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Original Article

Short-term carcinogenesis evaluation of *Casearia sylvestris*

Cleide A.S. Tirloni[a], Giseli K. Traesel[a], Francislaine A.R. Lívero[c], Salvador D.V. Neto[a], Ronaldo de Faria Junior[a], Thaís C. Paim[a], Joyce A. Santos[b], Silvia A. Oesterreich[a], Ariany C. Santos[a], Roosevelt I.C. Souza[a], Euclides L. Cardozo Junior[c], Arquimedes Gasparotto Junior[a,*]

[a] *Faculdade de Ciências da Saúde, Universidade Federal da Grande Dourados, Dourados, MS, Brazil*
[b] *Faculdade de Ciências Exatas e Tecnológicas, Universidade Federal da Grande Dourados, Dourados, MS, Brazil*
[c] *Laboratório de Farmacologia e Toxicologia de Produtos Naturais, Universidade Paranaense, Umuarama, PR, Brazil*

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ABSTRACT

Casearia sylvestris Sw., Salicaceae, is an important medicinal plant widely used in Brazil for the treatment of various cardiovascular disorders. This species was included as of interest by Brazilian Unified Health System. Although preclinical studies described cardiovascular protective effects and apparent absence of toxicity, no studies have evaluated its carcinogenic potential. In this study, we proposed a short-term carcinogenesis evaluation of *C. sylvestris* in Wistar rats, aiming to check the safety of this species to use it as proposed by Brazilian Unified Health System. *C. sylvestris* leaves were obtained and the crude extract was prepared by maceration from methanol/water. Wistar rats were orally treated for 12 weeks with 50, 250 or 500 mg kg^{-1} of crude extract or vehicle. Body weight, daily morbidity and mortality were monitored. Blood and bone marrow samples were collect for micronucleus test, comet assay and tumor markers evaluation. Vital organs were removed to macro and histopathological analyses. The crude extract did not induce mutagenic and genotoxic effects and no alterations were observed in important tumor markers. Finally, no detectable signs of injury through gross pathology or histopathological examinations were observed. Our results certify the absence of the crude extract toxicity, indicating its safety, even at prolonged exposure as proposed by Brazilian Unified Health System.

Introduction

It is estimate that about 80% of the world's population depends on traditional practices to primary health care, and 85% of this portion uses plants (WHO, 2011). Considering the difficult access of these populations to conventional forms of treatment, the World Health Organization (WHO) suggests the adoption of traditional practices as a tool for maintaining health and encourages the development of public policies to insert them into the official health system of its 191 member countries, including Brazil (WHO, 2011).

In this context, Brazilian Ministry of Health proposed actions for development of public policies for the inclusion of medicinal plants in the Brazilian Unified Health System (SUS), allowing the expansion of disease prevention, maintenance and recovery of health. The purpose is to expand the therapeutic options to users, ensuring access to medicinal plants in view of a complete health care (Carvalho et al., 2011). One of the strategies to attend this was the elaboration of Form Herbal Medicines of the Brazilian Pharmacopoeia, which supports the handling practices and dispensing in the SUS, contemplating 47 species of natural plants, including the *Casearia sylvestris* Sw., Salicaceae (Anvisa, 2011).

Casearia sylvestris is distributed in tropical regions and occurs in almost all Brazilian territory, where is popularly known as 'guaçatonga' (Torres and Yamamoto, 1986). Traditionally, its leaves and barks are used for diarrhea, fever, hyperlipidemia, inflammation, obesity, skin diseases and tonic health (Ferreira et al., 2011). Between pharmacological properties there are anti-cancer (Ferreira et al., 2016), antihyperlipidemic (Schoenfelder et al., 2008), antiinflammatory and antioxidant (Albano et al., 2013), antiatherogenic (Brant et al., 2014), besides no acute or prolonged toxicological effects (Ameni et al., 2015).

Despite the importance of medicinal plants for human care, their toxicological potential is frequently evaluated slightly and not as priority (Ferreira et al., 2007). Most of phyto-derivative commercialized medications, called "natural", are used by the population without having carried out a detailed study of the chemical

* Corresponding author.
E-mail: arquimedesjunior@ufgd.edu.br (A. Gasparotto Junior).

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composition, efficacy and safety of use. This result is limited knowledge about side effects, especially on the genetic material (Varanda, 2006).

Carcinogenic evaluation of the medicinal plants is essential to establish control measures in widespread use, before the plants being considered therapeutic agents. Carcinogenesis test are part of the regulatory process of new drug development, and life-span studies in rodents have been considered the gold standard since early in the 20th century. However, they require many financial resources, nearly thousand animals, and take approximately 2–3 years from initiation to report (Cohen and Arnold, 2016). Currently, short-term assays, (1–3 months of duration), have been proposed by regulatory authorities as inexpensive and rapid alternative to evaluate carcinogens substances (Bajpayee et al., 2005).

So, despite the *C. sylvestris* present important preclinical pharmacological effects no data on its carcinogenic potential have been published. Then, we proposed a preclinical short-term carcinogenesis evaluation of *C. sylvestris*, aiming to check the safety of this species to use it in herbal medicines as proposed by SUS.

