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STUDY OF ACUTE AND SUBACUTE TOXICITIES AND GENOTOXIC AND MUTAGENIC POTENTIALS OF THE LYOPHILIZED EXTRACT OF Campomanesia sessiliflora (O.Berg) MATTOS LEAVES IN WISTAR RATS

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Abstract

Campomanesia sessiliflora (O.Berg) Mattos is a Brazilian native plant species used in a popular medicinal tea for treating gastrointestinal, urinary, and dermatological pathologies. This study evaluated the toxicity of *Campomanesia sessiliflora* (O.Berg) Mattos via acute and subacute toxicity tests. It also analyzed mutagenic and genotoxic potentials by the micronucleus test, which detects genetic material damage indicating mutagenicity, and the comet assay, which assesses DNA damage levels as a genotoxicity indicator. The plant extract initially originated from the ultrasonic maceration of *Campomanesia sessiliflora* (O.Berg) Mattos leaves in a hydroethanolic solution. The involved animals were adult Wistar rats. Ten females were available to evaluate acute toxicity and estimate the LD50, receiving a dose of 2000 mg/kg. The subacute toxicity evaluation used 35 females and 35 males divided into seven groups: negative control (saline control – SC), positive control (cyclophosphamide control – CC), 125 mg/kg (125), 250 mg/kg (250), 500 mg/kg (500), 1000 mg/kg (1000), and the satellite group (ST). Genotoxicity and mutagenicity experiments applied bone marrow micronucleus and comet assays. Acute and subacute toxicity tests did not present behavioral, physical, and physiological changes ($p \geq 0.05$). Administering the *Campomanesia sessiliflora* (O.Berg) Mattos extract reduced spleen size in male and female animals, without histopathological changes. However, doses above 500 mg/kg showed significant genotoxic and mutagenic effects in the comet and micronucleus assays compared to the control group. The extract did not exhibit acute or subacute toxicity, but doses higher than 500 mg/kg indicated some level of genotoxicity and mutagenicity.

Keywords: Comet assay. *Campomanesia sessiliflora*. Medicinal plants. Micronuclei, toxicity.

1. Introduction

Campomanesia sessiliflora (O.Berg) Mattos is a plant species from the *Campomanesia* genus and the *Myrtaceae* family. *C. sessiliflora* (O.Berg) Mattos is popularly called green guabiroba in the Brazilian Cerrado biome region (de Jesus et al 2020; WFC 2023). *C. sessiliflora* species appear in central rural areas of Minas Gerais and Mato Grosso do Sul (Cardoso et al. 2010; Lescano et al. 2019), where the population widely consumes its fruits and uses its leaves as a medicinal tea (Castro et al. 2020) for treating gastrointestinal, urinary, and dermatological pathologies (Cardoso et al. 2010). Thus, the extensive application of *C. sessiliflora* promotes social, cultural, and economic relevance in the Brazilian Midwest region. Although it is still incipient, evidence suggests an antioxidant, photoprotective, antiproliferative, and antimicrobial action of *C. sessiliflora* (Catelan et al. 2019; Castro et al. 2020), increasing the interest in verifying the advantages of using the plant as an herbal medicine. Overall, there is a continuous search for new treatments capable of optimizing standard therapies (Verschaeve and Van Staden 2008).

In this context, plants are a promising source of metabolites of medicinal interest. They produce various specialized secondary metabolites, including numerous active or complementary compounds. This diversity is due to several factors, such as high plant biodiversity in many regions of the world and the ecological role of these compounds in plant physiology, as they face several challenges, including protection against herbivores, pathogens, stress (UV radiation protection), and other interactions that may promote pharmacological actions (Pirintsos et al. 2022). Another possibility is using different plant parts with potentially distinct metabolites. Thus, there has been an increasing interest in medicinal plants with promising pharmacological activity, usually related to defense mechanisms mediated by secondary metabolites with biological activities.

There is also growing attention to economically viable medicinal plants, which are easily accessible, have lower rates of adverse reactions, are reliable, and provide low production costs (Hamedi et al. 2022). Besides the highly sophisticated drugs from medicinal plants, medicinal plant applications are most significant in Brazilian primary health care. However, medicinal plant use is empirically based on traditional and colloquial applications without proof of therapeutic efficacy and possible adverse effects from their consumption (de Moura et al. 2020).

However, most medicinal plants have cytotoxic, mutagenic, and genotoxic components capable of interacting with human DNA, promoting cellular mutations (Verschaeve and Van Staden, 2008; Campos et al. 2016). Hence, public health and environmental agencies in several countries require mutagenicity and toxicity tests before introducing a drug into the market, even if it is herbal.

Therefore, analyses are needed to demonstrate cytotoxic, mutagenic, and genotoxic potentials, which may be investigated through analyses, such as the micronucleus test in the hematopoietic bone marrow of rats and the comet assay (Varanda 2006). The micronucleus test detects damage induced by the evaluated substance in the chromosomes or the mitotic apparatus of erythroblasts using the erythrocytes sampled in the bone marrow of rats. It aims to identify substances that cause cytogenetic damage by forming micronuclei, delayed chromosomal fragments, or entire chromosomes. An increased frequency of micronucleated polychromatic erythrocytes in treated animals indicates induced chromosomal damage (OECD 2016a).

The comet assay (*in vivo* single-cell alkaline gel electrophoresis) measures DNA strand breaks in eukaryotic cells. Hence, cell suspensions are made and incorporated into agarose slides treated with a lysis buffer to remove cell and nuclear membranes. Subsequently, the material undergoes electrophoresis at a high pH to detect single and double-strand breaks in DNA and chromosomal damage that promotes comet-like structures under fluorescent microscopy (OECD 2016b). Regulatory agencies widely accept both methodologies, and the scientific community extensively applies them to investigate the safety of herbal medicine consumption (Varanda 2006).

Considering the use of *Campomanesia sessiliflora* (O.Berg) Mattos as food (fruits) and popular medication (tea made from the tree's leaves and bark) and the potential for herbal medicine production based on its biological activity, use safety must be evaluated. Therefore, this study assessed *in vivo* acute and subacute toxicities, mutagenicity, and *in vivo* genotoxicity of *Campomanesia sessiliflora* (O.Berg) Mattos.

