Food and Chemical Toxicology 60 (2013) 463–470

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Evaluation of the cytotoxicity, genotoxicity and apoptotic induction of an aqueous extract of *Achyrocline satureioides* (Lam.) DC

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ARTICLE INFO

Article history:
Received 28 May 2013
Accepted 3 August 2013
Available online 12 August 2013

Keywords:
Achyrocline satureioides (Lam.) DC
Asteraceae
Cold aqueous extract
Cytotoxicity
Genotoxicity
Apoptosis

ABSTRACT

Achyrocline satureioides is widely consumed as infusion or aperitif and shows important therapeutic properties. Previously, we reported absence of genotoxicity of cold aqueous extract (CAE) of *A. satureioides* by *Allium* test. However, one test cannot predict the genotoxic effects of a substance. Thus, the aim of this work was to investigate cytotoxicity, genotoxicity and apoptotic ability of CAE of *A. satureioides*. In addition, CAE was chemically characterized. The cytotoxicity was evaluated by Trypan blue and MTT assays. The apoptotic capacity was evaluated by Hoechst staining and DNA fragmentation-analysis. The genotoxicity was studied by comet assay (CA) and micronucleus test. The identification and quantification of flavonoids were performed by HPLC–ESI–MS/MS. The cytotoxicity studies indicated low toxicity of CAE. In addition, CAE did not induce apoptotic effects on human PBMCs. CAE did not show genotoxicity in vitro against Vero cells, at 10–50 μg/mL. CAE did not induce in vivo genotoxic effects, but it showed at high concentrations cytotoxicity by micronucleus assay. CAE presented flavonoids such as quercetin, 3-*O*-methylquercetin and luteolin. In conclusion, *A. satureioides* at popularly concentrations used, in aperitif or infusion, can be consumed safely because did not show any cytotoxic or genotoxic effects.

1. Introduction

Achyrocline satureioides (Lam.) DC is a relevant species which belongs to Asteraceae family. This plant, commonly known as 'marcela del campo', is native from America and extends throughout the continent, as well as in Europe and Africa. Marcela grows in phytogeographic region named 'Monte'. In Argentina, it is often found in sandy and humid soils in the hills of Córdoba, San Luis and Buenos Aires (Instituto Nacional de Investigación Agropecuaria, 2004). Several medicinal properties have been attributed to this plant, such as anti-inflammatory, sedative, hepatoprotective, antioxidant, immunomodulatory, antimicrobial, antitumoural, antiviral and photoprotective (Zanon et al., 1999; Kadarian et al., 2002; Ruffa et al., 2002; Arredondo et al., 2004; Bettega et al., 2004; Hnatyszyn et al., 2004; Polydoro et al., 2004; Morquio et al., 2005; Calvo et al., 2006; De Souza et al., 2007; Sabini et al., 2010).

A. satureioides is widely used as medicinal herb in South America. This plant is popularly used in infusion, macerate in cold water, decoction and as syrup (García et al., 1990; Martínez Crovetto, 1981; Alonso Paz et al., 1992). It is one of the constituents of aperitif drinks widely consumed by the people (Petenatti et al., 2004).

Also, this plant is traditionally employed as an antispasmodic in cases of intestinal infections and various digestive disorders. Its infusion is widely utilized for the treatment of respiratory problems, including asthma, bronchitis and upper respiratory tract infections, as well as for viral infections. It has also been used in gynecological wash preparations and treatment of cardiovascular diseases (Filot Da Silva and Langeloh, 1994; Instituto Nacional de Investigación Agropecuaria, 2004; Taylor, 2005).

Several studies with *A. satureioides* reveal its great ethnobotanical potential. However, studies of cytotoxic and genotoxic effects of this vegetal species are necessary. In previous studies we reported absence of genotoxicity of cold aqueous extract of

Abbreviations: CA, comet assay; CAE, cold aqueous exctract; FCS, fetal calf serum; ME, mercaptoethanol; MN, micronucleus; MNPCE, micronucleated polychromatic erythrocytes; MTT, 3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; NCE, normochromatic erythrocytes; PBMCs, peripheral blood mononuclear cells; PCE, polychromatic erythrocytes.

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http://dx.doi.org/10.1016/j.fct.2013.08.005

A. satureioides by *Allium cepa* L test (Sabini et al., 2011). High concentrations of the extract induced cytotoxicity action, but this effect was reversed and was not associated with mutagenicity. On the other hand, genetic toxicology determines that one single test cannot predict genotoxic effects of a substance (Zúñiga González and Gómez Meda, 2006).

Therefore, the aim of the present study was evaluate the cytotoxicity, genotoxicity and apoptotic induction of cold aqueous extract obtained from *A. satureioides*. In addition, the chemical characterization of this aqueous extract was performed.

2. Materials and methods

2.1. Plant material and extraction

A. satureioides plants were collected manually from Villa Jorcoricó, southern Córdoba hills (32° 41′ S; 64° 43′ W; 800 m sea level) in May 2007. The plant material was identified by Dr Luis Del Vitto, Faculty of Pharmacy and Biochemistry, Universidad Nacional de San Luis, San Luis, Argentina. A voucher specimen was deposited in the Herbarium of the Universidad Nacional de San Luis (No. 6362).