Material and methods

Plant material and extract preparation

Leaves of *Casearia sylvestris* Sw., Salicaceae, were harvested in a botanical garden of Vera Cruz do Oeste, Brazil (620–650 m of altitude, S 25103028″–W 53152037″) during the summer of 2013. The specimen was identified by taxonomists and deposited in the Herbarium of Botanical Museum of Curitiba, Brazil (no. 359503). Leaves were dried in a forced draft oven (45 °C, 48 h). The crude extract was prepared by maceration from methanol/water (70:30 v/v) at room temperature for 15 days (Brant et al., 2014). The solvent was eliminated by a rotary evaporator and lyophilized (yielding 17.14%). The freeze-dried extract (MECS) was dissolved in filtered water before the experiments.

Liquid chromatography–mass spectrometry (LC–MS)

MECS was examined by ultra-high performance liquid chromatography. Samples at 2 mg ml^{-1} concentration were prepared in H_2O–MeOH (7:3 v/v) and the analysis was carried out on a reversed-phase HSS-C18 column. The binary solvent was composed of 0.1% aqueous formic acid and methanol (v/v). The linear solvent gradient at a flow rate of 0.4 ml min^{-1} was developed by increasing methanol percentage from 0% to 38% (10 min), then to 60% at 13 min and returning to initial condition at 14 min, re-equilibrated for 3 min. The column was heated at 60 °C and samples kept at room temperature. 2 μl was injected and compounds were detected at λ 200–400 by high-resolution mass spectrometry (HR-MS).

HR-MS analyses were developed on an electrospray ionization mass spectrometry operating in the negative ionization at atmospheric pressure. The source temperature was 350 °C and N_2 stream was used for sample desolvation with sheath gas and auxiliary flow rate at 60 and 20 arbitrary units, respectively. The ionization parameters were: spray voltage of 3.5 kV, capillary of −20 V and tube lens of −130 V. For mass accuracy, external calibration was performed and resolution was set at 30,000 FWHM (at *m/z* 400) in LC–MS mode. Acquisition was obtained in total ion current mode, with mass range of *m/z* 100–1000. Compound fragmentation was obtained by collision-induced dissociation using helium and energy of 20 eV.

Carcinogenesis and mutagenesis evaluation

Animals

Female and male Wistar rats, 12 weeks old, were housed at 22 ± 2 °C, 12 h light dark cycle, 55 ± 10% humidity conditions, with *ad libitum* access to water and chow. The ethical committee on animal use of the Federal University of Grande Dourados (UFGD) approved all the procedures (no. 11/2015).

Experimental design and sample collection

Rats were distributed into eight groups (n = 8–10) for treatment with MECS (50, 250 and 500 mg kg^{-1}, *p.o.*, though gavage, 1 ml kg^{-1}) or vehicle (filtered water, 1 ml kg^{-1}, control group) daily, during 12 weeks. The doses were selected based on previous data reporting cardiovascular effects of MECS (Brant et al., 2014). A ten-time lower dose from the highest dose was also tested.

Rats morbidity and mortality, aggressiveness, eyes and ear pallidness, convulsions, salivation, motor activity, breathing, heartbeat, diarrhea, coma and injury were monitored. Body weight (BW), consume of chow and water were weekly controlled.

At the end of the experiment, the animals were food-deprived for 12 h and anesthetized with isoflurane. Blood samples were collected from caudal vein for genotoxicity test and after decapitation for tumor markers analyses. Liver, spleen, kidneys and lungs were removed for determining the relative weights, gross pathology and histopathological analyses. The right femur was collected for micronucleus assay.

Gross pathology and histopathological evaluation

Samples were harvested, fixed in 10% formalin, dehydrated with alcohol and xylene, embedded in paraffin wax, sectioned (4 μm) and stained with hematoxylin/eosin. The slices were analyzed by a veterinary pathologist, with a slice scanner, at 10× magnification.

Micronucleus test

The proximal epiphyses were cut off from femurs and the spinal cord was removed by drain with fetal bovine serum. After centrifugation (5 min, 1000 × *g*) the pellet was homogenized, a drop of this was placed on a slide and the smear was performed. Slices were dried at room temperature and fixed in absolute methyl alcohol (10 min) and 24 h latter were stained with giemsa (15 min), washed in distilled water and blind analyzed. Two thousand polychromatic erythrocytes (MN/PCE)/animal were counted using an optical microscope (1000× magnification). The polychromatic/normochromatic erythrocytes ratio (PCE/NCE ratio) was calculated by analyzing 100 random erythrocytes/animal (OECD, 1997).

