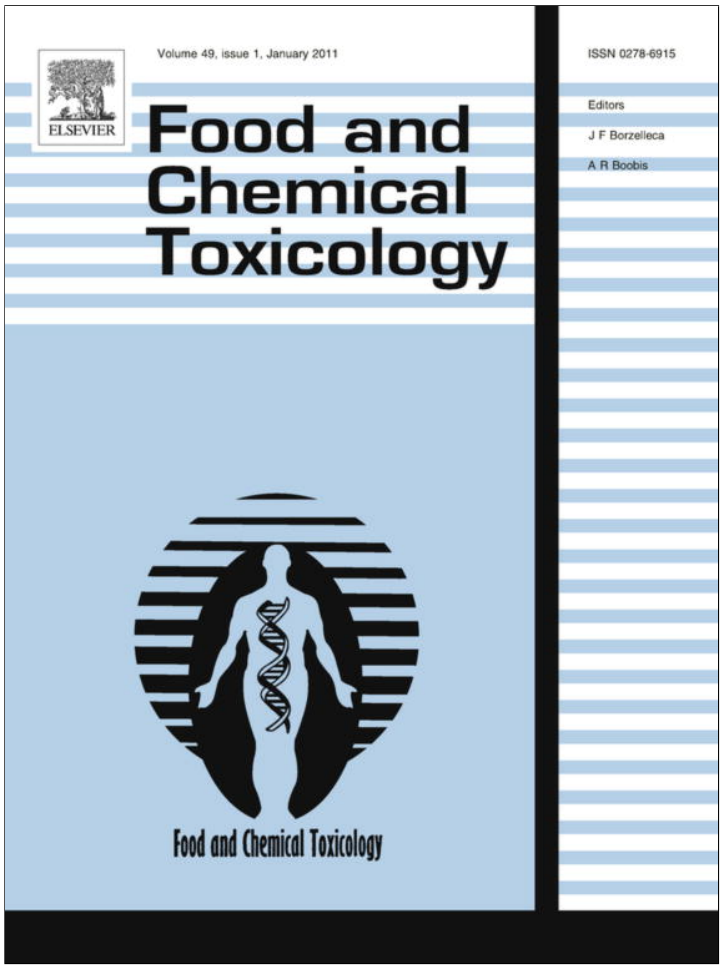

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Apoptosis and mutagenicity induction by a characterized aqueous extract of *Baccharis articulata* (Lam.) Pers. (Asteraceae) on normal cells

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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).

2.6. Statistical analysis

All of the values obtained in the assays carried out during this investigation were expressed as averages and standard deviation. The data obtained for citotoxicity assays were evaluated using the program GraphPad Prism version 5.00.288 (San Diego, USA, 2007) and compared with the parametric *t*-test for twin samples. Statistical differences were considered significant when the value was $p < 0.05$. The data obtained for micronucleus assays were submitted to a one way analysis of variance (ANOVA) and the Tukey multiple comparison test using the GraphPad Prism version 5.00.288 (San Diego, USA, 2007). The differences were considered to be statistically significant at $p < 0.05$.

3. Results

The effect of Ba-CAE on viability of human PBMCs from healthy individuals was studied. A dose-dependent decrease in the number of viable cells was observed by trypan blue dye exclusion assay. The cytotoxic concentration 50% (CC_{50}) of Ba-CAE was 165 μg/mL (Fig. 1).

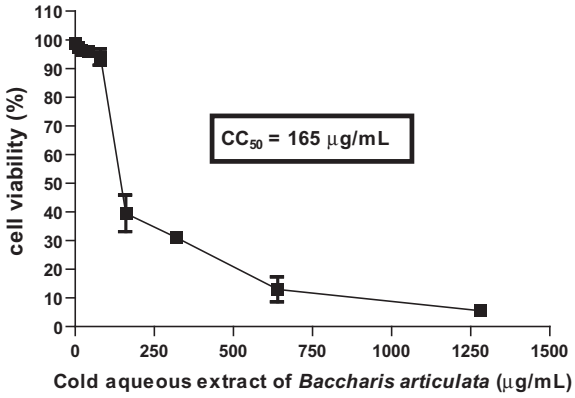

Fig. 1. Viability of human PBMCs from healthy individuals exposed to different concentrations of cold aqueous extract of *Baccharis articulata* (Ba-CAE) for 18–24 h. The results are presented as percentage (mean ± SD). Cell viability was evaluated by trypan blue dye exclusion method.