2. Material and Methods

Study location

All experiments occurred in the Animal Facility, the Laboratory of Toxicological Tests (LETOX), and the Laboratory of Surgical Techniques of the Faculty of Health Sciences (FCS) of the Federal University of Grande Dourados (UFGD – Dourados, MS, Brazil).

Animals

The Vivarium of the Federal University of Grande Dourados provided all Wistar rats (male and female) involved in the experiments. The animals remained in polypropylene plastic cages in a temperature-controlled environment (22 ± 2°C), on a 12-hour light/dark cycle, and treated with standard chow and water *ad libitum*. The experimental protocol received approval from the Animal Research Ethics Committee of the Federal University of Grande Dourados via protocol #04/2020. The experiments used 80 adult and albino Wistar rats aged eight to 12 weeks, with 45 females and 35 males weighing 250 to 300g.

Plant material and extract attainment

C. sessiliflora leaves were collected in Dourados (22º 14' 16" S and 54º 48' 02" W), MS, Brazil. Dr. Maria do Carmo Vieira identified the plant. The specimen was deposited in the herbarium of the Federal University of Grande Dourados (DDMS00005255). The collected leaves were dried and crushed, and the solutions were prepared at a ratio of 50 mg of dry leaves to 25 mL of solvent. The hydroethanolic extract (ethanol:water 80:20 v/v) was obtained via ultrasound extraction (two hours). The infusion was readied with hot water (95°C) muffled for 30 minutes. After the extraction, the samples went through filter paper and were reconstituted in a volumetric flask with the same volume as the amount of extracting solvent. Finally, filtration occurred, including a concentration in the rotary evaporator and extract drying via lyophilization (Kataoka and Cardoso, 2013). The lyophilized extract was reconstituted in filtered water for gavage.

Acute toxicity

The acute oral toxicity assessment followed the OECD Guideline 425 (OECD 2022). The animals were divided into two groups of five females each. One group received the lyophilized extract reconstituted in distilled water in a single 2000mg/kg dose. The control group underwent the same conditions with a 0.9% saline solution (saline control – SC). After administration, the groups were evaluated during the first 24 hours, initially at 15, 30, and 60 minutes, and then every four hours. From the second day onwards, the animals were evaluated once a day up to the 14^{th} day for standard animals and up to the 28^{th} day for the satellites. The verified and recorded parameters were clinical signs (piloerection, contortions, tremors, convulsions, cyanosis, ataxia, and diarrhea), physiological data (body weight, water, and feed consumption), and behavioral changes. Mortality was evaluated to estimate the LD50, the lethal dose for 50% of animals. The animals were euthanized on the 15^{th} day, with ketamine (ketamine/Ceva/Sespo/Brazil) and xylazine (2% xylazine hydrochloride/Syntec/Brazil) anesthesia at 25 and 10 mg/kg, respectively, and an inhaled overdose of isoflurane (Baxter/Herlan/USA). After confirming the death by analyzing vital signs and corneal reflex, exsanguination was performed by cardiac puncture. Macroscopic analyses and weighing of critical organs were performed.

Subacute toxicity

The acute oral toxicity evaluation followed the OECD Guideline 407 (OECD, 2008). This test used 70 animals divided into seven groups of ten (five males and five females). Four doses were administered daily by gavage for 28 days. Group 1 received 125 mg/kg (125), Group 2 received 250 mg/kg (250), Group 3 received 500 mg/kg (500), and Group 4 received 1000 mg/kg (1000). The negative control group received

0.9% saline (saline control – SC), and the satellite group had 1000 mg/kg of the extract for 28 days (ST), remaining untreated for another 14 days to observe late clinical signs of toxicity or the reversal of toxic signs. The latter group (positive control) received a saline solution and an intraperitoneal injection of 50 mg/kg of cyclophosphamide (cyclophosphamide monohydrate/Sigma-Aldrich - Brazil) 24 hours before euthanasia (cyclophosphamide control – CC). Physiological data (body weight, water, and feed consumption), abnormal behavioral changes, and clinical signs (piloerection, contortions, tremors, convulsions, cyanosis, ataxia, and diarrhea) were observed and recorded daily. The animals were euthanized on the 29th day, with ketamine anesthesia (ketamine/Ceva/Sespo/Brazil) and xylazine (2% xylazine hydrochloride/Syntec/Brazil) at 25 and 10 mg/kg, respectively, and an inhaled overdose of isoflurane (Baxter/Herlan/USA). After confirming the death by analyzing vital signs and corneal reflex, exsanguination was performed by cardiac puncture. Then, the blood was available for laboratory analysis. Vital organs (heart, lungs, liver, kidneys, spleen, ovary, uterus, testes, and epididymis) were macroscopically evaluated, weighed, and submitted to a histological section.

Micronucleus test

Micronucleus tests occurred parallel to animals treated in subacute toxicity. Test procedures followed the OECD Guideline 474 (OECD 2016a) and the protocol by Ribeiro et al. (2003). After euthanasia, each animal had the right femur removed and the bone marrow canal exposed through a cut at the end of the femur. Next, the medullary canal received 1 ml of fetal bovine serum to remove the cells, and the suspension was centrifuged for five minutes at 1000 RPM. The supernatant was removed until 0.5 ml remained in the tube, and the cells were placed on the slide with a single medium compression smear at a 45-degree angle. The slides were dried for 24 hours, fixed with methanol for five minutes, stained with pure Giemsa dye for three minutes, then with a 1:6 dilution for 15 minutes, passed in running water, and dried again for 24 hours. Finally, they were microscopically evaluated with a 40x immersion objective. Each animal had 2000 polychromatic erythrocytes analyzed.

Comet assay

The comet assay occurred parallel to animals treated for subacute toxicity. Test procedures followed the OECD Guideline 489 (OECD 2016b). Blood cells from the animals' bone marrow were inserted into slides prepared with agarose, subjected to electrophoresis, stained with ethidium bromide, and evaluated under a fluorescence microscope. One hundred fifty cells from each sample were analyzed. The classification of these cells considered tail size into four classes: Class 0: no damage; Class 1: low damage; Class 2: medium damage; Class 3: maximum damage; Class 4: apoptosis.

