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Apoptosis and mutagenicity induction by a characterized aqueous extract of *Baccharis articulata* (Lam.) Pers. (Asteraceae) on normal cells

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ABSTRACT

In a previous study we have demonstrated that cold aqueous extract of *Baccharis articulata* (Ba-CAE) induced the death of human peripheral blood mononuclear cells (PBMCs) and exerted low mutagenic effects on mice at 6 h after administration. The aim of this work was to investigate whether the PBMCs death induced by Ba-CAE is due to apoptosis, and whether this extract exerts mutagenic effects on mice at 24 and 48 h after administration. In addition, Ba-CAE was chemically characterized. PBMCs from healthy volunteers were exposed to extract (10, 20, 40, 80, 160, 320, 640 and 1280 μg/mL) for 18–24 h. Cell viability was determined by staining of trypan blue dye exclusion method. Apoptosis was determined by Hoechst 33258 staining, TUNEL, and DNA fragmentation analysis by agarose gel electrophoresis. BALB/c mice were injected with extract (1800, 900 and 450 mg/kg) and sacrificed at 24 and 48 h postinjection. Bone marrow samples were used to assess chromosome mutations by the micronucleus test. The extract induced PBMCs death by apoptosis and increased the frequency of micronuclei in bone marrow. The phytochemical study of Ba-CAE showed the presence of flavones as luteolin and acacetin, caffeoylquinic acids as chlorogenic acid, and tannins.

1. Introduction

The *Baccharis* spp. (Asteraceae) are herbs and shrubs native to South America. Approximately 400 species of *Baccharis* have been indentified and they grow in southern Brazil, Paraguay, Bolivia, Peru, Uruguay and northern and central Argentina (Giuliano, 2001). Many of these species are extensively used in folk medicine, mainly in the treatment of digestive disorders and also as febrifuge, aphrodisiac, antimicrobial, anti-inflammatory, and anti tumor agents, among others (Alonso and Desmarchelier, 2006). *Baccharis articulata* (Lam.) Pers., commonly known as "carqueja", is popularly used in infusion, decoction or maceration as digestive, cholagogue, diuretic, hepatoprotective, and anti-diarrheic agent, as well as in the treatment of respiratory and urinary infections (Martínez Crovetto, 1964; Perez and Anesini, 1994). Currently are marketed medicinal products containing "carqueja", both in countries where it originates and in the United States and Europe for different uses as slimming, anti-diabetes, for female infertility, male sexual impotence, and in the treatment of skin disorders as leprosy, and rheumatism (Bandoni et al., 1972; Mangiaterra, 2005). The *n*-butanol fractions obtained from aqueous extract of *B. articulata* showed potential antioxidant activity (De Oliveira et al., 2003). The ethanol extract of this species demonstrated antiviral activity on *Herpes suis* virus strain RC/79 (Zanon et al., 1999). In addition, Gene et al. (1992) demonstrated anti-inflammatory effects of an aqueous extract of *B. articulata.*

The majority of medicinal plants have not been studied in terms of their toxic and/or mutagenic potential. It is necessary early in the development of unknown products and chemicals to determine the potential cytogenotoxic effects. A balance between therapeutic versus toxicological effects of the compound is important when determining its applicability as a pharmacological drug. In a previous study, we demonstrated that the cold aqueous extract of *B. articulata* induced toxic effects on human PBMCs causing cell death, and low mutagenic effects on bone marrow of mice at 6 h after administration (Cariddi et al., 2010). Because it is necessary

Abbreviations: FCS, fetal calf serum; ME, mercaptoethanol; MN, micronuclei; MTT, 3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; NCE, normo chromatic erythrocytes; PBMCs, peripheral blood mononuclear cells; PCE, polychromatic erythrocytes; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

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to know the potential risks involved in the consumption of extracts of *B. articulata*, this study evaluated whether human PBMCs death induced by cold aqueous extract of *B. articulata* is due to apoptosis and whether this extract also exerts mutagenic effects on bone marrow of mice at 24 and 48 h after administration. In addition, we chemically characterized the aqueous extract.

2. Materials and methods

2.1. Plant material

Aerial parts of *B. articulata* (Lam.) Pers. (Asteraceae) were collected in April 2008, in Alpa Corral hills, Córdoba province, Argentina. This plant was identified and classified taxonomically by Dr. Margarita Grosso in the Area of Botany of the Universidad Nacional de Río Cuarto, and a voucher specimen was stored in the RCV (Río Cuarto Vasculares) herbarium as file #1810. The morphological characterization of the plant was executed macro- and microscopically to confirm the identity of this species.