In order to determine whether the cytotoxic effect of Ba-CAE was due to apoptosis, we analyzed DNA fragmentation as induced by the extract. Lymphocyte morphology was evaluated following Hoechst 33258 DNA staining. We observed in the fluorescence microscope that some nuclei of the PBMCs treated with Ba-CAE at 40–1280 μg/mL contained small bright blue dots representing chromatin condensation and/or nuclear fragmentation. In addition, other apoptotic figures were also observed, such as kidney shaped nuclei, formation of membrane blebs and apoptotic bodies. The nuclei of cells cultured in medium alone were uniformly blue (Fig. 2). We corroborated these results by TUNEL staining. In this assay we observed that PBMCs treated with Ba-CAE at concentrations of 40–1280 μg/mL also showed apoptosis compared to cells cultured in medium alone (7 ± 1%TUNEL + PBMCs). The percentage of

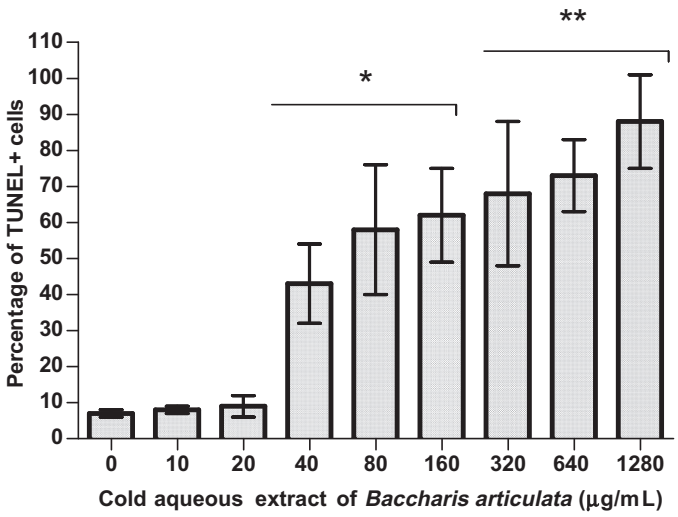

Fig. 3A. Quantification of apoptotic PBMCs from healthy individuals by TUNEL assay versus the *in vitro* treatment with different concentrations of Ba-CAE. $^{*}p < 0.01$ and $^{**}p < 0.001$ with respect to control (cells with medium alone).

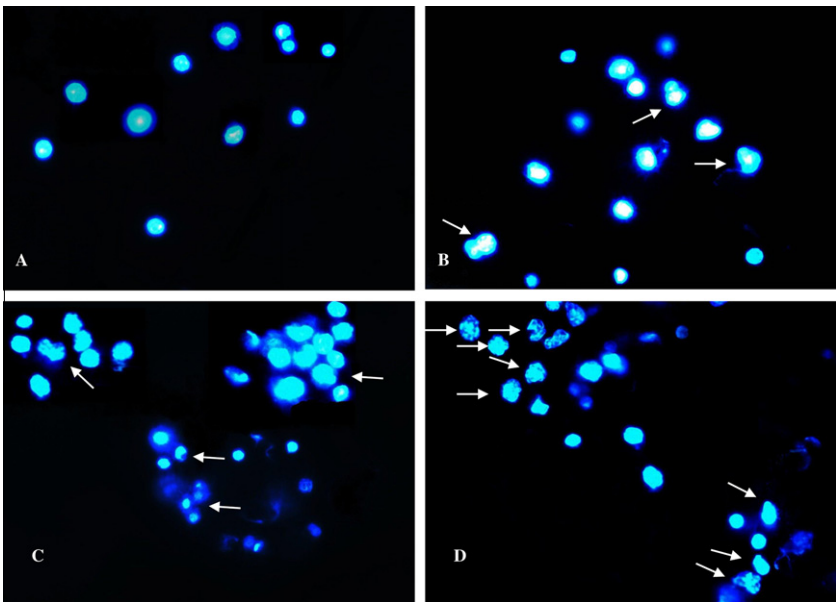

Fig. 2. Photomicrographs of the nuclear morphology of normal human PBMCs stained with Hoechst 33258 (100×). (A) PBMCs without treatment for 18–24 h (control). (B) PBMCs treated with Ba-CAE (40 μg/mL) for 18–24 h. (C) PBMCs treated with Ba-CAE (165 μg/mL) for 18–24 h. (D) PBMCs treated with Ba-CAE (1280 μg/mL) for 18–24 h. Arrows show apoptotic cells. These cells were identified by characteristic features of apoptosis (e.g. nuclear condensation, kidney shaped nuclei, formation of membrane blebs and apoptotic bodies).

