of diseases is as old as the human species, especially due to the ease of obtaining them and their low cost when used in natura [1]. The popular and traditional use is not sufficient to prove if herbal treatments are as effective and safe. Thus, medicinal plants are no different than any other synthetic xenobiotic and their use should be based on supporting experimental evidence of the risks they present to those who use them [1-3].

*Celtis iguanaea* (Jacq.) Sargent belongs to the family Cannabaceae, known in traditional medicine as “Esporão-de-galo” in Portuguese. It has a wide geographical distribution, occurring in the South America. This species is an angiosperm and dicotyledonous characterized as a shrub or small tree with a thorny plant, 6 m to 9 m high, with rounded crown and branches, having canopy in pyramid shape and an upright cylindrical trunk of 15 cm to 30 cm in diameter. It blooms from August to October, and its fruits ripen from February to March [4-6]. The infusion of its leaves is popularly used in the treatment of joint pain, asthma, cramps and poor digestion, as a diuretic, and in the treatment of gastric ulcers [4-7].

The phytochemical screening of the leaves and stem of *C. iguanaea* showed the presence of flavonoids, coumarins and mucilage [8]. Two pentacyclic triterpenes, the friedelin and epifriedelino, were isolated from the bark of *C. iguanaea* and may be responsible for interference in the physiological processes of the plant during the growth phase of some species [9]. Preliminary studies of Souza et al. [4] observed the gastroprotective effect after ulcer induced and treated hexane fraction of the *C. iguanaea* leaves. Preliminary data from Martins et al. [5] showed anticholinergic and secretory efficacy of hexane extract of leaf the plant suggesting anticholinergic and antihistaminergic mechanism. Hereafter, Martins et al. [6] demonstrated that the hexane extract of *C. iguanaea* leaves exhibits gastro protective activity in different gastric ulcer models.

However, so far no study about the toxic effects of the extract from the leaves *C. iguanaea* has been reported. Thus, the present study was undertaken to determine the possible harmful effects of the crude extract of the *C. iguanaea* (Jacq.) Sargent leaves after acute 14 day and sub-chronic 28 day oral administration to rat’s wistar.

Materials and Methods

Plant material

The leaves of *C. iguanaea* were collected in Jaboticaba (State of Rio Grande do Sul, Brazil) (coordinates 27°37’48”S, 53°16’55”W) in March of 2013. A dried voucher specimen is preserved in the herbarium of the Department of Biology at the Federal University of Santa Maria by register number SMBD 12.952.

Extract preparation

The leaves of the plant were dried in a stove at a controlled temperature (40°C) and powdered in a knife mill. The powder was then subjected to maceration with 70% ethanol at room temperature for seven days, with daily agitation. The solvent was renewed until the total extraction of the compounds. The material was filtered and concentrated under reduced pressure, in order to remove the ethanol. The aqueous extract was evaporated to dryness to provide the crude extract of the leaves.

Chemical, apparatus and general procedures

Methanol, acetic acid, gallic acid, ellagic acid and chlorogenic acid were purchased from Merck (Darmstadt, Germany). The quercetin and rutin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT re- procating pumps connected to a DDU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and the LC solution 1.22 SP1 software.

Quantification of compounds by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions by using C18 column (4.6 mm to 250 mm) packed with 5 µm diameter particles. The mobile phase consisted of water containing 2% acetic acid (A) and methanol (B), and the composition gradient was 5% of B until 2 min, 25% (B) until 10 min, 40, 50, 60, 70 and 80% (B) every 10 min; following the method described by Oboh et al. [10], with minor modifications. The *C. iguanaea* was analyzed in a dissolved way with the mobile phase at a concentration of 15 mg/ml. All the samples and mobile phase were filtered through a 0.45 µm membrane filter (Millipore) and, then, degassed by ultrasonic bath prior tose. Stock solutions of standard references were prepared in the HPLC mobile phase at a concentration range of 0.025 mg/ml to 0.300 mg/ml. The flow rate was of 0.7 ml/min, with an injection volume of 50 µl and the wave length was of 270 nm for gallic acid and elagic acid, 325 nm for chlorogenic acid and 366 nm for quercetin and rutin. The chromatography peaks were confirmed by comparing the retention time with those with reference standards and by DAD spectra (200 nm to 600 nm). Calibration curve for gallic acid: Y = 12574x + 1195.6 (r = 0.9998); chlorogenic acid: Y = 13472x + 1308.5 (r = 0.9997); ellagic acid Y = 11834x + 1359.2 (r = 0.9999), rutin: Y = 13149x + 1273.8 (r = 0.9997). All chromatography operations were carried out at ambient temperature and repeated three times. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 /S and 10 s/S, respectively, being s the standard deviation of the response and S is the slope of the calibration curve [11].

