SUPPLEMENTARY MATERIAL

***Schizocalyx cuspidatus* (A. St.-Hil.) Kainul. & B. Bremer extract improves antioxidant defenses and accelerates the regression of hepatic fibrosis after exposure to carbon tetrachloride in rats**

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Abstract

The aim of this study was to investigate the effect of leaves extract of *Schizocalyx cuspidatus* (A. St.-Hil.) Kainul. & B. Bremer on hepatic morphofunctional dysfunction induced by carbon tetrachloride (CCl₄). Liver lesions were induced via intraperitoneal administration of CCl₄ every 48 hours for 12 days. Forty-nine rats were randomized into 7 groups: G1: CCl₄; G2: CCl₄ (animals euthanized 24 hours after last CCl₄ application); G3: CCl₄ + DMSO; G4: SCE 400 mg/kg; G5: DMSO (700 µl); G6: CCl₄ + SCE 200 mg/kg and G7: CCl₄ + SCE 400 mg/kg. SCE administration resulted in reduction in hydroperoxide levels, lipidic droplets and necrosis compared to G1, G2 and G3. There was an increase in the amount of collagen fibers in G1, G2, and G3 compared to the groups. These results show that the extract of SCE leaves has great potential for the recovery of liver damage after the application of CCl₄.

Keywords: fibrosis; carbon tetrachloride; antioxidant activity; hepatotoxicity.

Experimental

Chemicals

Carbon tetrachloride (CCl₄), dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), formaldehyde and glutaraldehyde were acquired from the Sigma Chemical Co. (St. Louis, MO, USA). The diagnostic kits used in the evaluation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP) were acquired from Human In Vitro Diagnostics (Itabira, Brazil) and the kits for bilirubin measurement were obtained from Labtest (São Paulo, Brazil). Superoxide dismutase (SOD) activity was measured using a kit acquired from the Cayman Chemical Company (Michigan, USA). Distilled water purified by reverse osmosis was used in the HPLC analysis. Methanol (HPLC grade) was purchased from Vetec, whereas ethanol (analytical reagent grade) was obtained from Sigma-Aldrich.

Preparation of the ethanolic extract and phytochemical characterization

Samples of *S. cuspidatus* were collected in a biome of Brazilian Atlantic forest in the municipality of Araponga in Minas Gerais state, Brazil (20°43'00,0"S and 42°29'10,8"W, 1.200 m of altitude). Voucher material was deposited at the herbarium of the Federal University of Viçosa (UFV; number VIC 21.559). Leaf samples were separated, dried for 48 hours at room temperature in a darkened, well-ventilated room, pulverized in a knife mill and stored. The powdered air-dried leaves of *S. cuspidatus* (500g) were extracted exhaustively with 95% ethanol by the percolation method. The extract was concentrated under vacuum at 50°C using a rotary evaporator and then lyophilized until complete removal of the solvent was achieved, yielding an ethanolic extract (SCE) (139g). Phytochemistry of the extract was performed on chromatography plates coated with silica gel GF 254[®] (Merck, Darmstadt, Germany) using different mobile phases and detection reagents in accordance with the protocol described by Wagner (2009). To measure the total phenol and proanthocyanidin contents, powdered stem bark (1.0 g) was extracted with 200 mL of water at 100°C under reflux for 30 minutes. The concentration of total phenols was determined colorimetrically (absorbance at 760 nm) using the Folin-Ciocalteu method (Verza et al. 2007). The proanthocyanidin content was determined using the procedure of Price et al. (1980). The absorbance of the sample and standard were measured at 500 nm and the proanthocyanidin was expressed as milligrams of catechin equivalents per gram of dry matter. The content of total flavonoids was determined using rutin as the reference compound (Zabala et al. 2015).

High-performance liquid chromatography analysis was performed using the Shimadzu UFLC system equipped with an LC-20AD (Tokyo, Japan) binary pump, SPD-M20A diode array detector, an SIL-A0AHT auto sampler, a CBM-20A system communications bus module and a CTO-20A column oven. Chromatographic separation was achieved on a Shim-pack VP-ODS (150 x 4.6 mm i.d.; 4.6 μ m particle size) from Shimadzu with a guard column (10 x 4.6 mm). The mobile phase consisted of solvent A: pure water and solvent B: methanol with the following parameters: starting with a linear gradient, the initial percentage of A was decreased from 95-5% and B was increased from 5-95% from 0–65 min; this was followed by isocratic elution A (5%) and B (95%) from 65–70 min, and a linear gradient of A (5-95%) and B (95-5%) from 70-72 min; the column temperature was 40°C, flow rate was 1.0 ml/min and UV-diode array detection was at 254 nm. The Shimadzu Labsolutions software was used. Initially, the extract was resuspended in methanol (1.0 mg/20 mL), treated with activated carbon to remove chlorophyll, filtered and concentrated under reduced pressure to give a dry extract. Then, all extracts was diluted in methanol grade HPLC (20 mL) by sonication, were filtered over a cellulose membrane (0.45 μ m pore diameter) and a volume of 15 μ l was injected. The DAD detector was set to a scanning range of 200-400 nm.