The statistical analysis included the scores generated by each slide. This damage index corresponds to the total products of the multiplication of comets versus their class, calculated with the following formula:

Score = (number of cells class 0 x 0) + (number of cells class 1 x 1) + (number of cells class 2 x 2) + (number of cells class 3 x 3) + (number of cells class 4 x 4)

Damage frequency was the percentage of all class 1 to 4 comets relative to total comets.

Damage frequency = [(total number – class number 0).100] / total number.

Biochemical and hematological analyses

The biochemical evaluation analyzed total bilirubin, total cholesterol and fractions, triglycerides, glucose, electrolytes (sodium, potassium, and calcium), liver function markers (alanine aminotransferase - ALT and aspartate aminotransferase - AST), renal function markers (urea, creatinine, and uric acid), and the protein profile (albumin and total protein). The hematological evaluation considered differential counts of

leukocytes, erythrocytes, platelets, hemoglobin levels, hematocrit, and distribution width of red blood cells (Traesel et al. 2015).

Histopathological procedures

After blood collection, vital organs (heart, lung, kidney, liver, and spleen) and reproductive organs (testicles, epididymis, uterus, and ovary) were weighed and dissected. The samples were fixed with 10% buffered formalin. After fixation, the fragments were cleaved, dehydrated with increasing absolute concentrations of ethanol, cleared in xylenol, and embedded in paraffin. Sections were cut to a thickness of 5 µm, stained with hematoxylin and eosin (H&E), and mounted on glass slides for light microscopy examination (Martey et al. 2010).

Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) for each treatment group. The statistical evaluation used an analysis of variance (ANOVA) followed by the Bonferroni post-hoc test, with $p<0.05$ representing significant differences between treated and control groups.

3. Results

In the acute toxicity test, administering a 2000mg/kg dose for the group treated with *C. sessiliflora* extract and the negative control did not cause death or any other sign of immediate toxic effect on the animals. Daily assessments through Hippocratic screening did not show changes in the behavioral, physical, and physiological states of the treated animals and the negative control.

Regarding weight gain, the progression between the evaluated groups did not demonstrate significant differences ($p\geq0.05$) (Figure 1A). The linear regression model of weight progression also did not show significant differences between groups. It is worth noting the different trends between groups, evidenced by the lower position of the linear regression curve in the group receiving *C. sessiliflora* compared to the untreated group (data not shown). As for water and feed consumption, there were no differences between the average consumption of animals allocated in the treatment and control groups (Figure 1 B-C).

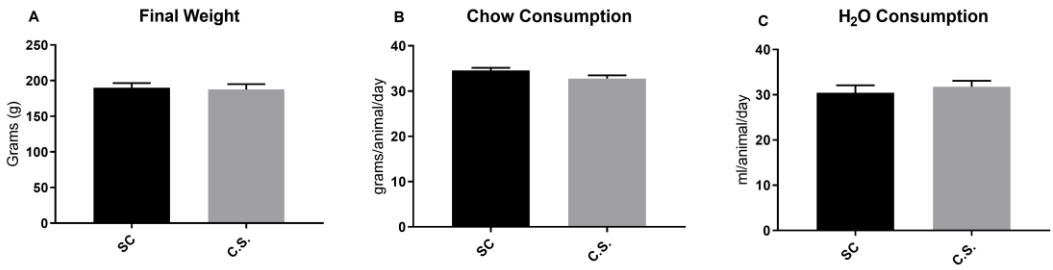


Figure 1. Final weight and chow and water consumption between saline control (SC) and *C. sessiliflora* (C.S) groups in acute toxicity. Values expressed as mean ± SEM.

The mean weight gain during the subacute experiment expressed possible toxicity. Only the 250mg/kg group of females significantly differed from the saline control (SC) and cyclophosphamide (CC) groups. Considering the absence of a dose-dependent effect or the verification of the same impact on the experiment with males, we attributed the effect to random data distribution (Type I statistical error). The progression of the animals' weight during the subacute toxicity test did not sustain significant differences in the weight gain curve between groups or the animals' sexes. Also, linear regression slope lines did not show significant differences (data not shown). There were also no significant differences in mean feed and water intake in males or females during the subacute toxicity experiment (Figure 2).

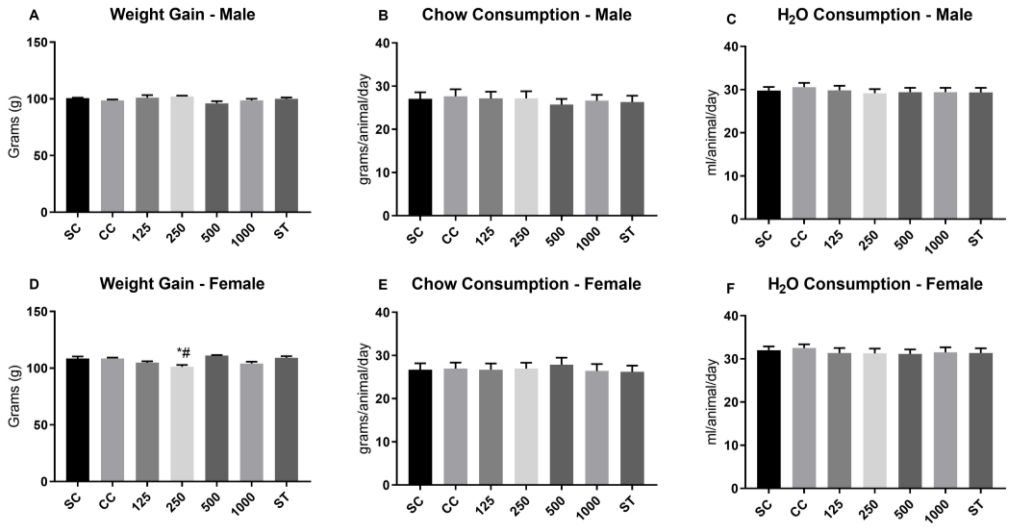


Figure 2. Weight gain and chow and water consumption between male and female rats in subacute toxicity. Values expressed as mean ± SEM.