Comet assay

Blood of rats (20 μl) treated with MECS during 12 weeks or cyclophosphamide (20 mg kg^{-1}, *p.o.*, through gavage, 1 ml kg^{-1}, 24 h before sample collection) was homogenized in 120 μl of 1.5% low-melting-point agarose gel and transferred to slices with 5% agarose gel. After immersion in a lysis buffer (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris [pH 10.0] with 1% Triton X-100 and 10% dimethyl sulfoxide) for 4 h, slices were incubated in an alkaline buffer (300 mM NaOH and 1 mM EDTA, pH >13, 4 °C) for 20 min. Nucleoids were electrophoresed (20 min, 25 V, 300 mA, in the dark at 4 °C), whereupon the alkali was neutralized with 0.4 M Tris (pH 7.5), in 3 cycles of 5 min. Then, slices were fixed with alcohol and dried overnight, stained with ethidium bromide solution (0.002 mg ml^{-1}), and 100 cells/animal were analyzed with a fluorescence microscope (excitation 420–490 nm, barrier 520 nm). Classifications: class 0 (no damage); class 1 (comet tail shorter than the nucleoid diameter); class 2 (comet tail once or twice the nucleoid diameter); and class 3 (comet tail greater than twice the

nucleoid diameter). Readings were used to calculate damaged cell (sum of 0–3 classes damage) and score of damage (the value of the damage multiplied by the class number and then the sum of the three classes) (OECD, 2014).

Tumor markers

Plasmatic levels of tumor markers CA 15-3 (breast), CA 19-9 (pancreas, biliary and digestive tract), CA 125 (ovaries and liver), CA 27-29 (breast), CA 72-4 (stomach and ovarian), alpha-fetoprotein (AFP; liver cancer), squamous cell carcinoma antigen (SCC; cervix squamous cell, head, neck, esophagus and lung); and thyrocalcitonin (TC; thyroid) were determined by electrochemiluminescence using an automated analyzer. Results are expressed as $U\,l^{-1}$ (CA 15-3, CA 19-9, CA 125, CA 27-29), $ng\,l^{-1}$ (AFP and SCC) or $pg\,ml^{-1}$ (TC).

Statistical analyses

Differences were determined by one-way analysis of variance (ANOVA) or Kruskal Wallis followed by Bonferroni's or Dunn's *post hoc*. Weigh gain of rats was analyzed by two-way ANOVA followed by Bonferroni's *post hoc*. The level of significance was 95% ($p < 0.05$). The data are expressed as mean ± standard error of the mean (S.E.M.). Graphs were drawn and statistical analysis was carried out using the GraphPad Prism software version 5.0 for Mac OS X (GraphPad® Software, San Diego, CA, USA).

Results

Phytochemical characterization

Bioactive clerodane diterpenes typical of genus *Casearia* were identified, highlighting the casearvestrins A–C and casearins B and G. Gallic acid derivatives such as isobutyl gallate-3,5-dimethyl ether and methyl gallate-3,5-dimethyl ether have also been identified. Furthermore, the fingerprint obtained from MECS showed five peaks with absorbance at 335 nm compatible with flavonoid glycosides. The peak Rt 6.093 is similar to rutin and its average content was found to be $20.2\,mg\,g^{-1}$ (Fig. 1).

Body weight, relative organs weight and clinical evaluation

Treatment with MECS did not alter the BW (g) of female or male rats (Fig. 2A and B, respectively). The relative weight of liver, spleen and kidneys were the same between all groups found (Supplementary Table). Moreover, no alterations in chow or water consumption, and behavioral were found.

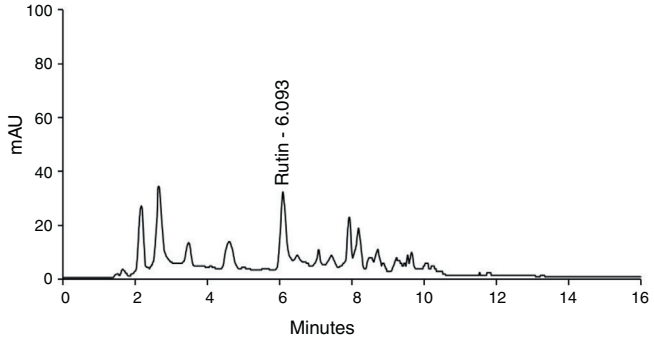

Fig. 1. UPLC analysis of MECS obtained from *Casearia sylvestris*.

Gross pathology and histopathological analyses

MECS did not induce lung, kidney or liver macroscopic alterations (data not shown). The histological examination confirmed the absence of damage in lung (A), kidney (B) or liver (C) in female or male rats treated with vehicle (Figs. 3 and 4, respectively), MECS $50\,mg\,kg^{-1}$ (Figs. 3 and 4, respectively), MECS $250\,mg\,kg^{-1}$ (Figs. 3 and 4, respectively) or MECS $500\,mg\,kg^{-1}$ (Figs. 3 and 4, respectively).