Aerial vegetal parts (leaves, stems and blooms) were submitted to extraction with water at 4 °C (15 g of dried and pulverized material per 700 mL of water) for 2 days. The suspension was filtered and lyophilized (LABCONCO freeze dry system 4.5, Labconco Corporation Kansas city, USA). This solution was identified as Cold Aqueous Extract (CAE). Stock was prepared at concentration of 100 mg/mL in phosphate buffered saline (PBS) and centrifuged at 10,000 rpm for 30 min. The extract was stored at −20 °C.

2.2. Cell culture

Bioassays were performed in Vero cells (*Cercopithecus aethiops* green monkey kidney epithelial cell line; ATCC CCL-81) grown in Eagle's minimal essential medium (EMEM; Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Natocor, Argentina), glutamine (30 μg/mL) and gentamicin (50 μg/mL) (all from Sigma–Aldrich, Italy). Cell cultures were maintained at 37 °C in a 5% (v/v) CO_2 humidified atmosphere.

2.3. Cytotoxicity assays

2.3.1. Cytotoxicity on Vero cells

The cytotoxic concentration of the extract which reduced the viable cell number by 50% (CC_{50}) was determined by Trypan blue dye exclusion method and MTT assay.

For cytotoxicity assays, cells were cultured in 96-well culture plates (Cellstar, Greiner Bio-One, Germany). After incubation for 24 h at 37 °C, cells were exposed to increasing concentrations of the extract (5-3600 μg/mL). Assays were carried out in triplicate. Monolayers incubated only with EMEM were the cellular viability controls.

To determinate CC_{50} by Trypan blue method and after cells were treated with CAE for 48 h, the monolayers were washed with PBS and dissociated with trypsin-EDTA 2X. Then, viable cell number was calculated by optic microscopy using Neubauer chamber.

The CC_{50} was also determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich) method (Mosmann, 1983). Briefly, monolayers treated with CAE for 48 h at 37 °C were incubated with MTT solution for 4 h at 37 °C. Subsequently, supernatant was removed and acid-isopropanol (0.04 N HCl in isopropanol) was added. After gently shaking for 15 min, the absorbance was read on a multiwell spectrophotometer (Bio-Tek, ELx800) at 570 nm. Percentage survival fraction was calculated considering optical density (OD) of cultures treated versus controls.

2.3.2. 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 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 Comité de Ética de la Investigación Científica (COEDI), Universidad Nacional de Río Cuarto. In accordance with ethical standards, the healthy volunteers were properly informed of the study and signed an agreement authorizing the test.

2.3.3. Cytotoxicity on human PBMCs

The cells (2×10^5 cells/well) in 200 μL of final volume, were cultured in sterile 96-well microplates containing RPMI-1640 medium, added with 25 mM Hepes, 2 mM L-glutamine, 5% FCS, 50 mM 2-mercaptoethanol, 100 μg/mL streptomycin and 100 μg/mL penicillin. Cells were exposed to different concentrations of CAE (500, 1000, 1500, 2000, 2500 and 3000 μg/mL). Cell cultures with only RPMI-1640 were used as control. The system was incubated at 37 °C with 5% CO_2 and humidified atmosphere for 18–24 h. After that time, cell viability was evaluated by two independent methods: (a) Trypan blue dye exclusion using Neubauer chamber for counting of viable cells, as described by Militão et al. (2006) and (b) colorimetric MTT assay, as described by Mosmann (1983). Each experiment was done in triplicate.

2.4. Apoptotic assays

2.4.1. 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 CAE (500, 1000, 1500, 2000, 2500 and 3000 μg/mL) for 18–24 h. After that time, cells were centrifuged and fixed with cold methanol (−20 °C). Then, cells were stained with Hoechst 33258 (20 μg/mL of final concentration), and incubated for 5 min at room temperature in the dark. Cells were examined with 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).

2.4.2. DNA fragmentation analysis

The isolation of fragmented DNA from cells cultivated in 96-well plates was carried out according to the procedure of Amirghofran et al. (2007) with modifications. In brief, cells (2×10^5 cells/well) were treated with different concentrations of CAE (500–3000 μg/mL) and then centrifuged at 2600 rpm, 15 min. In addition, cells cultured with media alone were used as control. The pellet was suspended 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, it was added 200 μL of NaCl 3 M and centrifuged at 3000 rpm for 15 min. After isopropanol addition, the DNA was precipitated with 70% ethanol. The samples were loaded into 2% agarose gel and electrophoresed. The DNA band pattern was visualized under UV light using ethidium bromide staining.