Table 1 shows the animals' biochemical data. Uric acid and triglyceride biochemical markers showed significant differences between groups and animals' sexes. All female groups treated with *C. sessiliflora* extract showed significantly lower uric acid levels than those in the control group. This reduction was higher at doses above 250 mg/kg. Only the 250 and 500mg kg doses showed a significant decrease in male animals, without a significant effect at the 1000mg/kg dose. It is worth noting that the effect of the cyclophosphamide intervention showed different behavior between the sexes. In females, the intervention significantly reduced uric acid levels in the saline control group. However, this effect did not occur among males. Another distinct effect between sexes was the behavior of plasma triglycerides (Table 1) in females. The arithmetic mean progressed with the evolution of the administered dose, with 1000 mg/kg presenting significantly higher values than that of the saline control group. Male animals did not experience this effect.

Table 1. Biochemical parameters in male and female rats in subacute toxicity.

Male	SC	CC	125	250	500	1000
Glucose	266.4±29.5	214.6±27.5	295.6±40.9	226.7±17.5	208.3±60.4	221.2±25.8
Total Protein	53.6±2.5	59.3±2.4	53.9±6.7	52.3±1.2	48.1±6.9	56.3±0.5
Creatinine	0.3±0.1	0.4±0.1	0.3±0.1	0.3±0.1	0.1±0.1#	0.4±0.1
Cholesterol	52.1±5.4	51.4±7.7	51.6±9.6	84.8±2.9	66.5±19.5	73.5±6.4
Albumin	35.9±1.5	37.7±1.6	34.2±3.8	30.5±1.9	31.3±5.1	34.1±1.8
Uric Acid	3.4±0.6	3.8±0.3	2.9±0.4	1.4±0.4*#	1.9±0.3#	3.0±0.5
Urea	32.2±3.2	33.8±4.4	31.1±2.3	29.9±3.0	26.0±3.2	40.3±4.1
ALT	138.4±23.5	127.9±25.4	109.3±33.3	155.1±33.3	53.9±13.4	166.0±62.5
AST	197.4±22.9	226.3±33.7	185.5±56.9	356.6±51.4	144.4±30.6	302.9±47.1
HDL	31.0±3.3	34.7±3.0	29.7±5.4	45.6±2.2	34.1±10.5	37.5±2.5
Triglycerides	37.8±5.1	28.2±6.1	59.3±14.1	77.8±17.0#	57.0±10.6	44.3±8.7

Female	SC	CC	125	250	500	1000
Glucose	224.3±27.7	152.5±31.8	234.9±27.2	245.1±28.3	196.9±27.7	218.4±49.9
Total Protein	59.2±1.7	50.9±9.8	57.8±2.0	56.8±0.6	53.3±3.5	46.7±7.3
Creatinine	0.4±0.1	0.2±0.2	0.4±0.1	0.2±0.1	0.1±0.1	0.1±0.1
Cholesterol	63.7±0.7	51.5±12.4	52.9±3.6	45.3±3.3	59.6±11.2	53.5±9.3
Albumin	39.4±1.8	32.5±6.6	39.4±1.1	39.2±1.1	33.1±2.0	28.0±5.2
Uric Acid	5.7±1.6	2.6±0.4*	3.0±0.3*	1.6±0.2*	1.7±0.6*	1.7±0.6*
Urea	38.4±1.8	29.1±3.8	27.6±2.5	34.7±3.3	31.7±5.2	26.2±4.8
ALT	44.3±10.5	41.6±6.8	33.7±2.9	31.4±1.4	62.4±13.7	32.4±6.2
AST	171.3±38.1	166.0±11.7	132.3±18.0	144.2±34.4	239.3±82.8	185.9±29.3
HDL	41.5±3.0	30.0±9.3	32.3±2.0	26.8±3.7	30.2±5.8	24.4±5.8
Triglycerides	35.6±3.8	39.0±10.1	38.2±3.1	52.6±9.7	61.9±17.2	88.6±15.8*#

Values expressed as mean ± SEM. (SC) Saline control; (CC) Cyclophosphamide control; (*) Statistically different from SC; (#) Statistically different from CC.

The animals' blood counts showed different effects between the sexes (Table 2). White blood cell counts were higher in females receiving a 1000mg/kg dose. However, this effect did not occur in males. Red blood cell, hemoglobin, and hematocrit counts showed significantly lower values than the control at 125 mg/kg. However, their expressions increased at 500 mg/kg, while this effect did not occur in females.

Table 2. Hematological parameters in male and female rats in subacute toxicity.