Micronucleus test

Cyclophosphamide increased the number of MN-PCE in female and male rats by 283.9% (Fig. 5A and B, respectively); and decreased the PCE/NCE ratio by 99% in female and male (Fig. 5C and D, respectively) when compared with control group. MECS did not alter the values of MN-PCE or PCE/NCE ratio in female (Fig. 5A and C, respectively) or male rats (Fig. 5B and D, respectively) when compared with C-. Illustrative image of normochromatic erythrocyte, polychromatic erythrocyte and micronucleus are presented in Fig. 5E (C-), F (MECS $50\,mg\,kg^{-1}$), G (MECS $250\,mg\,kg^{-1}$) or H (MECS $500\,mg\,kg^{-1}$).

Comet assay

Classes and number of damaged cells found in the comet assay of female and male are presented in Tables 1 and 2, respectively. No alterations were observed in the negative control group. Cyclophosphamide induced an intense damage in all samples. In contrast, MECS did not induced alterations.

Tumor markers

MECS did not alter the plasmatic levels of CA 15-3, CA 19-9, CA 125, CA 27-29, CA 72-4, SCC and TC of female or male rats

Table 1
Number of damaged cells and classes of damage of female rats treated with vehicle (C−, negative control), cyclophosphamide (C+, positive control) or freeze-dried extract of *Casearia sylvestris* 50, 250 and 500 $mg\,kg^{-1}$.

Groups	Damage cells (%)	Classes of damage				Score
		1	2	3	4	
C−	10.2 ± 0.64	89.8 ± 1.22	7.1 ± 0.70	2.7 ± 0.63	0.4 ± 0.16	13.7 ± 1.84
C+	87.0 ± 3.75[a]	8.5 ± 2.21[a]	20.1 ± 8.01[a]	63.1 ± 7.51[a]	8.52 ± 4.52[a]	194.1 ± 8.91[a]
MECS $50\,mg\,kg^{-1}$	8.7 ± 3.01	91.3 ± 0.94	6.5 ± 0.87	1.9 ± 0.31	0.3 ± 0.21	11.20 ± 2.61
MECS $250\,mg\,kg^{-1}$	7.8 ± 0.55	92.1 ± 1.41	5.5 ± 0.79	1.6 ± 0.43	0.7 ± 0.33	11.67 ± 2.34
MECS $500\,mg\,kg^{-1}$	7.2 ± 0.49	93.0 ± 0.88	5.2 ± 0.63	1.8 ± 0.33	0.2 ± 0.13	9.40 ± 2.75

Values expressed as mean ± S.E.M., $n = 8–10$. Class 0, no damage; class 1, tail of comet shorter than the diameter of nucleoid; class 2, tail of comet once or twice the diameter of nucleoid; class 3, tail of comet more than twice the diameter of nucleoid. C−, negative control, C+, cyclophosphamide, positive control. Differences between groups were evaluated by one-way ANOVA followed by Bonferroni's test.

[a] $p < 0.05$ when compared with C−.

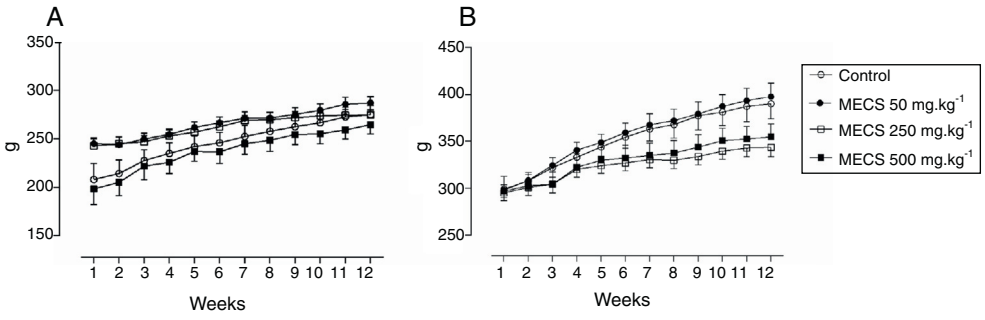

Fig. 2. Effect of *Casearia sylvestris* extract (MECS) on body weight gain (g) of female (A) or male (B) rats. Animals received (orally) vehicle (control group) or 50, 250 or 500 mg kg^{-1} MECS. Treatments were performed once a day, during 12 weeks. Values are expressed as means ± S.E.M. (n = 8–10). Statistical comparison was performed using two-way ANOVA followed by Bonferroni's test.