2.5. Genotoxicity assays

2.5.1. Single-cell gel electrophoresis (comet assay)

Comet assay (CA) was carried out following a method described by Singh et al. (1988) with slight modifications. Vero cells were cultured in 96-well culture plates in EMEM, supplemented with 10% (v/v) FCS, glutamine (30 μg/mL) and gentamicin (50 μg/mL). Cell monolayers were exposed to increasing concentrations of CAE (10, 25, 50, 250 and 500 μg/mL) for 4 h at 37 °C. Positive (Mitomycin C 0.01 mM) and negative (only EMEM) controls were included. Cell viability was determined using Trypan blue dye exclusion method, and DNA damage was analyzed only at those concentrations that exceeded 90% cell viability. The cell monolayers were washed with PBS, and disaggregated with trypsin-EDTA 2X for 5 min at 37 °C. A 20 μL aliquot of cells from each well (80 μL for concentration) was mixed with 100 μL 0.75% low melting point agarose at 37 °C. Immediately 75 μL was spread onto 2 microscope slides per concentration pre-coated with 0.75% normal melting point agarose. The slides were coverslipped and allowed to gel at 4 °C for 20 min. The coverslips were gently removed and 75 μL of 0.75% low melting point agarose at 37 °C was added. Again, the slides were coverslipped and allowed to gel at 4 °C for 20 min. The coverslips were removed and the slides were immersed in cold, freshly prepared lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), supplemented with 1% Triton X-100 and 10% DMSO (Merck). The slides, which were protected from light, were allowed to stand at 4 °C for 1 h. They were placed in a gel box, and left in high pH (>13) electrophoresis buffer (300 mM NaOH, 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM EDTA) at 4 °C for 20 min before electrophoresis to allow the DNA to unwind. Electrophoresis was carried out in ice bath (4 °C) for 20 min at 250 mA and 30 V (0.722 V/cm). The slides were submerged in neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 15 min, dried at room temperature and fixed in 100% ethanol for 10 min. The slides were dried and stored overnight or longer before staining. For staining the slides were briefly rinsed in distilled water, covered with 25 μL 1X ethidium bromide staining solution prepared from a 200 μg/mL 10X stock solution, and coverslipped. The material was evaluated immediately at 400× magnification using fluorescence 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), with 515–560 nm excitation filter and a 590 nm barrier filter. From each treatment, images from 100 "nucleoids" were captured with a camera attached to the fluorescent microscope and linked to the CometScore® 1.5 software. Highly damaged cells were not included in the scoring (clouds were not analyzed). Tail moment (TM) was used to estimate DNA damage (arbitrary units).

Statistical analysis was performed using GraphPad Prism program, version 5.00.288 (San Diego, USA, 2007). Kruskal Wallis test and Dunns multiple comparison as "a posteriori" test were used in all the experiments. The Spearman statistical test was used to examine possible dose-response effects.

2.5.2. Animals and treatment

Male and female Balb/c mice aged 8–12-weeks old, (weighing 20–25 g) were obtained from the Central Bioterio of the Universidad Nacional de Río Cuarto. Animals had access to food and water *ad libitum* and were housed in a temperature-controlled environment on a 12 h light/12 h dark cycle throughout the experimental period. All experimental procedures were conducted in accordance with recent legislation. This study was approved by Comité de Ética de la Investigación Científica (COEDI), Universidad Nacional de Río Cuarto. Five groups of mice were inoculated by intraperitoneal injection with CAE at concentrations of 50, 100, 250, 500 and 750 mg/kg body weight (b.w.) dissolved in saline solution and 2 control groups were included. The negative control group received saline solution and the positive control group received 20 mg/kg b.w. of cyclophosphamide (Sigma–Aldrich, St. Louis, US). Each treatment group consisted of six animals.

2.5.3. Lethal dose 50% (LD_{50})

The mortality rate of mice treated with CAE was assessed at 24 h post intraperitoneal injection (i.p.), according to Reed and Muench (1932).

2.5.4. Micronuclei test in mouse bone marrow

The assay was carried out following standard protocols as recommended by Schmid (1975). The animals were sacrificed by cervical dislocation at 24 h post-injection. The bone marrow samples of femoral bone were obtained with FCS, were homogenized, centrifuged, and plated on slides which were fixed by soft flutter. Then, the slides were stained with May-Grunwald–Giemsa. To establish genotoxic capacity of CAE, the frequency of micronuclei in 1000 polychromatic erythrocytes per slide was determined. To detect possible cytotoxic effects, the ratio of polychromatic erythrocyte/normochromatic erythrocyte (PCE/NCE) in 1000 polychromatic erythrocyte was calculated. The slides were scored using a light microscope at a 1000× magnification. Average number of micronucleated polychromatic erythrocytes (MNPCE) in individual mice was used as the experimental unit, with variability based on differences among animals within the same group.

2.6. Statistical analysis

Values were expressed as the mean ± standard deviation (SD). The data obtained for cytotoxicity assays were evaluated using GraphPad Prism program, version 5.00.288 (San Diego, USA, 2007) and compared with the parametric *t*-test for twin samples. Results obtained by micronucleus assays were submitted to a one-way analysis of variance (ANOVA) and the Tukey's multiple comparison test using the GraphPad Prism software. The level of significance was established at $p < 0.05$.

2.7. Identification and quantification of polyphenol and flavonoid derivatives by HPLC–ESI–MS/MS

2.7.1. Preparation of extract

With the aim to determine the presence of typical polyphenol and flavonoid derivatives of *A. satureioides* (De Souza et al., 2002 and Retta et al., 2012), CAE (100 mg) was dissolved in H_2O (3 mL) and was extracted three times with 2 mL of diethyl ether (Ether). Thus, an extract rich in both components (polyphenols and flavonoids) was obtained from CAE complex matrix. The ethereal phase obtained (CAE-Ether) was evaporated to dryness.