Animals

Adult male Wistar rats (160 g to 200 g) were obtained from the Biotério Central da Federal University of Santa Maria (UFSM) and were arbitrarily distributed in different experimental groups. The animals were housed in polypropylene cages at an ambient temperature of (24 ± 2)°C and 45% to 55% relative humidity, with a 12:12 h light/dark cycle and were also adapted to local conditions for at least 72 h before the experiment. The animals were provided commercial food pellets and water ad libitum. All experimental protocols were approved by the Ethics and Animal Welfare Committee of the Federal University of Santa Maria (CEUA UFSM; Protocolo 103/2013).

Acute toxicity study

The acute oral toxicity of the *C. iguanaea* was evaluated in Wistar male rats as preconized by the OECD 423 Guideline [12], with some modifications. The experimental procedures were performed twice...
using three male Wistar rats per group at each step. The control group received distilled water (10 mL/kg) and the test group a single dose of 2000 mg/kg of *C. iguanaea* dissolved in water. Both treatments were made by gavage and the dose of 2000 mg/kg corresponds to the maximum dose recommended by the protocol adopted. The animals were observed 6 h and 12 h after administration and every day for 14 days. The weight and food intake of the animals were monitored, as well as cases of death, occurrence of tremors, convulsions, changes in skin and fur, somatomotor activity and behavior. On the 15th day, after a short period of fasting, the animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), received tramadol analgesia (8 mg/kg i.p.) and euthanized by cardiac puncture and subjected to gross pathological examination of all the major internal organs such as brain, heart, lung, liver, kidney, spleen, adrenals and gonads.

**Subacute toxicity study**

The subacute toxicity study was developed following the guidelines of OECD 407 Guideline [13], with minor modifications. The *C. iguanaea* was dissolved in water and administered by gavage, each morning for 28 consecutive days. The animals were divided in four groups, totaling 20 animals used in the experiment (n = 5), as follows:

- **Group I**: control group treated with water (10 mL/kg);
- **Group II**: treated with 100 mg/kg of *C. iguanaea*;
- **Group III**: treated with 200 mg/kg of *C. iguanaea*;
- **Group IV**: treated with 400 mg/kg of *C. iguanaea*.

The body weight of the animals was identified by daily monitoring throughout the study period. On day 29 of the study, all the animals were subjected to a short fasting period. After this period, rats were the anesthetized with pentobarbital sodium (50 mg/kg, i.p.), received tramadol analgesia (8 mg/kg i.p.) and were euthanized by cardiac puncture. Blood was collected in two tubes: one with the anticoagulant ethylenediaminetetraacetate (EDTA) to analyze hematological parameters and the other without any additive to analyze biochemical parameters. A slice of hepatic and renal tissue was removed, dissected, and fixed in 10% formalin. Another slice of the liver and the kidney of each animal were removed, homogenized in 50 mM Tris-HCl, pH 7.4, centrifuged and the supernatant used to measure aminolevulinate dehydratase enzyme activity, thio-barbituric acid reactive substances and enzyme catalase activity.