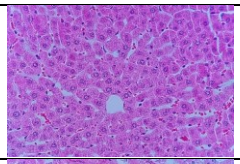
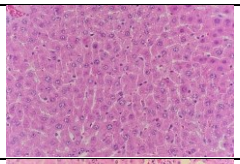
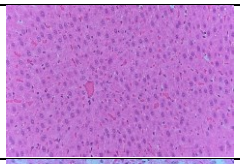
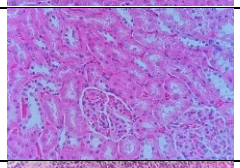
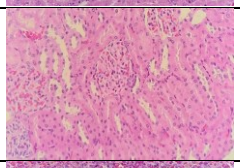
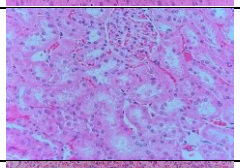
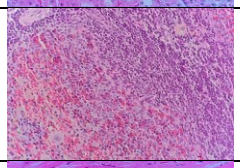
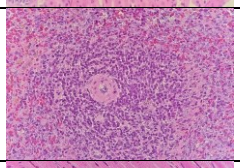
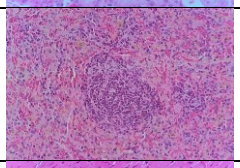
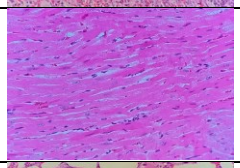
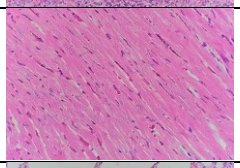
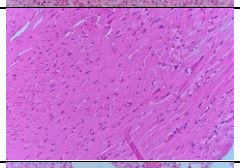
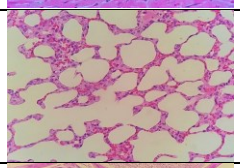
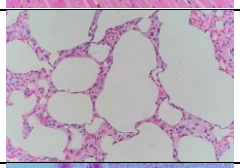
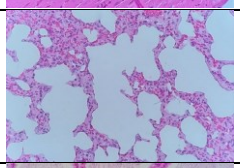
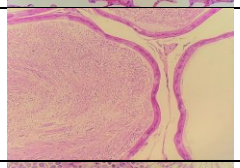
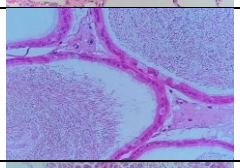
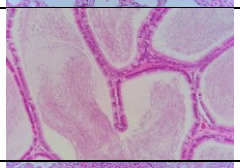
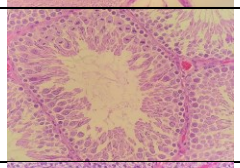
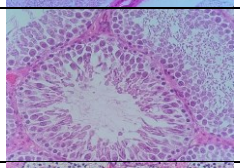
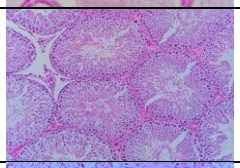
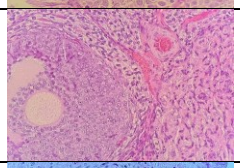
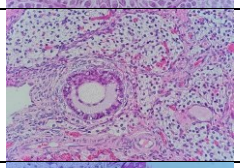
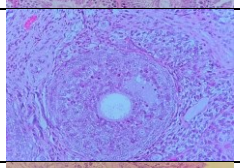
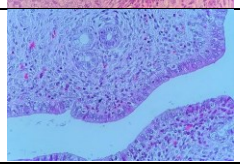
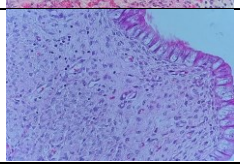
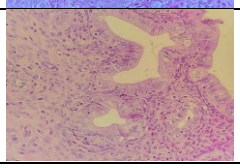
Male	SC	CC	125	250	500	1000
WBC	2.5±0.3	2.7±1.2	3.8±0.5	7.9±3.2	6.1±1.4	3.8±0.6
RBC	6.9±0.2	7.0±0.1	4.5±0.1*#	7.1±0.2	7.3±0.3	6.6±0.3
HGB	12.8±0.1	13.4±0.1	8.8±0.3*#	13.5±0.3	14.8±0.7*	12.8±0.3
HCT	34.8±0.5	36.1±0.4	23.1±0.9*#	36.3±1.0	39.6±1.7*	33.7±1.2
MCV	50.8±0.6	51.5±0.4	51.4±1.2	51.1±0.5	54.3±1.0*	51.4±0.5
MCH	18.7±0.4	19.1±0.3	19.5±0.6	19.1±0.3	20.3±0.5	19.5±0.5
MCHC	36.8±0.4	37.0±0.3	37.9±0.4	37.3±0.3	37.3±0.3	38.0±0.7
PLT	896.5±27.0	862.3±42.7	580.0±22.7*#	936.3±70.6	688.6±86.7	904.3±50.6
LYM%	57.0±4.7	62.0±2.7	69.6±3.6	49.7±8.7	59.2±0.7	45.8±4.6
NEUT%	36.3±4.4	33.8±2.7	27.0±3.5	45.0±8.0	34.4±0.7	47.3±4.6
RDW-SD	28.1±0.5	27.8±0.1	27.1±0.3	27.9±0.1	28.8±0.2	28.2±0.3
RDW-CV	12.8±0.5	12.4±0.2	11.4±0.7	12.4±0.1	12.4±0.3	12.6±0.4
MPV	7.6±0.1	7.5±0.1	11.4±0.7*#	7.4±0.2	8.1±0.1	8.2±0.1
EOS	3.0±0.4	2.5±0.3	1.4±0.2*	2.3±0.3	3.4±0.2	3.3±0.5
MONO	3.8±0.5	1.8±0.5*	2.0±0.3*	3.0±0.6	3.0±0.3	3.8±0.5#
Female	**SC**	**CC**	**125**	**250**	**500**	**1000**
WBC	3.0±0.3	3.0±1.2	2.5±0.4	3.9±0.3	2.6±0.4	7.2±0.1*#
RBC	5.6±0.5	5.8±0.1	4.7±0.4	5.3±0.5	5.3±1.1	7.1±0.2
HGB	12.3±1.2	12.2±0.3	9.8±1.0	11.2±1.2	11.4±2.2	13.5±0.4
HCT	31.1±2.9	32.0±0.3	24.7±2.1	28.6±3.0	29.6±5.8	36.7±1.2
MCV	55.3±0.3	55.4±1.2	53.0±1.1	54.2±0.7	55.5±0.4	51.7±2.7
MCH	21.9±0.4	21.1±0.1	21.0±0.6	21.3±0.3	21.4±0.2	19.1±1.0*
MCHC	39.6±0.6	38.1±0.8	39.6±0.5	39.3±0.2	38.6±0.2	36.9±0.1*
PLT	785.5±82.6	823.3±77.2	534.5±93.1	599.8±84.0	524.5±284.5	880.5±62.5
LYM	55.0±4.9	57.3±4.9	65.3±4.5	63.8±4.9	63.5±0.5	56.5±1.5
NEUT	41.3±4.2	38.3±4.3	30.5±4.4	32.0±4.2	31.5±0.5	38.5±1.5
RDW-SD	26.2±0.2	27.6±1.1	26.2±0.1	26.3±0.3	25.9±0.5	27.5±0.8
RDW-CV	9.3±0.3	10.9±0.8	9.9±0.1	9.8±0.3	9.1±0.8	11.7±1.7
MPV	7.2±0.1	7.8±0.2*	7.4±0.1	7.4±0.1	7.3±0.1#	8.1±0.2*
EOS	1.3±0.3	1.7±0.3	1.5±0.3	2.0±0.6	2.0±0.1	2.5±0.5
MONO	2.5±0.6	2.7±0.3	2.8±0.3	2.3±0.5	3.0±0.1	2.5±0.5

Values expressed as mean ± SEM. WBC: White Blood Cell count (106/µL); RBC: Red Blood Cell count (106/µL); HGB: Hemoglobin concentration (g/dL); HCT: Hematocrit (%); MCV: Mean Corpuscular Volume (fL); MCH: Mean Corpuscular Hemoglobin (pg); MCHC: Mean Corpuscular Hemoglobin Concentration (g/dL); PTL: Platelet count (103/µL); LYM: Lymphocytes (%); NEUT: Neutrophils (%); RDW-SD: Red Cell Distribution Width - Standart Deviation; RDW-CV: Red Cell Distribution Width - Coefficient of Variation; MPV: Mean Platelet Volume; EOS: Eosinophils (%); MONO: Monocytes (%).