2.7.2. Sample preparation

Three individual solutions of the CAE-Ether in MeOH (1.8 mg/mL) were prepared to carry out qualitative and quantitative analysis by HPLC–ESI–MS/MS. MeOH-HPLC grade (Merck) which was filtered through 0.45 μm Millipore membrane was used in all samples.

2.7.3. HPLC–ESI–MS/MS instruments and chromatographic conditions

An Agilent Series 1200 LC System (Agilent, USA) coupled to a MicrOTOF Q II (Bruker Daltonics, USA) was used for HPLC–ESI–MS/MS analysis. The HPLC system consisted in a micro-vacuum degasser, binary pumps, an autosampler (40 μL sample loop), a thermostated column compartment and a diode array detector. The mass spectrometer equipped with electrospray ion source and qTOF analyzer was used in MS and MS/MS mode for the structural analysis of phenolics and flavonoids.

HPLC analysis was performed on a thermostated (40 °C) Hypersil 5 column C_{18} (30 × 4.6 mm, Phenomenex) at a 0.4 mL/min flow rate using MeOH–Formic Acid 0.16 M (53:47) as mobile phase (De Souza et al., 2002). The injection volume was 40 μL.

ESI–MS detection was performed in negative ion mode with mass acquisition between 100 and 1500 Da. Nitrogen was used as drying and nebulizer gas (7 L/min and 3.5 bar, respectively), and 180 °C for drying temperature. For MS/MS experiments fragmentation was achieved by using Auto MS^2 option. DAD analyses were carried out in the range between 200 and 700 nm.

Calibration standard samples were prepared from the stock solutions in MeOH to obtain appropriated dilutions which were filtered through Millipore membrane before use. MS analysis was used for quantification of the compounds with specific calibration curve. When reference compounds were not available, as in the case of 3-*O*-methylquercetin, the calibration of structurally related substance was used (quercetin). Compounds concentrations were calculated in triplicate and reported as mean ± standard deviation in each case.

3. Results

3.1. Cytotoxicity assays

The viability tests were performed in order to assess the toxicity of CAE of *A. satureioides*. Vero cells and human PBMCs viability was determined by Trypan blue dye exclusion method and MTT assay. The effect of CAE on viability of human PBMCs showed a dose-dependent decrease in the number of viable cells by Trypan blue dye exclusion method. On the contrary, MTT assay did not show cell viability decrease (Fig. 1). Studies on Vero cells demonstrated that CAE exerted a dose-dependent decrease in the number of viable cells by both methods (Fig. 2). Table 1 shows the CC_{50} values obtained from CAE of *A. satureioides*.

3.2. Apoptotic assays

To determine if cytotoxic effect of high concentrations of CAE was due to apoptosis, DNA fragmentation was analyzed. Lymphocyte morphology was evaluated by Hoechst 33258 DNA staining. Observation by fluorescence microscope revealed that few nuclei of the PBMCs treated with CAE at 2500 and 3000 μg/mL contained small bright blue dots representing chromatin condensation and/or nuclear fragmentation. The nuclei of cells cultured in medium alone were uniformly blue (Fig. 3).

Furthermore, agarose gel electrophoresis of DNA demonstrated that any of concentration of CAE showed the typical DNA laddering in agarose gels (Fig. 4).

3.3. Genotoxicity assays

3.3.1. Single-cell gel electrophoresis (comet assay)

Vero cell viability, tested by Trypan blue, was above 90% at every concentrations of CAE of *A. satureioides* employed for CA.

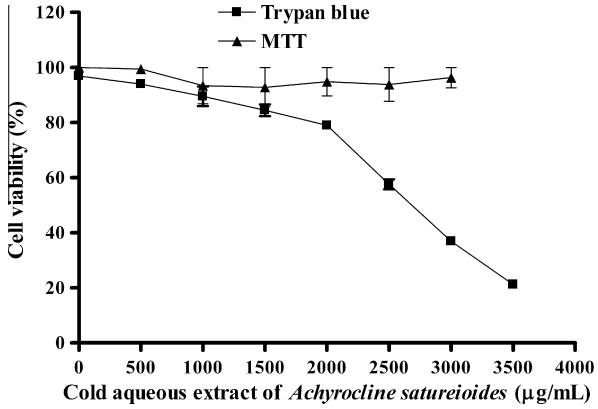

Fig. 1. Viability of human PBMCs from healthy individuals exposed to different concentrations of cold aqueous extract (CAE) of *Achyrocline satureioides* for 18–24 h. The results are presented as percentage (mean ± SD). Cell viability was evaluated by trypan blue dye exclusion method and MTT assay.

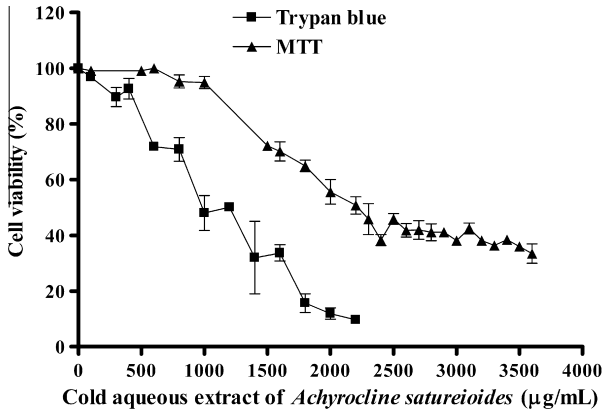

Fig. 2. Viability of Vero cells exposed to different concentrations of cold aqueous extract (CAE) of *Achyrocline satureioides* for 48 h. The results are presented as percentage (mean ± SD). Cell viability was evaluated by trypan blue dye exclusion method and MTT assay.