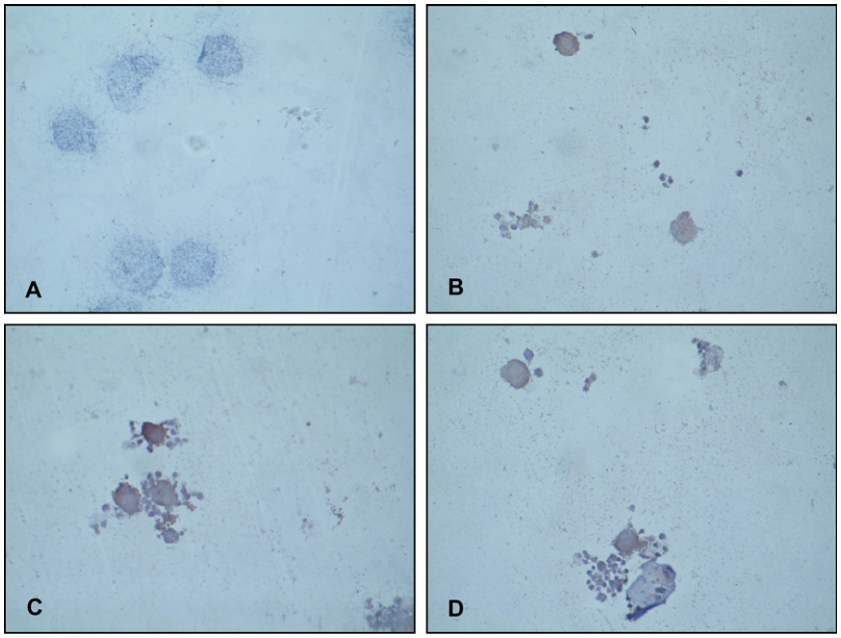

Fig. 3B. Photomicrographs of human PBMCs from healthy individuals stained with TUNEL (100×) using the commercial kit ApopTag® Plus Peroxidase In Situ Apoptosis (Chemicon International, USA). (A) PBMCs with medium alone at 18–24 h (control). (B), (C) and (D) PBMCs treated with Ba-CAE (1280 μg/mL). Apoptotic cells are shown in brown.

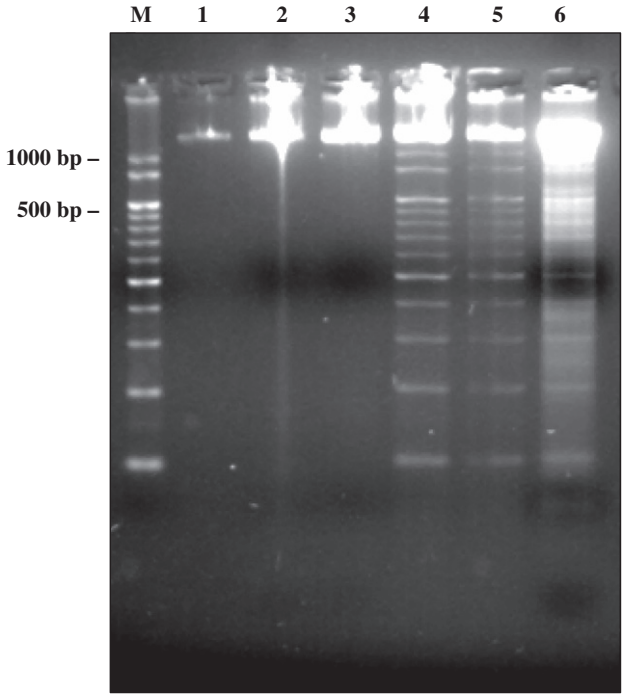

Fig. 4. Electrophoresis in agarose gel showing the DNA laddering of human PBMCs from healthy individuals treated with cold aqueous extract of *Baccharis articulata* (Ba-CAE). M: DNA marker 100 bp, 1: control (cells with medium alone), 2–6: cells treated with Ba-CAE 10, 20, 40, 165 and 1280 μg/mL, respectively.