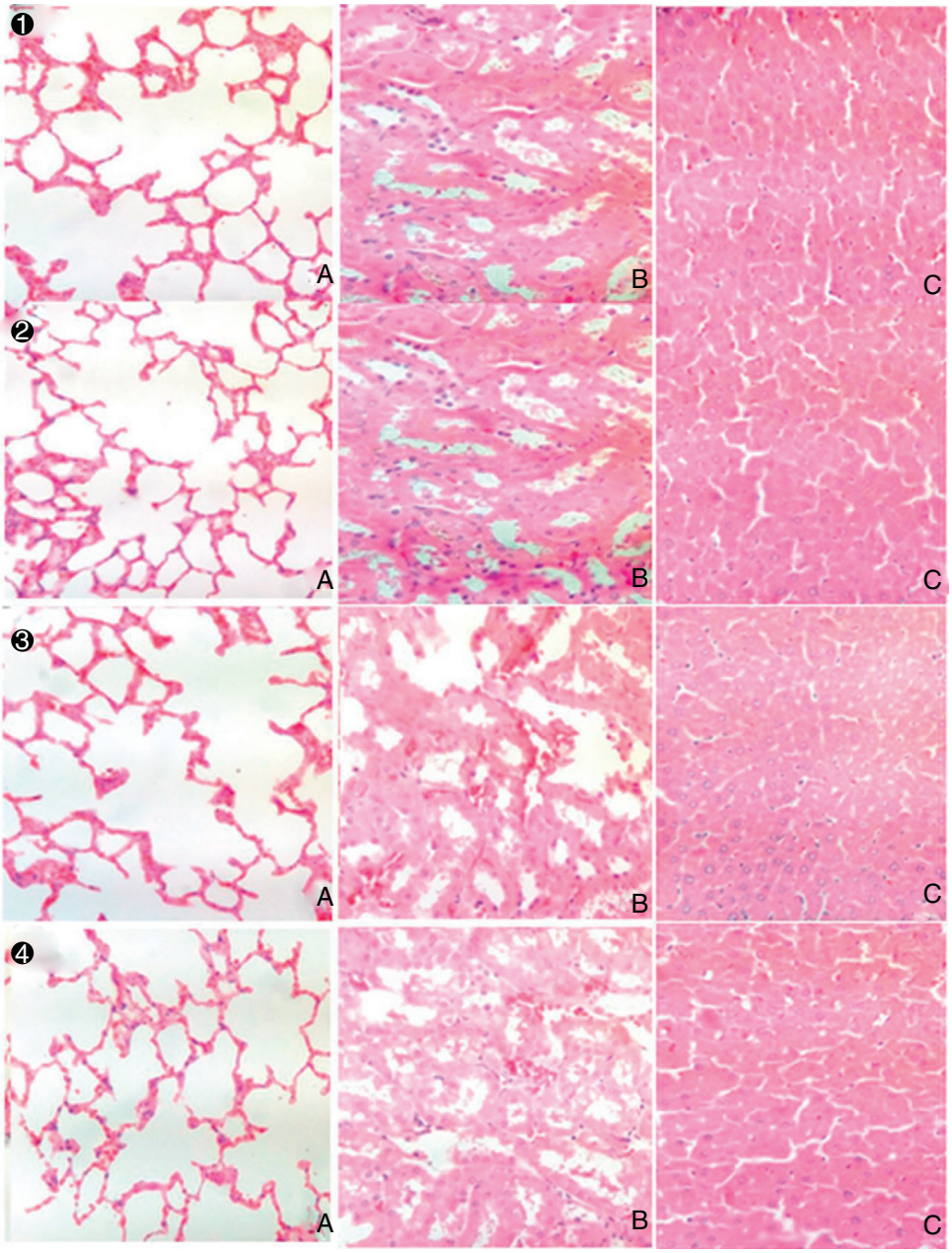

Fig. 3. Lung (A), kidney (B) or liver (C) histology of female rats treated with vehicle or C. sylvestris extract (MECS). Animals received (1) vehicle (control group), (2) 50 mg kg^{-1} MECS, (3) 250 mg kg^{-1} MECS or (4) 500 mg kg^{-1} MECS. Treatments were performed once a day, during 12 weeks. Slides were stained with Hematoxylin and Eosin. 40× magnification.