Table 1
Cytotoxicity of cold aqueous extract (CAE) of *Achyrocline satureioides* against Vero cells and human PBMCs determined by different methods.

Extract	Cytotoxicity			
	Vero cells (CC_{50} μg/mL)		Human PBMCs (CC_{50} μg/mL)	
CAE	AT	MTT	AT	MTT
	1080	2189.5	2660	>3000

The results of the CA, expressed as data of Tail moment of Vero cells treated with 10, 25, 50, 250 and 500 μg/mL, negative and positive controls are shown in Fig. 5. Mitomycin C (positive control) induced a significant increase in DNA migration when compared to negative control ($p < 0.001$). It was not significant difference of cells treated with 10, 25 and 50 μg/mL of CAE respect to negative control. A statistically significant increase in DNA of damaged cells was observed for CAE at 250 and 500 μg/mL respect to the undamaged cells of negative control ($p < 0.001$, Dunns Test). Fig. 6 exhibits cells with or without DNA damage.

3.3.2. Lethal dose 50% (LD_{50})

During this trial physiological changes could not be demonstrated in the animals inoculated with concentrations of 50, 100 and 250 mg/kg b.w. of CAE of *A. satureioides*. These animals had normal behavior, similar to negative control group, with no change in feeding habits during 24 h post-treatment. By contrary, the animals inoculated with the highest concentrations of the extract, 500 and 750 mg/kg, showed 50 and 83.33% of mortality, respectively. The survivor animals of the groups treated with 500 and 750 mg/kg showed toxicity signs such as weakness and hypothermia. Mortality values recorded in the treatments with CAE demonstrated certain dose-response relationship. The LD_{50} of the extract was 500 mg/kg in Balb/C mice.

3.3.3. Micronuclei test in mouse bone marrow

The results of clastogenic ability of CAE are shown in Table 2. Basal level of 3.88 ± 0.99 micronucleated erythrocytes (MNE)/1000 analyzed cells was observed in the animals of negative control group. In the animals treated with 50 and 100 mg/kg b.w.

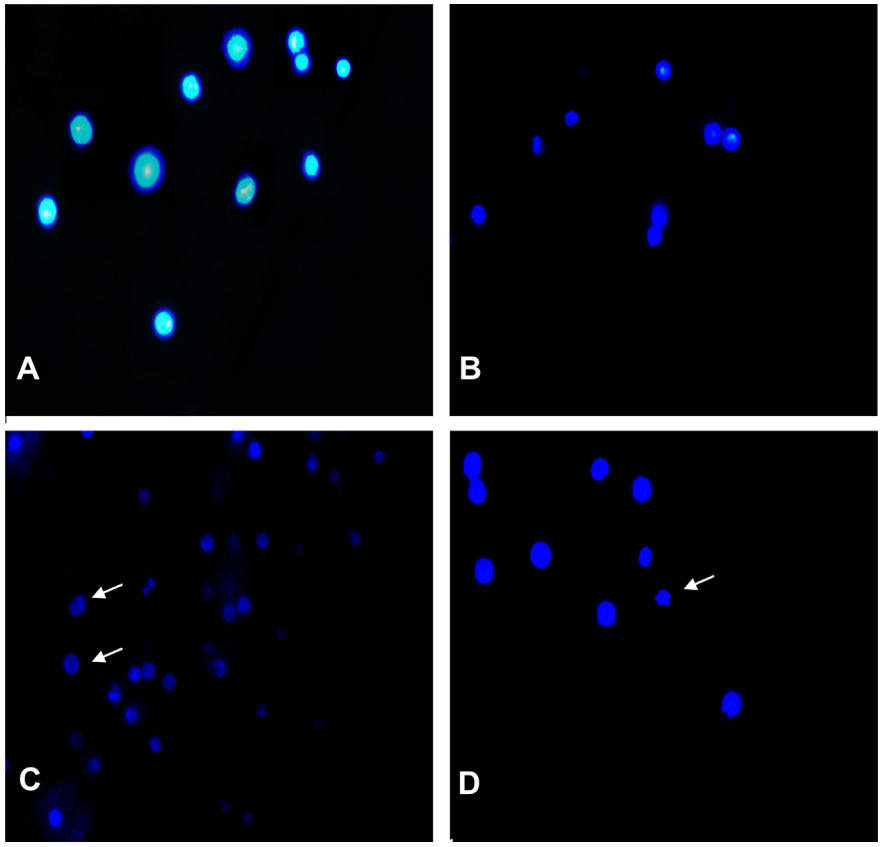

Fig. 3. Photomicrographs of the nuclear morphology of normal human PBMCs treated with CAE for 18–24 h and stained with Hoechst 33258 (100×). (A) PBMCs without treatment (control), (B) PBMCs treated with CAE (500 μg/mL), (C) PBMCs treated with CAE (2500 μg/mL), (D) PBMCs treated with CAE (3000 μg/mL) Arrows show apoptotic cells. These cells were identified by characteristic features of apoptosis (e.g. nuclear condensation and formation of membrane blebs).