**Biochemical and hematological parameters**

The blood without the anticoagulant was allowed to clot before centrifugation (4000 rpm for 10 min) to obtain serum, which was utilized for the assessment of glucose (GLU), blood urea nitrogen (URE) levels, creatinine (CRE), aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) activities analyzed by use of standard methods on the Cobas MIRA® (Roche Diagnostics, Basel, Switzerland) automated analyzer. The anticoagulated blood was analyzed immediately for hematological parameters: erythrocytes (RBC), hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cells distribution width (RDW) leukocytes (WBC), differential leukocytes and platelet (PLT) counts were determined with the use of an automatic counter veterinary Mindray BC 2800.

**Histopathology of subacute toxicity**

We analyzed 19 liver samples and 18 kidney samples, fixed in 10% buffered formalin. The samples underwent standard histological processing, embedded in paraffin, sectioned at 8 μm sections and stained with hematoxylin and eosin. In the liver analysis were photomicrographed 4 random fields/blade in 4 histological slides per sample. The nuclear area and nuclear density of hepatocytes and the densities of kupfer cells were analyzed. The analyzes were made in the software Image Plus Pro 4®, we used a grid of 200 μm²; dividing this grid in 12 subfields of equal size, with subsequent analysis by standardized sampling four subfields. In renal analysis were photomicrographed 5 random fields / blade in the area of the renal cortex, 5 blades per sample. In each histological blade, 10 randomly renal glomeruli were analyzed. The variables measured were glomerular area and nephron area. The subcapsular area was estimated by subtracting the area of the glomerulus by the nephron area. Renal analysis is also performed by the software Image Plus Pro 4®.

**Statistical analysis**

The data are expressed as means ± S.D. All the results were analyzed by one-way ANOVA, and Tukey test were used in order to compare any significant differences between the control group and the treated group. The differences between the groups were considered to be significant when p < 0.05. The chromatographic analyses were performed using the software R version 3.1.1 (R Core Team, 2014). The experiment was laid out in a Completely Randomized design and replicated three times. Data were collected on the following parameter: number of days to bud break for each sprouted cutting, number of leaves per cuttings, numbers of branches per cuttings, number of rooted plants which was counted at the end of the experiment, number of roots per rooted cuttings, longest length of root per cutting, and root weight.

**Results**

**HPLC analysis**

HPLC profile of *C. iguanaea* revealed the presence of the phenolics acids: gallic acid (t<sub>R</sub> = 9.75 min; peak 1), chlorogenic acid (t<sub>R</sub> = 19.43 min; peak 2) and ellagic acid (t<sub>R</sub> = 24.17 min; peak 3), and the flavonoids: rutin (t<sub>R</sub> = 28.01 min; peak 4) and quercetin (t<sub>R</sub> = 32.89 min; peak 5) (Figure 1 and Table 1).
Figure 1: Representative high performance liquid chromatography profile of C. iguanaea, detection UV was at 327 nm. Gallic acid (peak 1), chlorogenic acid (peak 2), ellagic acid (peak 3), rutin (peak 4) and quercetin (peak 5).

| Compounds        | C. iguanaea (mg/g) | LOD (%) | LOQ (μg/mL) |
|------------------|-------------------|---------|-------------|
| Gallic acid      | 2.71 ± 0.03 a     | 0.27    | 0.021       |
| Chlorogenic acid | 5.03 ± 0.01 b     | 0.50    | 0.007       |
| Ellagic acid     | 17.68 ± 0.01 c    | 1.76    | 0.013       |
| Rutin            | 15.94 ± 0.02 d    | 1.59    | 0.019       |
| Quercetin        | 10.83 ± 0.03 e    | 1.08    | 0.011       |

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at p < 0.05.

Table 1: Composition of C. iguanaea CEL.

**Acute toxicity study**

In the acute toxicity study, an oral dose of up to 2000 mg/kg of the C. iguanaea extract was administered and monitored for 14 days. Clinical observations did indicate neither toxicity nor mortality. Furthermore, no alteration in body weight, food and water consumption was observed among treated and untreated animals (data not shown). In addition, necropsy revealed no gross pathological signs in any organs.