TUNEL-positive cells treated with Ba-CAE per 400 cells was: 10 μg/mL (8 ± 1%); 20 μg/mL (9 ± 3%), 40 μg/mL (43 ± 11%), 80 μg/mL (58 ± 18%), 160 μg/mL (62 ± 13%) 320 μg/mL (68 ± 20%), 640 μg/mL (73 ± 10%), 1280 μg/mL (88 ± 13%). Statistically significant differences were found in cells treated with Ba-CAE with respect to cells cultured in medium alone (Ba-CAE at 40, 80 and 160 μg/mL: $p < 0.01$; Ba-CAE at 320, 640 and 1280: $p < 0.001$) (Fig. 3A and 3B). Furthermore, we confirmed these results by agarose gel electrophoresis of DNA. As is shown by the typical DNA laddering in agarose gels, apoptosis was induced at extract concentrations of 40–1280 μg/mL (Fig. 4). Human PBMCs treated with Ba-CAE at concentrations of 10 or 20 μg/mL did not show apoptosis signals in the three apoptotic assays performed. In all apoptosis assays we observed that cell death increased with concentration of Ba-CAE. These results strongly suggest that Ba-CAE causes apoptosis in human PBMCs.

Table 1 shows the results of the micronucleus assay obtained for BALB/c mice treated with Ba-CAE. There was a significant increase in the frequency of MN in PCE from the positive control group treated with cyclophosphamide. Clinical signs of behavioral toxicity, as hypothermia, were observed in four of the six animals treated with Ba-CAE at 1800 mg/kg at 24 h, and two of six animals died. In all treatments with Ba-CAE, both at 24 and 48 h, there was an increase in the frequency of MN in PCE, in comparison to the saline group (Tukey test, $p < 0.05$). The number of MN was increased at 48 h post-administration of Ba-CAE at 900 and 450 mg/kg (Tukey test, $p < 0.05$). In all treatments with Ba-CAE, only at 24 h could be observed a decrease in the PCE/NCE ratio; however, it was not statistically significant as compared to the saline group, demonstrating the absence of cytotoxicity on bone marrow. Fig. 5 shows photomicrographs of erythrocytes from bone marrow of mice treated with Ba-CAE (1800, 900 and 450 mg/kg) 48 h post-treatment. In this figure are observed NCE and both PCE without MN as micronucleated PCE.

The ether extract, obtained by partitioning the aqueous extract, gave a positive result for the reagent Shinoda (yellow coloration), indicating the preliminary presence of flavones (Geissman, 1962), according to previously reported (Abad and Bermejo, 2007; Palacios et al., 1983). By PC, two compounds (spots) of the ether extract were identified as luteolin and acacetin, since their coloration and R_F values were coherent with those of reference substances in three solvent system (BAW, TBA and 15% HOAc).

Preparative PC of the ether extract, developed in TBA, showed two main spots (A with $R_F = 0.75$ and B with $R_F = 0.90$), that were eluted with MeOH and were characterized by their UV absorption

Table 1
Mutagenic effects of cold aqueous extract of *Baccharis articulata* (Ba-CAE) on erythrocytes from bone marrow of mice.

Treatments	Animals[a]	PCE/NCE ± SD (TI)[b] 24 h	PCE/NCE ± SD (TI)[b] 48 h	MNPCE[c] (‰) 24 h	MNPCE[c](‰) 48 h
Negative control (Saline solution)	6	1.25 ± 0.18	1.16 ± 0.02	4.75 ± 1.77	4.25 ± 1.06
Cold aqueous extract of B. articulata (Ba-CAE) (mg/kg)					
1800	6	1.03 ± 0.04	1.12 ± 0.17	19.50 ± 3.54*	29.00 ± 2.12*
900	6	0.72 ± 0.16	1.08 ± 0.16	11.50 ± 0.71*(a)	21.50 ± 2.12*(b)
450	6	0.86 ± 0.36	1.20 ± 0.14	10.25 ± 0.35*(c)	18.75 ± 1.77*(d)
Positive control (cyclophosphamide)	6	1.20 ± 0.60	1.37 ± 0.38	26.50 ± 8.02**	30.50 ± 9.01**

*$p < 0.05$ **$p < 0.001$, statistically significant difference from saline group.(a) *vs* (b) $p < 0.05$, (c) *vs* (d) $p < 0.05$ (ANOVA Tukey test).
[a] Six mice were used per experimental group both at 24 h as at 48 h of treatment; At 24 h of treatment, two of six animals died after injection of Ba-CAE at 1800 mg/kg.
[b] Toxicity Index. In all cases 2000 polychromatic erythrocytes (PCE) were analyzed.
[c] Micronucleated polychromatic erythrocytes.