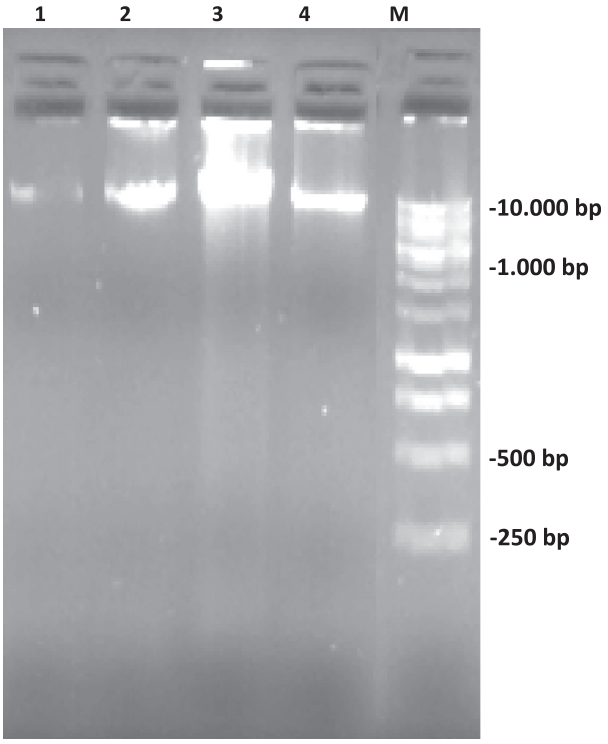

Fig. 4. Electrophoresis in agarose gel showing the DNA laddering of human PBMCs from healthy individuals treated with cold aqueous extract of *Achyrocline satureioides* (CAE). M: DNA marker 250 bp, 1: control (cells with medium alone), 2–4: cells treated with CAE 500, 2500 and 3000 μg/mL, respectively.

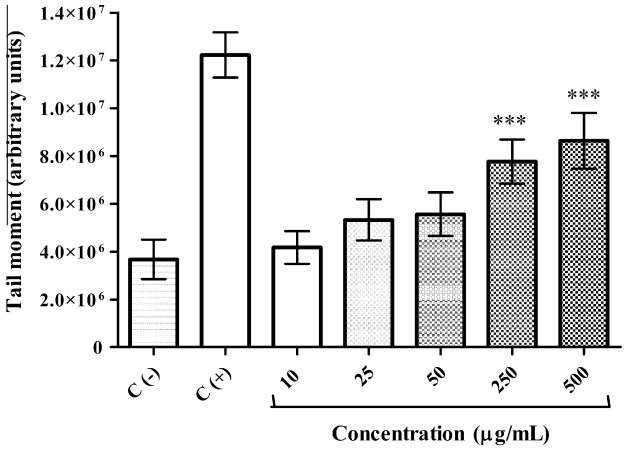

Fig. 5. Vero cells were treated with 10, 25, 50, 250 and 500 μg/mL of cold aqueous extract of *Achyrocline satureioides*. Results are expressed as tail moment. Data shown are means of four replicate cells samples. (***$P < 0.001$; Dunns test).

the number of MNE/1000 analyzed cells was 4.75 ± 1.71 and 2.75 ± 1.14, respectively. The group treated with 250 mg/kg showed a value of 1.42 ± 0.90. There was a decrease in the number of micronuclei at the highest concentration. The treatments of 50 and 100 mg/kg did not show significant difference respect to negative control group but they showed significant difference ($p < 0.001$, Tuckey test) respect to the cyclophosphamide group (11.7 ± 1.7).

For the other hand, the values of toxicity index (ratio of PCE/NCE) did not show significant difference between the group treated with 50 mg/kg b.w. of CAE respect to negative control. In contrast, a decrease in the PCE/NCE ratio was observed in the treatments of 100 and 250 mg/kg b.w. These treatments showed significant difference respect to negative control group ($p < 0.001$, Tuckey test). The study of the toxic capacity of CAE showed a dose-response relationship. There was no sex-dependent difference in any treatment.

Data of genotoxicity and toxicity (Table 2) indicate a logic correlation between them because the highest concentration of extract increased the toxicity and consequently it decreased micronuclei numbers counted.

3.4. HPLC–ESI–MS/MS analysis of cold aqueous extract

The chemical evaluation of CAE obtained from *A. satureioides* was oriented towards the search of active principles which have been previously reported (De Souza et al., 2002 and Retta et al., 2012). Among the flavonoids, quercetin (t_R = 18.3, $[M-1]^-$ = 301), 3-*O*-methylquercetin (t_R = 21.3, $[M-1]^-$ = 315) and luteolin (t_R = 21.6, $[M-1]^-$ = 285) could be identified by qualitative HPLC–ESI–MS/MS analysis of this extract (Fig. 7). Although the latter two compounds have similar t_R and the UV–V detector cannot separate them, the SM detector allowed their identification by parent ion $[M-1]^-$ (315 and 285 *m/z*). In addition, the MS/MS analysis of the parent ion $[M-1]^-$ = 315 showed a product ion at 300 *m/z* that corresponds to a loss of $-CH_3$ group, corroborating the identification of 3-*O*-methylquercetin. The amount of each compound in the extract under study is showed in Table 3 (quantification analysis). Data are expressed as mean ± standard deviation (SD) of three separate experiments. Thus, we established that luteolin is the compound found in high proportion, followed by 3-*O*-methylquercetin and quercetin, with the lowest percentage.