Animals

Male Wistar rats with a mean weight of 235 grams were kept in an experimental animal house under controlled light (12 hour light/darkness cycles) and temperature ($21 \pm 2^\circ\text{C}$) conditions, with relative air humidity of 60 to 70%. Water and standard rat chow were supplied *ad libitum*. Ethics Committee for the Care and Use of Laboratory Animals of the Federal University of Minas Gerais (UFMG) (protocol 122/2008).

Experiment protocol

Liver lesions were induced in the animals of groups 1, 2, 3, 6 and 7 by intraperitoneal (i.p.) administration of CCl₄ (1 ml/kg, 60% in olive oil, v/v) every 48 hours for 12 days (Xiao-Yan et al. 2011). Immediately after the final application of CCl₄, *S. cuspidatus* extract (SCE) (resuspended in 700 μ l DMSO [w/v]) was administered to the animals by gavage in two different doses: 200 mg/kg (SCE 200) and 400 mg/kg (SCE 400) of body weight, for 12 days. One group (G5) received only DMSO vehicle, for 12 days. The same protocol used for the groups receiving DMSO was followed in the groups receiving SCE (Group 4). Forty-nine rats were randomized into 7 treatment groups. G1: CCl₄ (animals euthanized 12 days after last CCl₄ application); G2: CCl₄ (animals euthanized 24 hours after last CCl₄ application); G3:

CCl₄ + DMSO (vehicle control); G4: SCE 400 mg/kg; G5: DMSO (700 µl); G6: CCl₄ + SCE 200 mg/kg; G7: CCl₄ + SCE 400 mg/kg.

Biochemical and histopathological analysis

Twenty-four hours after completing the different treatments, animals were anesthetized with ketamine (10 mg/kg of body weight) and xylazine (2 mg/kg of body weight) and euthanized by cardiac puncture. Blood Aliquots were collected and centrifuged at 175 G for 15 minutes. Serum was immediately used for AST, ALT, ALP and GGT analysis. For histopathological analysis and biochemical evaluation of the liver tissue, a median incision was made and the liver was removed intact and weighed using analytical scales. Next, two fragments (3-5mm) of the liver of each animal were quickly removed, one of which was frozen in liquid nitrogen (-196°C) and the other immersed in Karnovsky's fixative solution. Aliquots of 500 mg of the frozen liver fragments were homogenized in phosphate buffered saline (PBS) and centrifuged at 3.500 G at 5°C; the supernatant was used for superoxide dismutase (SOD) and rate of decomposition of hydrogen peroxide (H₂O₂) (Sridevi et al., 2011). Lipid peroxidation in the liver homogenate was assessed by measuring lipid hydroperoxides (Nourooz-Zadeh et al. 2004). Total protein levels were measured using the Bradford method (1976). For histopathology, fragments of liver were fixed in Karnovsky's solution, dehydrated in ethanol, cleared in xylol and embedded in paraffin. Semi-serial 4-µm-thick histological sections were obtained using a rotary microtome (Leica Multicut 2045[®], Reichert-Jung Products, Germany), using one out of every ten sections to avoid repeating analysis of the same histological area. The sections were stained with hematoxylin and eosin to analyze the area of necrosis and lipid droplets and were stained with Sirius red (Sirius red F3B, Mobay Chemical Co., Union, New Jersey, USA) to analyze the fibrosis (Junqueira et al. 1979). Seventy photomicrographs taken under x20 magnification were obtained for each group using a BX-60[®] light microscope and polarizing microscopy (Olympus, Tokyo, Japan). For this analysis, a grid containing 300 points within a standard test area (AT) of 73 x 103 µm² was superimposed over each image to quantify the proportion of necrotic areas and lipid droplets and fibrosis. The stereological parameter of fibrosis was based on the birefringence properties of the collagen fibrils, under polarization of the thick collagen fibers. The Image Pro-plus 4.5[®] software (Media Cybernetics, Silver Spring, USA) was used for all of these analyses (Lessa et al. 2010).

Statistical analysis

The data were expressed as measures of central trend, means and standard deviations (S.D.). The normalcy of data distribution was verified using the Kolmogorov-Smirnov test. Based on this test, the biochemical data were submitted to unifactorial analysis of variance (one-way ANOVA), followed by Tukey's test for multiple comparisons. In addition, the Kruskal-Wallis test was used to analyze the histological area of the lipid droplets and fibrosis, with statistical significance established at $p < 0.05$. All the tests were performed using the GraphPad Prism 5.0[®] statistical software (GraphPad Software, Inc., California, USA).

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Figure S1. Photomicrographs of the liver of rats exposed to CCl_4 and treated with *S. cuspidatus* extract (SCE 200 and 400 mg/kg) showing fibrosis areas (A-F, Sirius red staining under polarizing microscopy; bars= 60 μm). A= G1 (CCl_4 + 12 days); B= G2 (CCl_4); C= G3 (CCl_4 + DMSO); D= G4 and G5 (SCE and DMSO, respectively); E= G6 (CCl_4 and SCE 200); F= G7 (CCl_4 and SCE 400).