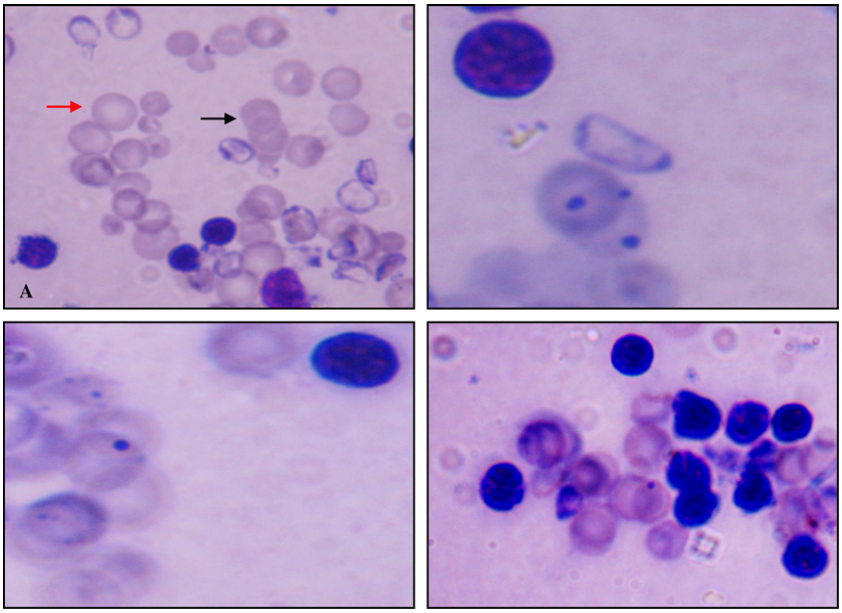

Fig. 5. Photomicrographs of erythrocytes from bone marrow of mice treated with Ba-CAE at 48 h. (A) Normal erythrocytes (black arrow indicates the PCE and red arrow NCE). (B), (C) and (D) Micronucleated PCE from bone marrow of mice treated with Ba-CAE at 1800, 900 and 450 mg/kg, respectively. May Grünwald and Giemsa stain (100×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spectra. Thus, UV spectral data of compound A were similar to luteolin (λ_{max} nm: 243sh, 255, 269, 293sh, 353) and UV spectrum of the compound B was like acacetin (λ_{max} nm: 268, 306sh, 328), in concordance with (Mabry, 1970). By means of PC and TLC, a compound in the aqueous extract was identified as chlorogenic acid by comparing its color and R_F values with reference substance. By UV–vis spectrophotometry, we established a 6.20 ± 0.40% w/w of caffeoylquinic acids expressed like chlorogenic acid in the aqueous extract. Finally, the aqueous extract was positive for reaction with 1% Cl_3Fe, by developing a blue-green color that confirms the presence of tannins.

4. Discussion

It is known that a balance between therapeutic and toxicological effects of any substance is important for its applicability as a pharmacological drug. *B. articulata* (Lam.) Pers. is widely used in the treatment of different disorders, but few studies refer to its toxic effects. In this work, we evaluated the toxic effects of cold aqueous extract of this species (Ba-CAE) on normal cells both *in vitro* and *in vivo* assays. In the *in vitro* assays, we demonstrate that Ba-CAE had toxic effects as revealed by the death of human PBMCs observed by the trypan blue dye exclusion assay. These results are in agreement with a previous study, in which the toxic effect of Ba-CAE on human PBMCs by trypan blue dye exclusion method was also evaluated and a CC_{50} = 150 μg/mL was reported (Cariddi et al., 2010), which is a similar value to that obtained in this work (CC_{50} = 165 μg/mL).