4. Discussion

This work was carried out in an effort to evaluate the cytotoxicity, genotoxicity and the apoptotic induction of CAE of *A. satureioides*. In relation to the cytotoxic studies, the CC_{50} values indicated low toxicity of the aqueous extract obtained from *A. satureioides* because every CC_{50} values were higher than 1000 μg/mL. The high values of CC_{50} of *A. satureioides* support its safety. The results of both methods indicated that the CAE exhibited more toxicity against Vero cells that against lymphocytes. The CC_{50} obtained by Trypan blue assay was lower than the CC_{50} obtained by MTT method, through both cell systems, indicating that CAE affects cytoplasm membranes before mitochondrial respiratory chain. The results obtained with Vero cells are consistent with previous findings, in which CAE showed values of CC_{50} of 960 and >1900 μg/mL by Neutral red uptake and MTT assays, respectively (Sabini et al., 2010). These results indicate that high concentrations of CAE are necessary to cause damage in Vero cells, which agree with results obtained in the present study. Similarly, Zapata et al. (2010) demonstrated that essential oil of *Achyrocline alata,* other species of *Achyrocline* genus, was not cytotoxic on Vero cells at concentrations of 25–200 μg/mL.

The apoptosis assays demonstrated that CAE does not induce this type of cell death because DNA fragmentation was not observed. To our knowledge, studies on the apoptosis of the *Achyrocline* genus were not previously performed. However, investigations have demonstrated that other species of Asteraceae family induce apoptosis on human and mice PBMCs (Mercer et al., 2007; Ogata et al., 2010).

Respect to the lethal dosis of CAE in Balb-C mice, it was possible determine the LD_{50} value which was 500 mg/kg b.w. Rivera et al. (2004) studied the acute toxicity in mice inoculated intraperitoneally with an aqueous extract of *A. satureioides* (aerial parts), collected in Uruguay (also collected in autumn). Their results

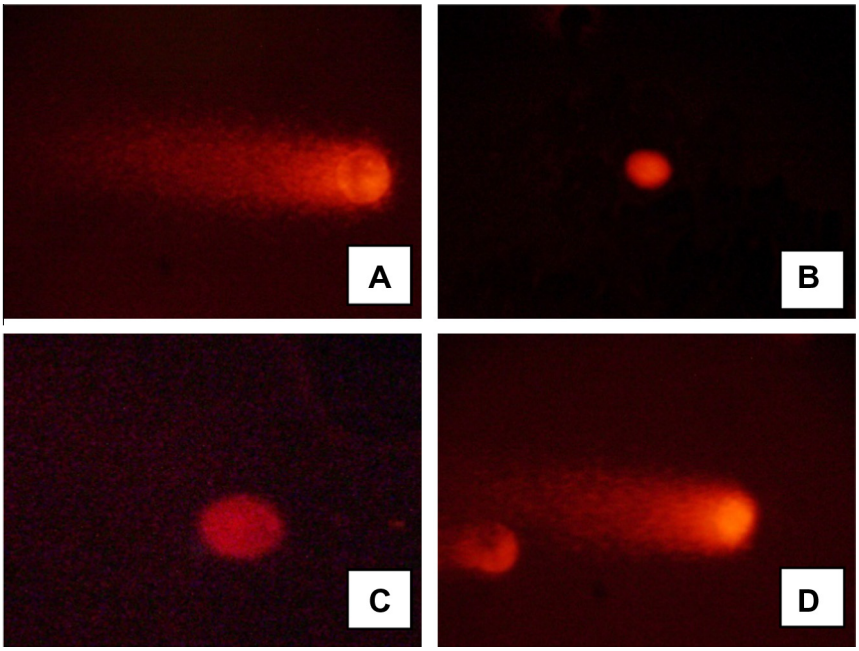

Fig. 6. Photomicrographs of nucleus of Vero cells analyzed by comet assay treated for 4 h with (A) Mitomycin C 0.01 mM; (B) Negative control; (C) 50 μg/mL of CAE of *A. satureioides*; (D) 500 μg/mL of CAE of *A. satureioides*.

Table 2
Genotoxicity and toxicity indexes determined in bone marrow cells of Balb/C mice treated with cold aqueous extract (CAE) of *Achyrocline satureioides* for 24 h, and respective controls.