In females, doses above 1000 mg/kg significantly increased the expression of mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and mean platelet volume, while this effect did not occur in male animals. The 125mg/kg group showed atypical behavior not corresponding to the dose-response in hemogram parameters.

The histopathological report did not show relevant changes between groups (Table 3). The lungs showed mononuclear inflammatory infiltration with alveolar wall thickening. These changes are consistent with nonspecific interstitial pneumonia, possibly from administering substances by the superior route (gavage). Testicular fragments presented fixation artifacts due to the used material (formaldehyde instead of Bouin or modified Davidson's solution).

Table 3. Histopathological analysis at the end of subacute toxicity.

	SC	CC	1000
Liver			
Kidney			
Spleen			
Heart			
Lung			
Epididymis			
Testicle			
Ovary			
Uterus			

There were no identified histopathological abnormalities.

Organ weights indicated that the *C. sessiliflora* extract treatment significantly reduced spleen weight in males and females (Figure 3). Also, 1000mg/kg doses among males significantly reduced the testicles and increased the epididymis and kidneys.

The comet assay and damage frequency demonstrated that *C. sessiliflora* extract doses above 500 mg/kg have potential genotoxic effects (Figure 4). However, these doses present significantly lower damage than the cyclophosphamide intervention (CC group). The cell count and classification of the micronucleus test determined the mutagenic effect (Figure 4). In this test, the treatments did not change the total count,

polychromatic erythrocytes, and micronucleated polychromatic erythrocytes in females and males compared to the saline control group.

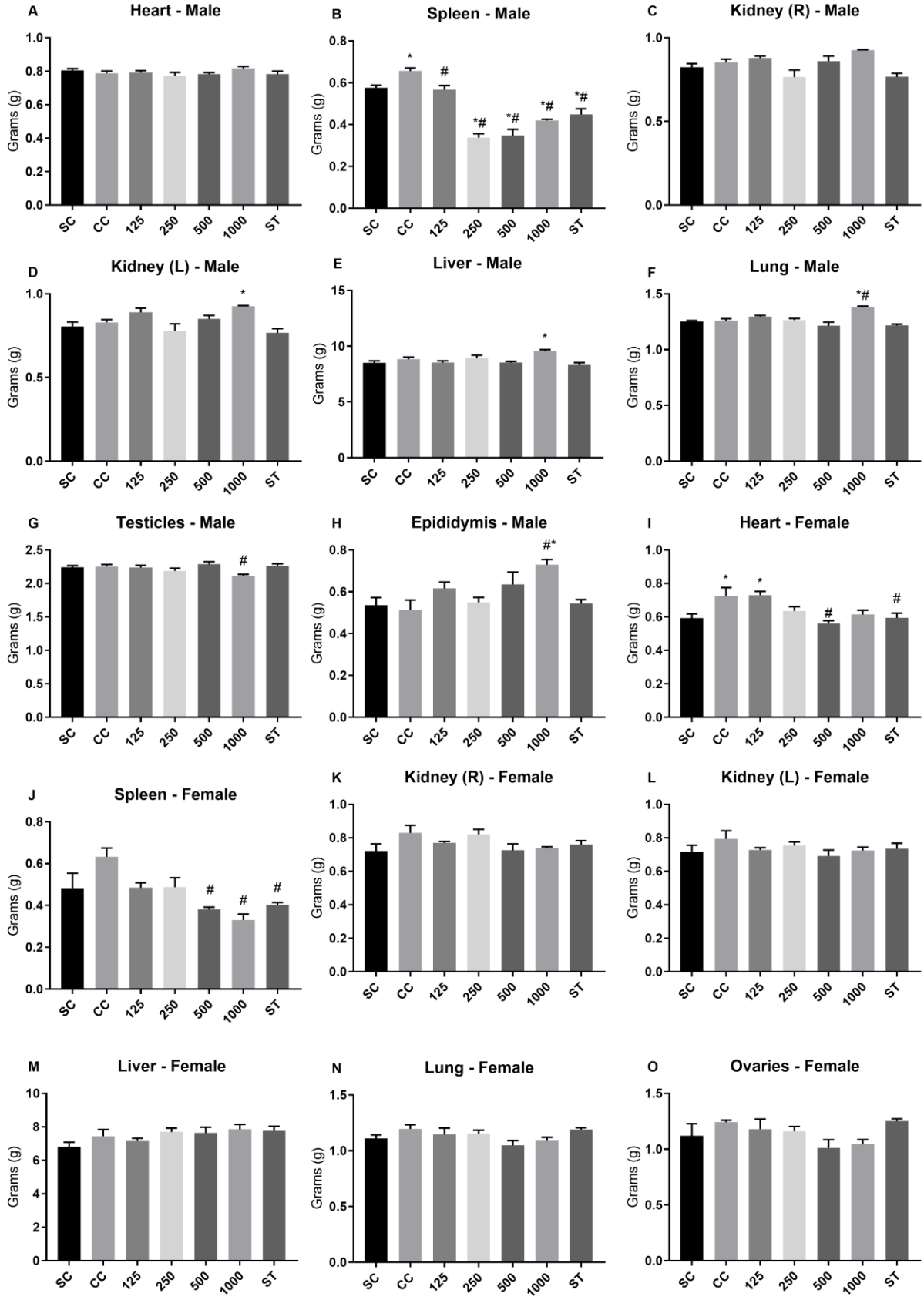


Figure 3. Organ weight in male and female rats at the end of subacute toxicity. Values expressed as mean ± SEM. (*) Statistically different from SC; (#) Statistically different from CC.

