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Dragon's blood *Croton palanostigma* induces genotoxic effects in mice

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ABSTRACT

Ethnopharmacological relevance: Dragon's blood is a dark-red sap produced by species from the genus *Croton* (Euphorbiaceae), which has been used as a famous traditional medicine since ancient times in many countries, with scarce data about its safe use in humans. In this research, we studied genotoxicity and clastogenicity of *Croton palanostigma* sap using the comet assay and micronucleus test in cells of mice submitted to acute treatment.
Material and methods: HPLC analysis was performed to identify the main components of the sap. The sap was administered by oral gavage at doses of 300 mg/kg, 1000 mg/kg and 2000 mg/kg. For the analysis, the comet assay was performed on the leukocytes and liver cells collected 24 h after treatment, and the micronucleus test (MN) on bone marrow cells. Cytotoxicity was assessed by scoring 200 consecutive polychromatic (PCE) and normochromatic (NCE) erythrocytes (PCE/NCE ratio).
Results and conclusion: The alkaloid taspine was the main compound indentified in the crude sap of *Croton palanostigma*. The results of the genotoxicity assessment show that all sap doses tested produced genotoxic effects in leukocytes and liver cells and also produced clastogenic/aneugenic effects in bone marrow cells of mice at the two higher doses tested. The PCE/NCE ratio indicated no cytotoxicity. The data obtained suggest caution in the use of *Croton palanostigma* sap by humans considering its risk of carcinogenesis.

1. Introduction

An assessment of the cytotoxic and genotoxic potential of medicinal plants is necessary because plants in general can synthesize toxic substances to protect themselves against infections, insects and herbivores in nature, but also may often affect the organisms that feed of them.

Croton palanostigma is a plant that belongs to the Euphorbiaceae family. This traditional medicine is derived from a fast growing tree that is known by different names in various countries: in Peru it is called “sangre de grado”, in Ecuador, “sangre de drago”, and in several other countries “dragon's blood”. Species from this genus contain a red viscous sap that can be obtained from making cuts in the bark (Duke and Vasquez, 1994). This bark sap is used for wound healing (Porras-Reyes et al., 1993), antimicrobial (Ubillas, 1994), controlling diarrhea (Gabriel et al., 1999; Holodniy et al., 1999), gastric ulcer healing, as a treatment for intestinal inflammation that are common in the rainforest regions (Miller et al., 2000), as an anti-cancer agent (Sandoval et al., 2002), as an anti-emetic and anti-itch (Miller et al., 2008), among others (see Gupta et al., 2008 for a review).

Despite of some reports that several compounds can be found in the dragon's blood sap (Cai et al., 1993a,b), it has been reported that the bark sap contains fundamentally the alkaloid, Taspine (Bettolo and Scarpati, 1979), and the lignin 3′4′-*O*-dimethylcedrusin, which has been claimed to be the active principles responsible for the anti-inflammatory and wound healing activities (Perdue et al., 1979; Vaisberg et al., 1989; Pieters et al., 1993). The complete chemical constituents present in dragon's blood from several sources can be found in a review of Gupta et al. (2008).

In our continuing efforts to evaluate the genotoxic potential of medicinal plants found in Brazil, and due to the fact that have been no studies on the likely genotoxic effects of the *Croton palanostigma* sap, the present study evaluated the *in vivo* genotoxicity and clastogenicity/aneugenicity potential of this sap in different cells of mice using the comet and micronucleus assays.

2. Materials and methods

2.1. Chemicals

The alkylating agent cyclophosphamide (CPA, CAS No. C 0768, Sigma) was used as the DNA damaging agent in comet and micronucleus assays. The other main chemicals were obtained

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from the following suppliers: dimethyl sulphoxide (DMSO, Merck, CAS No. 67-68-5); ethidium bromide (Sigma, CAS No. 1239-45-8); ethylenediaminetetraacetic acid (EDTA, Merck); low melting point (LMP) agarose (Invitrogen 15517-014:); normal melting point (NMP) agarose (Invitrogen 15510-019); sodium *N*-lauroylsarcosine (Sigma L-5125:); tris(hydroxymethyl)aminomethane (TRIS, Merck, CAS No. 77-86-1) and Triton X-100 (Merck).

2.2. Croton palanostigma sap collection

The sap of *Croton palanostigma* was obtained in Belém City, State of Pará, Brazil, in July of 2008. For obtainment of sap, cuts were made in the bark of plant of mode similar to the metodology used by native population of North of the Brazil. A sample of vegetable material was compared with voucher specimen and description reported in compendium "Flora Brasiliensis" (available in http://florabrasiliensis.cria.org.br) by Renê Artur Ferreira of the Universidade do Vale do Itajaí, Itajaí, Brazil; and was identified as *Croton palanostigma*.

2.3. Phytochemical characterization.

The phytochemical characterization was performed using as solvents ethyl acetate (Synth), deionized water, methanol (Tedia, HPLC grade) and trifluoracetic acid (Synth). It was used a Thermo Scientific HPLC System, equipped with Accela 600 bomb, Accela automatic injector, 80 HZ Accela PDA Detector and a reverse phase column C18 Hyperosil ODS (250 mm × 4.6 mm internal diameter). Data were registered and analyzed using ChromQuest Software 5.0. The solid phase extraction was realized using SupelcoSupelclean LC-18 3 mL cartridges.

2.3.1. Sample preparation

The sample SCp1 was prepared using 100 μL of *Croton palanostigma* sap transferred to a 5 mL volumetric flask and completed with water. The sample was filtered using a 45 μm filter.