Treatments	Dose (mg/kg)	MNPCE (‰) (mean ± SD)			PCE/NCE (TI) (mean ± SD)		
		M	F	Total	M	F	Total
Negative control (saline solution)	0	3.50 (±1.09)	4.25 (±0.89)	3.88 (±0.99)	3.62 (±0.78)	3.19 (±0.84)	3.40 (±0.81)
A. satureioides CAE	50	6.00 (±2.17)	3.50 (±1.25)	4.75 (±1.71)	2.74 (±0.50)	2.83 (±0.54)	2.78 (±0.52)
A. satureioides CAE	100	3.00 (±1.21)	2.50 (±1.07)	2.75 (±1.14)	2.09 (±0.33)	2.12 (±0.29)	2.15* (±0.31)
A. satureioides CAE	250	1.33 (±0.81)	1.50 (±0.99)	1.42 (±0.90)	1.47 (±0.41)	1.63 (±0.43)	1.55* (±0.42)
Positive control (cyclophosphamide)	20	12.0 (±1.7)*	11.3 (±2.1)*	11.7 (±1.7)*	1.67 (±0.2)	1.72 (±0.1)	1.69 (±0.15)

SD = standard deviation; MNPCE = Micronucleated polychromatic erythrocytes; PCE = polychromatic erythrocytes; NCE = normochromatic erythrocytes; M = male; F = female.
* Significantly different from negative control ($p < 0.001$) (ANOVA Tukey test); IT = Toxicity Index. In all cases 2000 polychromatic erythrocytes (PCE) were analyzed.

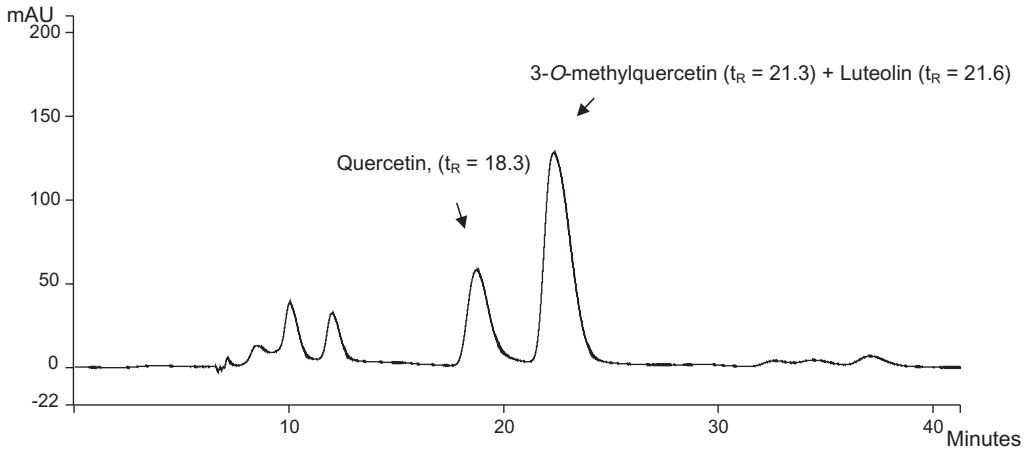

Fig. 7. Qualitative HPLC analysis of cold aqueous extract of *Achyrocline satureioides*.

indicated that mice treated with doses above of 180 mg/kg showed signs of toxicity. However the assays performed in the present study clearly show that treatment with CAE at 250 mg/kg did not induce signs of toxicity. On the other hand, Simoes (1988) demonstrated that 500 mg/kg of ethanol and aqueous extracts of *A. satureioides* did not induce acute toxicity in mice administered intravenously. These results differ from our results which indicate that 500 mg/kg was LD_{50}.

Table 3
Chemical composition of CAE of *Achyrocline satureioides*.

CAE	% *P*/*P* (mg compound/100 mg CAE)		
Compound	Luteolin	Quercetin	3-*O*-methylquercetin
	0.6028 ± 0.0002	0.0224 ± 0.0009	0.0405 ± 0.0003

The study of genotoxic damage by CA allowed us to detect that CAE at the higher concentrations showed genotoxic activity *in vitro* on Vero cells. The CA assay has high sensitivity. Also, this test detects damage that can be reversed by repair processes (Collins et al., 2008). In addition, we performed the genotoxicity *in vivo* by MN assay in mice which indicated that CAE did not induce micronuclei at any concentration tested, so the extract did not have genotoxic capacity. These results are consistent with those obtained with the same extract by *Allium cepa* L test reported in previous studies (Sabini et al., 2011). By the contrary, Vargas et al. (1991) reported that an aqueous extract of *A. satureioides* showed positive result in genotoxicity assay by Ames test. In the present study, the genotoxicity was correlated to the presence of tannins and flavones with certain hydroxylation patterns such as quercetin or kaempferol.

The cold aqueous extract of *A. satureioides* showed the presence of flavonoids such as quercetin, 3-*O*-methylquercetin and luteolin. Other investigations informed that these compounds have not been toxics (Duke, 2000), results coincident with the obtained with CAE.

In our research, the MN test indicated that a concentration of 50 mg/kg b.w. of CAE have no toxic neither genotoxic effect on the stem cells in bone marrow mouse; this concentration is safe for use and consumption.

In conclusion, in this study we showed that *A. satureioides*, medicinal herb species, induced toxic effects on normal cells both *in vitro* and *in vivo* at high concentrations. Therefore, its use as medicinal herbal should be controlled. However, the concentrations which are popularly used in aperitif drinks or in infusion can be consumed safely because they did not show any cytotoxic or genotoxic effects. This vegetal species at non toxic concentrations can be also utilized for medicinal purpose with guarantees of safety.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by Grants from CONICET, PICTOR 8/20325 BID 1728/OC-AR, Agencia Córdoba Ciencia-Nación, Resolution #222, years 2007/10, and SeCyT, Resolution #763, years 2009/11, from Universidad Nacional de Río Cuarto.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.08.005.

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