Zanon et al., 1999 evaluated the toxic capacity of ethanol extract of *B. articulata* on Vero cells monolayers and reported a non cytotoxic maximum concentration (NCMC) of 2.20 mg/mL. On the other hand, some authors have reported several bioactivities of other *Baccharis* species and have studied also its cytotoxicity. Paul et al. (2009) demonstrated that aqueous extract of *B. trimera* in concentration of 25, 50 and 100 mg/mL presented cytotoxic effects causing the death of human lymphocytes. Another study showed that hexane extract of *B. dracunculifolia* did not exhibit any apparent cytotoxic effects on murine macrophages at 20 μg/mL (Johann et al., 2010).

In this study we also demonstrate that Ba-CAE induces apoptosis in human PBMCs from healthy individuals. By the trypan blue dye exclusion method, we determined that Ba-CAE at concentrations of 10–80 μg/mL did not cause cell death. However, in apoptosis assays, this extract stimulated cell death starting at 40 μg/mL. This result could be due to the fact that trypan blue dye exclusion is an assay that detects cell death after breach of the cell membrane, but it is well known that the apoptosis process is a cascade

of events that begins before cell has completely lost membrane integrity (Gottlob et al., 2001; Montaner et al., 2000; Sharma et al., 2007). Therefore, some cells exposed to Ba-CAE at 40 and 80 μg/mL, which were probably counted as living cells in the trypan blue dye exclusion method, may have already had entered apoptosis. This is the first report of apoptotic effects induced by *B. articulata* on human PBMCs of healthy individuals, but the mechanism of induction is still unknown. It has been shown that other species of the Asteraceae family cause apoptosis in human and mouse lymphocytes (Mercer et al., 2007; Ogata et al., 2010). There is information on cell death induced by an extract of *Baccharis coridifolia*. This extract may have compounds that interact with DNA and induce oxidative stress lead to cellular death. Some of these compounds could be macrocyclic tricothecens (roridins, verrucarins and baccharinoids), which show high toxicity towards eukaryotic organisms (Mongelli et al., 1997, 2000). Apoptosis can be induced by diverse stimuli, such as common signaling mediators, oxidative stress, among others. Studies to elucidate whether the main compounds found in Ba-CAE are responsible for cytotoxic effects and mechanism by which they induce apoptosis on human PBMCs are now in progress.

In the *in vivo* assays, we demonstrate that Ba-CAE, in all concentrations tested, exerted mutagenic effects on bone marrow due to an increase in the frequency of the micronucleated PCE, both at 24 and 48 h. In a previous study we demonstrated that Ba-CAE, at 6 h post-injection and at higher doses (900 and 1800 mg/kg), increased the number of micronucleated PCE in comparison to the saline group (Cariddi et al., 2010). In addition, we observed that the time of exposure to the extract seems to have influenced the increase of micronucleated cells in comparison to the previous study (Cariddi et al., 2010) (6 h < 24 h < 48 h).

There are some studies that have demonstrated that diverse extracts obtained from other species of genus *Baccharis* exert mutagenic effects (Munari et al., 2010; Rodrigues et al., 2009a,b; Vargas et al., 1991). The genotoxic effects may be produce by certain compounds as flavonoids, as quercetin and rutin as well as caffeic acid (da Silva et al., 2002; Pereira et al., 2006). Although the mechanism by which these compounds induce a mutagenic effect is still unknown, some studies have shown that flavonoids can act as pro-oxidants, in certain concentrations, depleting the nuclear antioxidant defense systems and leading to oxidative DNA damage (da Silva et al., 2002). Some of main compounds identified in Ba-CAE could be responsible for mutagenic effect observed in this study. Further studies are needed to elucidate if the main compounds found in Ba-CAE are responsible for genotoxic effects.

In conclusion, Ba-CAE induced apoptosis and mutagenic effects on normal cells. The induction of apoptosis in human PBMCs was observed both at high and low doses and the genotoxic effects on erythrocytes from bone marrow of mice were observed in all doses tested. The positive results for cytotoxicity and mutagenicity demonstrated by Ba-CAE in this study and in a previous study indicate that although the species is a medicinal herb; its phytotherapy use in infusion, decoction or maceration should be limited as it may cause serious damage to cells when used improperly. Studies of cytotoxic and genotoxic effects of other extracts of *B. articulata* on normal cells are now in progress.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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