**Sub-acute toxicity study**

Daily oral administration of C. iguanaea extract for 28 days did not produce any obvious symptoms of toxicity or mortality up to the highest dose level of 400 mg/kg. Furthermore, no alterations in food intake and no modifications in body weight gain between the control and treated groups were observed (data not shown).

In relation to the assessment of biochemical parameters, we observed an increase of GLU in the groups treated with C. iguanaea in the dose of 200 mg/kg and 400 mg/kg (202.80 ± 9.31 and 196.80 ± 4.76) when compared to the control group (171.72 ± 8.34). In contrast, treatment with the extract of the plant decreased ASAT activity in the group treated with the higher dose in the control group (107.71 ± 4.11 dose 400 mg/kg; 120.66 ± 8.33 control). Other parameters evaluated as URE, CRE and ALAT showed no changes in their levels when comparing different doses of C. iguanaea with the control group (Table 2).

| Biochemical parameters | Study groups | Control | 100 mg/kg | 200 mg/kg | 400 mg/kg |
|------------------------|-------------|---------|-----------|-----------|-----------|
| GLU (mg/dL)            |             | 171.72 ± 8.34 | 175.00 ± 10.53 | 202.80 ± 9.31* | 196.80 ± 4.76* |
| URE (mg/dL)            |             | 49.01 ± 8.24  | 45.18 ± 8.93  | 38.24 ± 9.09  | 45.10 ± 4.74  |
| CRE (mg/dL)            |             | 0.65 ± 0.10   | 0.70 ± 0.14   | 0.63 ± 0.18   | 0.80 ± 0.18   |
| ASAT (U/L)             |             | 120.66 ± 8.33 | 107.80 ± 8.13 | 113.42 ± 3.73 | 107.71 ± 4.11* |
| ALAT (U/L)             |             | 49.11 ± 6.90  | 44.66 ± 4.90  | 48.00 ± 5.93  | 52.50 ± 6.69  |

Data are expressed as mean ± S. D. One-way ANOVA followed by Tukey test, when appropriate (n = 5). Blood sugar levels, blood urea nitrogen (URE), blood creatinine (CRE), aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT). Differences between the groups were considered to be significant when p < 0.05. (*) Different from the control.

Table 2: Effects of sub-acute administration of C. iguanaea for 28 days on biochemical parameters in rats.

With regard to hematological findings after the treatment for 28 days with C. iguanaea, the averages of RBC, HGB, HCT as well as the erythrocyte indices (VCM, HCM and CHCM) and platelets, behaved within the established normal ranges in relation to the control. In relation to the average values white series, there was no significant difference in the variables, when correlated with the control group (Table 3).

In relation to histological analysis of the kidney, approximately 900 glomerulus were analyzed. Both the parameters area and nephrotic glomerular area presented significant differences between the 400 mg/kg group and control group (Figures 2A and 2B). In the overview, there was a compensatory reduction in nephron area and corpuscle, without change in the subcapsular area. The histological analysis of the liver was approximately 5600 hepatocyte area and cell density of 300 courses. For all parameters, there were significant differences between
the 400 mg/kg group and the control group. There was an increase in average hepatocyte area with a reduction in the density of hepatocytes and Kupfer cells (Figures 2C and 2D). This result indicates a significant increase in hepatic metabolism. A result of increased hepatocyte metabolism suggests a concomitant increase in the cytoplasmic area, justifying the reduction in the average nuclear density in the group 400 mg/kg. The groups treated with 100 mg/kg and 200 mg/kg of *C. iguanaea* showed no difference in histological liver and kidney from the analysis of the control group (data not shown).