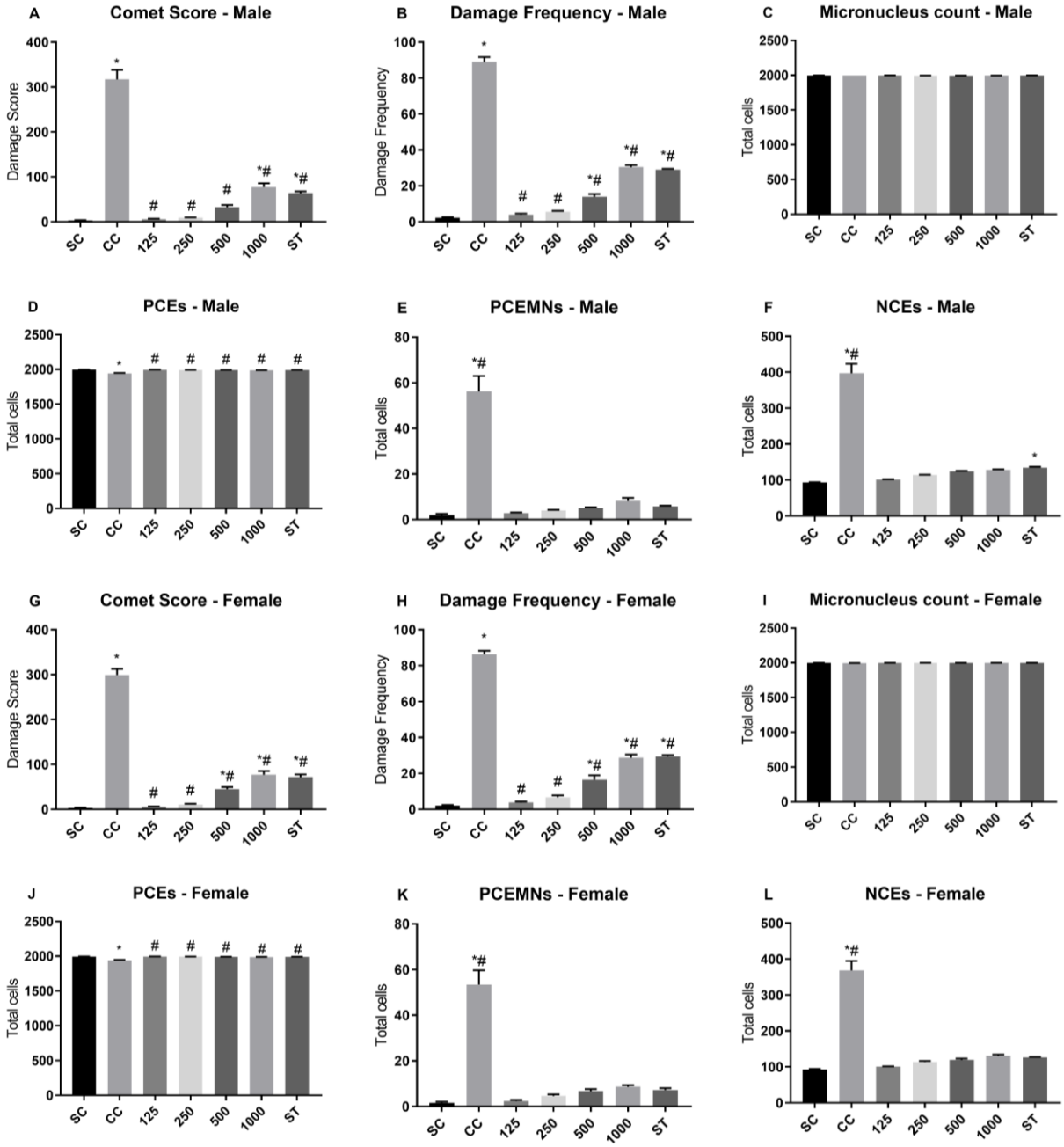


Figure 4. Comet and micronucleus assays in male and female rats at the end of subacute toxicity. Values expressed as mean ± SEM. (*) Statistically different from SC; (#) Statistically different from CC.

4. Discussion

Several health agencies worldwide, including the Brazilian Health Surveillance Agency (Anvisa), have encouraged phytotherapy and the research of new plant-based drugs with biological activity. Considering the extensive Brazilian biodiversity, the country has a high dormant potential for discovering new herbal medicines. However, the research of these new drugs must be rigorous to guarantee the selection of safe and effective herbal medicines. Hence, the World Health Organization has recommended several toxicity tests for plants used in traditional medicine, publishing guidelines for conducting these tests since 2004 (WHO 2004; Nakakaawa et al. 2023). That strengthened the performance of pharmacovigilance sectors in obtaining increasingly safe herbal medicines. Besides evaluating the safety of plant-based drugs of interest, the tests allow agencies to establish safe usage doses.

Thus, assessing toxicity patterns of medicinal plant extracts intended for clinical and/or popular use is crucial to determining consumption safety (Villas-Boas et al. 2021). Monitoring body weight gain and feed and water intake is relevant for assessing acute and subacute toxicities, as they provide essential information about altered physiological responses.

This study did not find signs of acute or subacute toxicity from behavior patterns, weight gain, and water and feed consumption during *C. sessiliflora* administration. Overall, *C. sessiliflora* fruits are usually consumed fresh. Also, teas made from its leaves and bark are disseminated in traditional medicine in the Brazilian Midwest region, suggesting inherent safety in their use/consumption. Furthermore, the Hippocratic screening did not show deaths or alterations, evidencing the absence of changes in the behavioral, physical, and physiological states in the treated animals and the negative control. As the extract of *C. sessiliflora* was neither toxic nor deadly in the acute trial, and its LD50 was higher than the threshold dose of 2000 mg/kg, this herbal drug is a promising candidate for herbal medicine.