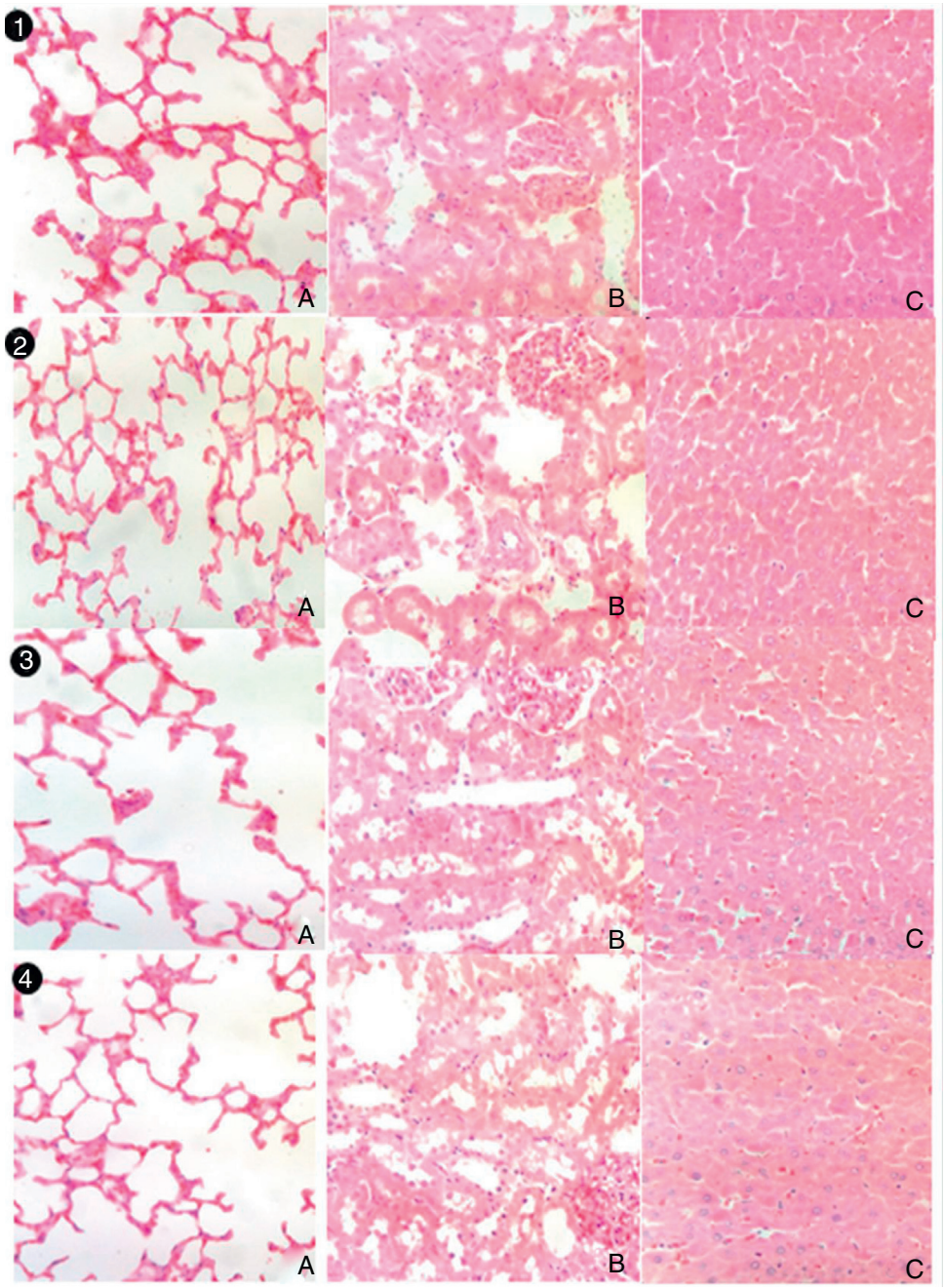

Fig. 4. Lung (A), kidney (B) or liver (C) histology of male rats treated with vehicle or *C. sylvestris* extract (MECS). Animals received (1) vehicle (control group), (2) 50 mg kg^{-1} MECS, (3) 250 mg kg^{-1} MECS or (4) 500 mg kg^{-1} MECS. Treatments were performed once a day, during 12 weeks. Slides were stained with Hematoxylin and Eosin. 40× magnification.

Table 2
Number of damaged cells and classes of damage of male rats treated with vehicle (C−, negative control), cyclophosphamide (C+, positive control) or freeze-dried extract of *Casearia sylvestris* 50, 250 and 500 mg kg^{-1}.

Groups	Damage cells (%)	Classes of damage				Score
		1	2	3	4	
C−	11.99 ± 0.74	87.9 ± 0.75	9.39 ± 0.56	1.8 ± 0.33	0.8 ± 0.25	15.39 ± 3.62
C+	91.0 ± 2.91[a]	9.0 ± 2.91[a]	19.6 ± 7.82[a]	62.0 ± 9.6[a]	9.40 ± 3.57[a]	171.8 ± 2.91[a]
MECS 50 mg kg^{-1}	10.59 ± 0.61	89.5 ± 1.08	7.19 ± 0.91	2.5 ± 0.52	0.9 ± 0.40	14.89 ± 3.14
MECS 250 mg kg^{-1}	10.49 ± 0.76	89.6 ± 1.16	8.39 ± 1.22	1.8 ± 0.36	0.3 ± 0.15	12.89 ± 3.45
MECS 500 mg kg^{-1}	9.73 ± 0.55	91.7 ± 0.76	6.79 ± 0.52	2.44 ± 0.90	0.5 ± 0.22	13.17 ± 3.20

Values expressed as mean ± S.E.M., $n = 8–10$. Class 0, no damage; class 1, tail of comet shorter than the diameter of nucleoid; class 2, tail of comet once or twice the diameter of nucleoid; class 3, tail of comet more than twice the diameter of nucleoid. C−, negative control, C+, cyclophosphamide, positive control. Differences between groups were evaluated by one-way ANOVA followed by Bonferroni's test.

[a] $p < 0.05$ when compared with C−.