2.1.1. Preparation of plant extract

Twenty grams of dried plant material were extracted with 1 L of doubly distilled water for 48 h at 4 °C. The final product, which was called cold aqueous extract of *B. atriculata* (Ba-CAE), was lyophilized and stored at −20 °C. At the time of use, it was dissolved in RPMI-1640 medium (Sigma–Aldrich, St. Louis, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin, streptomycin and neomycin) to obtain an initial concentration of 10 mg/mL of extract.

2.2. Isolation of human PBMCs

Peripheral blood was drawn from healthy volunteers (18–25 years old). PBMCs were isolated from blood samples using Hystopaque®-1077 centrifugation (Sigma–Aldrich, St. Louis, USA). From an optimal suspension of 1×10^6 cells/mL, cell viability was determined by trypan blue dye exclusion assay (Mongini and Waldner, 1996). The study was approved by the Universidad Nacional de Río Cuarto Institutional Review Board. In accordance with ethical standards, the healthy volunteers were properly informed of the study and signed an agreement authorizing the test.

2.2.1. Cell viability assay

PBMCs (2×10^5) in a final volume of 200 μl, were cultured in a sterile 96-well microplates containing RPMI-1640, added with 25 mM Hepes, 2 mM L-glutamine, 5% FCS, 50 mM 2-ME, 100 μg/mL streptomycin, 100 μg/mL penicillin and 100 μg/mL of neomycin. Cells were exposed to different concentrations of Ba-CAE (10, 20, 40, 80, 160, 320, 640 and 1280 μg/mL). Cell cultures with RPMI-1640 alone were performed as control. The system was incubated at 37 °C with 5% CO_2 and humidity for 18–24 h. After that time, cell viability was evaluated by trypan blue dye exclusion using Neubauer chamber for counting of viable cells, as described by (Militao et al. (2006)). Each experiment was done in triplicate.

2.2.1.1. Analysis of apoptosis by Hoechst staining. Cell morphology was evaluated by fluorescence microscopy following Hoechst 33258 DNA staining (Sigma–Aldrich, St. Louis, USA), as described (Montaner et al., 2000) with modifications. Briefly, PBMCs were cultured as described previously and exposed to Ba-CAE (10, 20, 40, 80, 160, 320, 640 and 1280 μg/mL) for 18–24 h. After that time cells were centrifuged and fixed with cold methanol (at −20 °C). Then, cells were stained with Hoechst 33258 to a final concentration of 20 μg/mL, and incubated for 5 min at room temperature in the dark. After incubation, cells were examined with a light microscope (Axiophot, Carl Zeiss, Germany), attached to the image-analysis system (Powershot G6, 7.1 megapixels, Canon INC, Japan with software AxioVision Release 4.6.3, Carl Zeiss, Germany). Apoptotic cells were identified by characteristic features of apoptosis (e.g. nuclear condensation, formation of membrane blebs and apoptotic bodies).

2.2.1.2. TUNEL assay. The number of apoptotic human PBMCs treated with Ba-CAE at different concentrations was also assessed by TUNEL staining using ApopTag® Plus Peroxidase In Situ Apoptosis Kit (Chemicon International, USA), as described by (Song et al. (2009)) with modifications. Briefly, PBMCs were cultured as described previously and exposed to Ba-CAE (10, 20, 40, 80, 160, 320, 640 and 1280 μg/mL) for 18–24 h. After that time cells were centrifuged and fixed in slides with acetic acid and methanol (1:4). Cells were then incubated with 20 μg/ml of proteinase k (Sigma–Aldrich, St. Louis, USA), for 15 min at room temperature, and treated with hydrogen peroxide at 3%, 5 min. The slides were incubated with DNA-terminal deoxynucleotidyl transferase (TdT) at 37 °C with humidity for 1 h. After that, the slides were incubated with antidigoxigenin antibody conjugated to peroxidase which was used to label the incorporated digoxigenin-labeled nucleotides, and added with the substrate supplied by the manufacturer. The slides were counterstained with Harris hematoxylin. Apoptotic cells were then assessed as the percentage of TUNEL-positive cells per 400 cells in each slide using a light microscope (Axiophot, Carl Zeiss, Germany), attached to the image-analysis system (Powershot G6, 7.1 megapixels, Canon Inc., Japan with software AxioVision Release 4.6.3, Carl Zeiss, Germany).