This study found potential biochemical and hematological alterations related to the sex of the animal receiving the *C. sessiliflora* extract during the subacute toxicity protocol, potentially indicating a different response from male and female consumption and potential interaction with hormonal factors. In the acute and subacute trials, the high dosage did not affect general health parameters, such as weight gain and water and feed consumption. This finding agrees with previous studies showing low toxicity from *C. sessiliflora* extracts (Castro et al. 2022; Silva et al. 2022). Other investigations involving plants of the *Campomanesia* genus, such as *Campomanesia velutina* (Cambess) O. Berg and *Campomanesia pubescens* (D.C.) O. Berg, also showed little influence on general health parameters, such as death, weight gain, and water and feed intake (Araújo et al. 2017; Villas Boas et al. 2018).

Regarding the biochemical outcome of the subacute toxicity protocol, the effects of *C. sessiliflora* call attention to hematological parameters. Deviations in biochemical and hematological parameters are relevant for reflecting changes in the hematopoietic system, vital for maintaining an individual's life and health (Jacob Filho et al. 2018). From a toxicological point of view, deviations in hematological parameters are critical because they threaten life and health. Although some parameters showed significant differences, others did not present a dose-dependent behavior, hindering the establishment of an assertive cause-effect. Future studies with chronic toxicity protocols might specifically address these issues.

The observed cyclophosphamide effects on monocytes, especially in male animals, corroborates the literature. Cyclophosphamide is a chemotherapy agent known for inducing significant immunosuppressive effects, potentially decreasing monocyte levels. This association has been reported and documented by McBride et al. (1987), Moschella et al. (2013), and Yadav et al. (2020). These studies highlight the drug's impact on the immune system, specifically its ability to induce acute reductions in monocyte counts.

These authors suggest that such reduction in monocytes (part of the broader category of leukocytes) may compromise the body's immune response, particularly in males. The broader implications of this reduction in overall immune function and the potential influence on therapeutic and adverse effects of cyclophosphamide must be considered. However, the 125mg/kg dose used in this study also significantly affected male animals. These same effects did not occur when administering the protocol in females. This study hypothesized a sex differentiation in biochemical and hematological responses to *C. sessiliflora* extract administration, as other alterations were also restricted to a specific sex.

Considering the small relationship of dose-dependent alterations, *C. sessiliflora* is safe from hematological and, consequently, hematopoietic points of view. However, the consistency of these alterations and a better characterization of the effect’s validity would be better explored in a chronic toxicity study, whose test structure would favor higher data reliability and the confirmation/refutation of the raised hypotheses.

Administering *C. sessiliflora* extracts at doses higher than 500 mg/kg significantly reduced the spleen size of animals of both sexes. Immunotoxicity studies report that immune system suppressant substances increase spleen size, i.e., splenomegaly, as during urethane intervention (Robles 2014). Buchan et al. (2018) demonstrated that animals with splenomegaly induced by a high-fat diet showed significant spleen size reduction with physical exercise and genistein (isoflavones) interventions. Thus, it seems that *C. sessiliflora* may have a potential regulatory effect on splenic function and morphology due to its biochemical composition (Castro et al. 2020; Castro et al. 2022). Liu et al. (2017) and Vidal and Whitney (2014) discuss that significant changes in testicular and epididymal weight may be associated with potential changes in male reproductive function. These patterns indicate that such changes may cause adverse effects on fertility, suggesting the need for additional studies to explore these issues further. An in-depth investigation might focus on how these testicular and epididymal weight changes correlate to reproductive health, including

sperm quality and reproductive capacity of males (reduced testicular size and enlarged epididymis – Figure 3).

The comet assay and damage frequency demonstrated that doses above 500 mg/kg of *C. sessiliflora* extract have potential genotoxic effects. However, these doses present significantly lower damage than the cyclophosphamide intervention. A study with an aqueous extract of the *Campomanesia xanthocarpa* leaf at doses similar to our investigation did not show a significant genotoxic effect compared to the PBS/saline control group (de Sousa et al. 2019). A genotoxic study with *Campomanesia pubescens* extract also did not show a genotoxic effect from the comet assay (Villas Boas et al. 2018).

Doses higher than 500 mg/kg evidenced a mutagenic potential in males receiving *C. sessiliflora* extract. Villas Boas et al. (2018) found no differences between the groups treated with *Campomanesia pubescens* extract and the saline control group (negative control). Considering the divergence of research with other species of the *Campomanesia* genus, future reproducibility studies and toxicity comparisons between species are especially encouraged.

5. Conclusions

This study demonstrated that the *Campomanesia sessiliflora* (O. Berg) Mattos leaf extract does not present detectable acute or subacute toxicity *in vivo* regarding changes in behavior, weight gain, and feed and water consumption.

However, the biochemical responses (uric acid, triglycerides, and hematological parameters) seemed influenced by the animal's sex. The data suggest that the treatment with *C. sessiliflora* extract and cyclophosphamide caused different reactions between the sexes, indicating an influence of hormonal or metabolic factors on the efficacy of these treatments.

Moreover, doses higher than 500 mg/kg presented some genotoxicity and mutagenicity *in vivo* in comet and micronucleus assays, respectively. While these changes contribute to a higher understanding and safety of popular herbal medicines, further studies might explore the biochemical differences between sexes, the progression and/or support, and the potential clinical relevance of genotoxicity and mutagenicity testing in a chronic toxicity approach experiment.

Authors' Contributions: BURIGATO, A.A.: conception and design, animal experiment, data acquisition, and article drafting; SILVA, J.V.: conception and design, animal experiment, data acquisition, data analysis and interpretation, article drafting, and critical review; MUELLER, L.P.: animal experiment, data acquisition, data analysis, interpretation and critical review; ARAÚJO, F.H.S.: animal experiment, data acquisition and critical review; CARDOSO, C.A.L.: conception and design, data acquisition and critical review; SOUZA, R.I.C.: data analysis and interpretation and critical review; SOUZA, A.R.: animal experiment, data acquisition, data analysis and interpretation and critical review; JUNIOR, F.F.B.: data analysis, interpretation and critical review; OESTERREICH, S.A.: conception and design, data analysis and interpretation and article drafting. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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