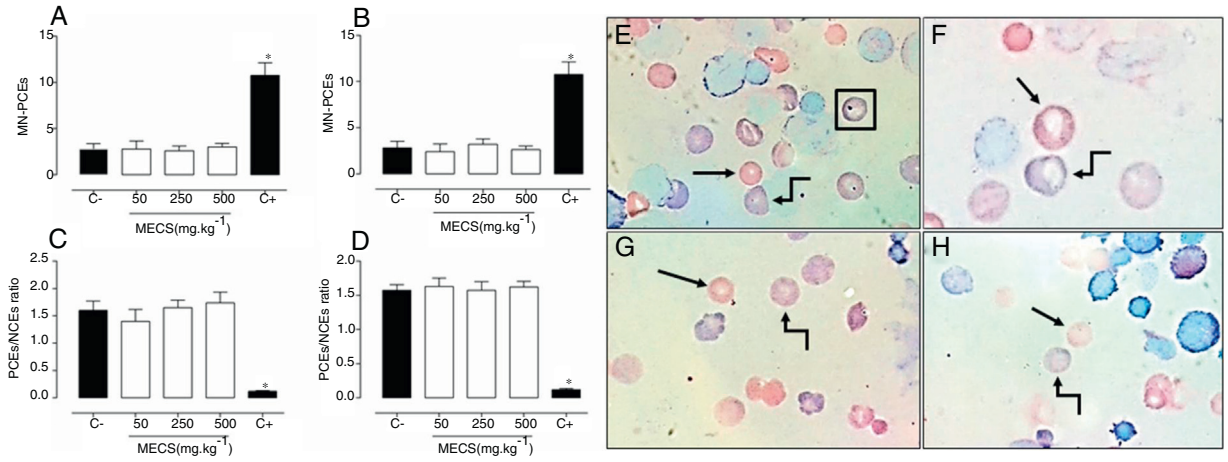

Fig. 5. Effect of *Casearia sylvestris* extract (MECS) on the counts of (A and C) female and (B and D) male MN-PCEs and PCE/NCE ratio, respectively. Cytotoxicity of vehicle and 50, 250 or 500 mg kg^{-1} MECS is presented in panels E, F, G and H, respectively. Arrows indicate normochromatic erythrocyte; angled arrows indicate polychromatic erythrocytes; and square indicate micronucleus in polychromatic erythrocyte. Slides were stained with Giemsa and shown with 1000× magnification. Statistical comparison was performed using one-way ANOVA followed by Bonferroni's test. Values are expressed as mean ± S.E.M. (n = 8–10). * $p < 0.05$ when compared with control group. MN-PCE, micronucleated polychromatic erythrocytes; PCE/NCE, polychromatic to normochromatic erythrocytes ratio. C−, control group; C+, cyclophosphamide, positive control group.

Table 3
Tumor markers of female rats treated with vehicle (control group) or freeze-dried extract of *Casearia sylvestris* 50, 250 and 500 mg kg^{-1} during 12 weeks.

Tumor marker	Groups			
	Control	MECS 50 mg kg^{-1}	MECS 250 mg kg^{-1}	MECS 500 mg kg^{-1}
CA 15-3	1.08 ± 0.07	1.07 ± 0.05	0.96 ± 0.05	1.01 ± 0.06
CA 19-9	10.01 ± 0.29	9.12 ± 0.35	9.55 ± 0.45	9.34 ± 0.33
CA 125	13.34 ± 0.55	13.91 ± 0.41	14.40 ± 0.69	14.33 ± 0.62
CA 27-29	6.20 ± 0.49	5.23 ± 0.37	5.09 ± 0.28	5.22 ± 0.43
CA 72-4	1.34 ± 0.12	1.43 ± 0.19	1.42 ± 0.13	1.21 ± 0.12
AFP	1.73 ± 0.15	1.26 ± 0.07[a]	1.21 ± 0.07[a]	1.18 ± 0.07[a]
SCC	0.35 ± 0.05	0.30 ± 0.04	0.37 ± 0.04	0.42 ± 0.04
TC	4.36 ± 0.37	4.00 ± 0.25	4.35 ± 0.39	4.05 ± 0.26

Values expressed as mean ± S.E.M., n = 8–10. CA, cancer antigen; AFP, alpha-fetoprotein; SCC, squamous cell carcinoma antigen; TC, thyrocalcitonin. Unit: CA 15-3, CA 19-9, CA 125, CA 27.29 and CA 72-4: U l^{-1}; AFP: ng ml^{-1}; SCC: ng ml^{-1}; TC: pg ml^{-1}. Differences between groups were evaluated by one-way ANOVA or Kruskal Wallis followed by Bonferroni's or Dunn's test.

[a] $p < 0.05$ when compared with control.

Table 4
Tumor markers of male rats treated with vehicle (control group) or freeze-dried extract of *Casearia sylvestris* 50, 250 and 500 mg kg^{-1} during 12 weeks.