The sample SCp2 was prepared using 100 μL of *Croton palanostigma* sap transferred to a 25 mL volumetric flask and completed with water. Afterwards, the solution was partitioned with 3 portions of ethyl acetate. After the phase's separation, the portions were combined and evaporate under reduced pressure. This residue was solubilized in a methanol:water (2:8) solution. The sample was eluted with the solvents to a 5 mL volumetric flask until complete the volume. The sample was filtered using a 45 μm filter.

The samples SCp1 and SCp2 were analyzed by HPLC. The mobile phase used a gradient mode with water (solvent A) and acetonitrile (solvent B), acidified with 0.1% of trifluoracetic acid, with the proportion 10–18% B (10 min); 18–25% B (10–23 min); 25–34% B(23–26 min); 34–42% B (26–30 min); 42–65% B (30–33 min), returning to the initial proportion at 38 min. The final elution time resulted in 38 min with 1 mL/min flux. It was injected 10 μL of each sample.

2.3.2. HPLC fingerprint conditions

The analysis were conducted using HPLC system (Waters) equipped whit a 600-F pump, 717 plus autosampler equipped with a UV–vis detector (PDA 2996). A C18 column (Luna Phenomenex, 25 cm, 4.6 mm i. d.; 0.5 μm film thickness and 100 A) was employed at a temperature of 25 °C. Gradient system used consisted of solvents A (methanol) and B (water acidified with acetic acid at pH 3,56) mixed, starting with 5% A, linearly increasing to 70% A in 25 min, 90% A in 30 min, until the end of the run, with flow rate of 0.6 mL/min. After each analysis, 5% mobile phase A was pumped and maintained for 10 min to re-equilibrate the system for baseline stability. UV–vis spectra were recorded in wavelength 200–400 nm (detection wavelength was 254 nm). The methanol was HPLC grade (Tedia, Fairfield, Ohio, USA) and the water was purified using a Milli-Q system (Millipore, Billerica, Massachusetts, USA).

All solvents were degassed in an ultrasonic bath (Unique, Santo Amaro, São Paulo, Brazil) and all solutions were filtered through at 0.45 μm Millipore Millex cellulose membrane (Schleicher and Schuell, Maidstone, Kent, UK) before injection.

2.4. Animals and dosing

The experiments were carried out on 12-week-old male Swiss albine mice (*Mus musculus*), weighing 25–30 g. The animals were acquired from the animal house of the Universidade Estadual Paulista (UNESP), Botucatu, São Paulo State, Brazil, and kept in polyethylene boxes, in a climate-controlled environment (25 ± 4 °C, 55 ± 5% humidity) with a 12-h light/dark cycle (7:00 am to 7:00 pm). Food (Nuvilab CR1—Nuvital) and water were available *ad libitum*. The mice were divided into five experimental groups of six animals each. *Croton palanostigma* resin was dissolved in distilled water. A fixed volume of 0.3 mL was administered in a single dose by gavage. The doses administered were 300 mg/kg, 1000 mg/kg, and 2000 mg/kg body weight, chosen on the basis of our acute toxicity studies in mice (>2000 mg/kg) and the limit dose recommended by OECD 420 for acute treatments in toxicology assays. The negative control group received distilled water, and the positive control group received an intraperitoneal injection of CPA at 50 mg/kg. The Animal Bioethical Committee of the Faculdade de Medicina de Marília, in the town of Marilia, Brazil, approved the present study on October 15, 2008 (protocol No. 468/08).

2.5. Comet assay

The comet assay (SCGE) was carried out by the method described by Speit and Hartmann (1999) and reviewed by Burlinson et al. (2007). Peripheral blood (before euthanasia) and liver (after euthanasia) samples were obtained from animals of each group, 24 h after treatment. Liver samples were washed in saline solution, in an ice bath. A small portion was transferred to a Petri dish containing 1 mL of Hank's solution (pH 7.5) and then homogenized gently with small pinches and a syringe to avoid clumps of cells. An aliquot of 20 μL of these cells suspensions was removed to determine cell viability. Cell counting was performed using a hemocytometer. Cell viability was determined by trypan blue dye exclusion. The number of trypan blue-negative cells was considered the number of viable cells, and was greater than 90%. Another equal aliquot of cells from each animal was mixed with 120 μL of 0.5% low melting point agarose at 37 °C, and immediately spread onto two microscope slides per animal, pre-coated with 1.5% normal melting point agarose. The slides were coverslipped and allowed to gel at 4 °C for 20 min. The coverslips were gently removed and the slides were then immersed in cold, freshly prepared lysing solution consisting of 89 mL of a stock solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH set to 10.0 with ~8 g solid NaOH, 890 mL of distilled water and 1% sodium lauryl sarcosine), plus 1 mL of Triton X-100 (Merck) and 10 mL of dimethyl sulfoxide (Merck). The slides, which were protected from light, were allowed to stand at 4 °C for 1 h and then placed in the gel box, positioned at the anode end, and left in a high pH (>13) electrophoresis buffer (300 mM NaOH-1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM, pH 10.0, EDTA) at 4 °C for 20 min prior to electrophoresis, to allow the DNA to unwind. The electrophoresis run was carried out in an ice bath (4 °C) for 20 min at 300 mA and 25 V (0.722 V/cm). The slides were then submerged in a neutralization buffer (0.4 M Tris–HCl, pH 7.5) for