2.2.1.3. Analysis of DNA fragmentation. The isolation of fragmented DNA from cells cultivated in 24-well plates was carried out according to the procedure of (Amirghofran et al., 2007) with some modifications. In brief, cells (2×10^5 cells per well) were treated with all concentrations of the plant extract and then collected by centrifugation (2600 rpm, 15 min). In addition, cells cultured with media alone were performed as control. The pellet was resuspended in 0.5 ml of DNA lysis buffer (2% SDS, 10 mM EDTA, 10 mM Tris–HCl, pH 8.5). The lysate was immediately incubated with 0.1 mg/ml proteinase k (Sigma–Aldrich, St. Louis, USA) and 0.5 mg/ml RNase A (Boehringer Mannheim, Germany) for 3 h at 37 °C, added with 200 μl of ClNa 3 M and centrifuged at 3000 rpm, for 15 min. After addition of isopropanol, the DNA was precipitated with 70% ethanol. The samples were then loaded into 2% agarose gel and electrophoresed. The DNA band pattern was visualized under UV light using ethidium bromide staining.

2.3. Animals

Male and female BALB/c mice aged 8–12-weeks old, (weighing 20–25 g), were obtained from the Bioterio Central of the Universidad Nacional de Río Cuarto. Animals were maintained in a temperature and humidity controlled room, with a 12-h light–dark cycle and were allowed food and water *ad libitum*. All experimental procedures were conducted in accordance with recent legislation. This study was approved by the Universidad Nacional de Río Cuarto Institutional Review Board.

2.4. Genotoxicity assay

This trial was carried out using the micronucleus test in mouse bone marrow as described by (Schmid (1975)) with modifications. Briefly, BALB/c mice were separated into groups of 6 (3 males and 3 females), and injected intraperitoneally in independent trials. Three concentrations of Ba-CAE diluted in saline solution (1800, 900 and 450 mg/kg) were used. The negative control group received saline solution by the same route, and the positive control group received 30 mg/kg body weight of cyclophosphamide (Sigma–Aldrich, St. Louis, US). The animals were sacrificed by cervical dislocation at 24 and 48 h post-injection. The bone marrow samples of femoral bone obtained with FCS were fixed with ethanol and stained with May–Grünwald and Giemsa. To evaluate the mutagenic properties induced by the plant extract, the presence of erythrocytes with micronuclei (MN) was observed in a total of 2000 polychromatic erythrocytes (PCE) per treatment. Furthermore, to obtain a measure of toxicity of Ba-CAE on bone marrow, we calculated the toxicity index (TI) by the PCE/NCE ratio in 1000 cells.

2.5. Phytochemical studies of aqueous extract

A phytochemical study of aqueous extract was performed as suggested by (Harborne, 1984). In order to obtain the non-polar compounds present in the complex matrix of the aqueous extract, this was extracted with ethylic ether. By means of the color reaction of flavonoids (Shinoda) (Geissman, 1962), flavones were preliminarily identified in the ether extract. With the aim to identify luteolin and acacetin in the ether extract, a paper chromatography (PC) in Whatman N° 1 was performed against reference substances. The R_F values (mobility relative to front) were calculated in three different mobile phase (MP): (a) BAW = *n*-butanol–acetic acid–water (4:1:5) (Harborne, 1984), (b) TBA = *t*-butanol–acetic acid–water (3:1:1) (c) 15% HOAc = acetic acid in distilled water (Mabry et al., 1970). PC was observed with UV light and NH_3 vapors under UV light. By preparative PC (Whatman N° 3), the ether extract was developed in TBA, and the spots with R_F values coincident with those of luteolin and acacetin were eluted with MeOH and were characterized by UV–vis spectrophotometry. Considering the frequent occurrence of caffeoylquinic acids, especially chlorogenic acid, in the genus *Baccharis* (Martino et al., 1989), we carried out the determination of this metabolite in the aqueous extract. Thus, another fraction of the dried aqueous extract was dissolved in EtOH and was analyzed by two different chromatographic technique against chlorogenic acid as reference substance, by using 15% HOAc in PC and ethyl acetate–methanol–water (100:17:10) in thin layer chromatography (TLC). Compounds (spots) in PC and TLC were detected with UV light and NH_3 vapors under UV light. Caffeoylquinic acids quantification in the aqueous extract, expressed as chlorogenic acid, was determined by UV–vis spectrophotometry (Martino et al., 1989). A calibration curve (EtOH), which was previously made in concentrations ranging from 1.1428×10^{-5} to 4.5712×10^{-5} M of a stock chlorogenic acid solution, was used to obtain the $\varepsilon_{(322\ nm)} = 16533\ M^{-1}\ cm^{-1}$. All measurements were carried out in triplicate, employing a Varian Cary 50 diode array spectrophotometer.

In addition, the tannins characterization in another aqueous extract aliquot was performed by the color reaction with ferric salts (1% Cl_3Fe) (Harborne, 1984).