Tumor marker	Groups			
	Control	MECS 50 mg kg^{-1}	MECS 250 mg kg^{-1}	MECS 500 mg kg^{-1}
CA 15-3	1.01 ± 0.05	1.02 ± 0.12	0.94 ± 0.03	1.04 ± 0.06
CA 19-9	10.17 ± 0.31	9.82 ± 0.37	9.74 ± 0.46	9.80 ± 0.37
CA 125	14.18 ± 0.43	13.71 ± 0.42	13.12 ± 0.40	13.09 ± 0.49
CA 27-29	6.00 ± 0.46	5.37 ± 0.42	5.37 ± 0.39	5.67 ± 0.27
CA 72-4	1.26 ± 0.09	1.36 ± 0.13	1.38 ± 0.10	1.39 ± 0.09
AFP	1.10 ± 0.06	1.13 ± 0.08	1.35 ± 0.08	1.21 ± 0.06
SCC	0.35 ± 0.03	0.35 ± 0.04	0.45 ± 0.04	0.40 ± 0.05
TC	4.00 ± 0.25	4.53 ± 0.37	4.55 ± 0.38	4.30 ± 0.30

Values expressed as mean ± S.E.M., n = 8–10. CA, cancer antigen; AFP, alpha-fetoprotein; SCC, squamous cell carcinoma antigen; TC, thyrocalcitonin. Unit: CA 15-3, CA 19-9, CA 125, CA 27.29 and CA 72-4: U l^{-1}; AFP: ng ml^{-1}; SCC: ng ml^{-1}; TC: pg ml^{-1}. Differences between groups were evaluated by one-way ANOVA or Kruskal Wallis followed by Bonferroni's or Dunn's test.

(Tables 3 and 4, respectively). The plasmatic levels of AFP were significantly reduced in female rats treated with all doses of MECS.

Discussion

In this work, a short-term carcinogenesis study was conducted with a popular herbal preparation obtained from *C. sylvestris*. Through a series of detailed protocols, we observed that the MECS did not induce any mutagenic or genotoxic effects after 12 weeks of treatment. Furthermore, no significant increase in tumor markers levels, signs of tumor or malignant cells in gross pathology nor histopathological changes in vital organs of Wistar rats were detected.

Although *C. sylvestris* are intended for large-scale distribution to the Brazilian public health services, studies on its genotoxic and carcinogenic potential remain unknown. This fact deserves adequate attention because in the last 16 years, 458 studies evaluated the genotoxic potential of medicinal plants and genotoxic activity was

reported in 28.4% of them (Sponchiado et al., 2016). This high incidence of positive results should alert to the importance of assessing the genotoxic and mutagenic potential of herbal medicines before applying them as therapeutic agents.

Important markers of the action of genotoxic compounds are the presence of micronuclei or the decompression of parts of cellular DNA. The micronuclei are originated from chromosome fragments that are acentric or that are delayed in relation to the others in their migration toward the poles of the mitotic spindle. These changes may be induced by agents that are capable of breaking DNA or interfering with spindle formation. Similarly, the presence of simple breaks, alkaline labile sites and crosslinks resulting from the action of genotoxic compounds, alters the cell structure of the DNA, which is normally supercoiled and strongly compacted, causing relaxation in parts of the molecule (Tafazoli et al., 2017). In view of the abovementioned data, the Organization for Economic Co-Operation and Development (OECD) recommends the micronucleus test and the comet assay as an important tool capable of identifying potentially genotoxic agents (OECD, 1997, 2014). Therefore, the absence of significant changes in these assays provided very consistent data on the low genotoxicity of the MECS.

Another important aspect of this study was the investigation of the carcinogenic potential of MECS. For this, we initially opted for the dosages of different tumor markers, and later, for a detailed histopathological analysis of different vital organs. Tumor markers (or biological markers) are proteins or pieces of protein present in tumors, blood or other biological fluids to which changes in their concentrations are related to the genesis and growth of neoplastic cells. In general, tumor markers can be produced by normal cells as well as by cancer cells, although are produced at very high levels by tumor cells (Duff, 2001). These markers may be useful in the diagnosis and prognosis of different types of tumors, as well as assist in the development of new treatment modalities (Touitou and Bogdan, 1998).

In our study, none of the animals receiving MECS showed a significant increase in different tumor markers levels when compared to animals treated only with the vehicle. On the other hand, female rats treated with MECS (at all doses) showed a significant reduction in AFP levels. AFP is an important fetal serum protein, which is normally synthesized in the liver, yolk sac and fetal gut with functions of plasma transport and maintenance of oncotic pressure. In general, levels above 500 ng/ml are highly suggestive of liver malignancy and values well below that level do not, on their own, have considerable clinical significance (Sauzay et al., 2016). Generally, tumor markers are complementary tests and should always be used accompanied by other methods for diagnosis or therapeutic modification. In our case, in a complementary way to tumor markers, all the vital organs of animals treated with the MECS were examined through detailed histopathological analysis, and, in none of the cases, morphoanatomic alterations suggestive of tumor and malignancy were observed.

Taken together, our study indicates that the methanolic extract from *C. sylvestris* is safe at the tested doses, with no mutagenic, genotoxic or carcinogenic effects.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Authors' contributions

CAST: *in vivo* experiments and data analyses. ELCJ: LC–MS analyses. SDVN, RDFJ and TCP: *in vivo* experiments. JAS, GKT and SAO: mutagenicity analyses. ACS and RICS: histopathological analyses. FARL: data analysis, data discussion and manuscript preparation. AGJ: data discussion and manuscript correction.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2017.05.009.

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