| Hematological parameters | Control | 100 mg/kg | 200 mg/kg | 400 mg/kg |
|--------------------------|---------|-----------|-----------|-----------|
| RBC (× 10^6/ul)          | 8.48 ± 0.28 | 7.71 ± 0.20 | 7.87 ± 0.33 | 8.12 ± 0.40 |
| HGB (g/dL)               | 17.00 ± 0.62 | 16.00 ± 0.44 | 15.83 ± 0.15 | 16.30 ± 0.57 |
| HCT (%)                  | 46.53 ± 2.65 | 43.00 ± 0.62 | 43.16 ± 0.64 | 44.17 ± 1.83 |
| MCV (fL)                 | 54.87 ± 1.30 | 55.90 ± 1.64 | 54.96 ± 2.23 | 54.55 ± 1.09 |
| MCH (pg)                 | 15.71 ± 1.28 | 16.05 ± 0.75 | 14.98 ± 1.02 | 15.37 ± 1.35 |
| MCHC (g/dL)              | 36.53 ± 1.75 | 37.20 ± 0.37 | 36.60 ± 0.65 | 36.77 ± 0.69 |
| RDW (%)                  | 14.56 ± 0.57 | 14.60 ± 1.02 | 13.20 ± 1.80 | 14.10 ± 1.62 |
| WBC (× 10^3/ul)          | 8.66 ± 1.51 | 10.90 ± 0.00 | 17.30 ± 10.39 | 13.27 ± 1.17 |
| Lymphocytes (%)          | 75.66 ± 10.02 | 77.00 ± 0.00 | 74.33 ± 2.51 | 75.25 ± 7.13 |
| Neutrophils (%)          | 23.00 ± 9.53 | 22.00 ± 0.00 | 23.00 ± 1.00 | 22.00 ± 6.98 |
| Monocytes (%)            | 0.66 ± 1.15 | 1.00 ± 0.00 | 0.66 ± 1.15 | 2.00 ± 0.81 |
| Eosinophils (%)          | 0.66 ± 0.57 | 0 ± 0 | 0 ± 0 | 0.75 ± 0.50 |
| PLT (× 10^3/ul)          | 1024.66 ± 147.65 | 1139.00 ± 67.45 | 1045.33 ± 52.20 | 1083.00 ± 38.74 |

Data are expressed as mean ± S.D. One way ANOVA followed by Tukey test, when appropriate. Red Blood Cells counts (RBC), Hemoglobin (HGB), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Cell Hemoglobin (MCH), Mean Cell Corpuscular Hemoglobin Concentration (MCHC), Red cells Distribution Width (RDW), Leukocytes (WBC) and Platelet (PLT). Differences between groups were considered to be significant when p < 0.05.

Table 3: Effects of the sub-acute administration of *C. iguanaea* on red cells, white cells, differential leukocytes and platelets in rats.
Discussion

Medicinal plants are discriminately used in the treatment of various diseases and presumed to be safe. Moreover, usage is based on popular knowledge, passed on by cultural groups that have a direct contact with nature and explore their potential, passing on the knowledge from generation to generation [3]. The species C. iguanaea, popularly known as “Esporão-de-galo” in Portuguese, is a plant used for the treatment of joint pain, poor digestion, as a diuretic, and in the treatment of gastric ulcers [4]. The present study demonstrated a comprehensive toxicological evaluation by performing acute and sub-acute oral toxicity studies in Wistar rats.

By observing the chromatographic analysis of C. iguanaea, it was possible to identify five mostly phenolic compounds present in the plant: gallic acid, chlorogenic acid, ellagic acid, quercetin and rutin. The presence of these phenolic compounds is corroborated by the data present in studies conducted by Paula et al. and Trevisan et al. [8,9].

In toxicity, systemic toxicity signals are defined by the reduction in body mass of experimental animals, as well as reduced weight gain. Systemic toxicity is manifested by reduction in water and food consumption, behavioral changes, apathy and poor coat condition, such as the presence of goose bumps, can also be expressed by contact with nature and explore their potential, passing on the knowledge from generation to generation [3]. The species C. iguanaea, popularly known as “Esporão-de-galo” in Portuguese, is a plant used for the treatment of joint pain, poor digestion, as a diuretic, and in the treatment of gastric ulcers [4]. The present study demonstrated a comprehensive toxicological evaluation by performing acute and sub-acute oral toxicity studies in Wistar rats.

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