IDECEFYN
Molecular Medicinal Chemistry
vol 21 January-April 2010, 4-7 http://www.idecefyn.com.ar ISSN 1666-888X

First approaches in the study of cytotoxic and mutagenic damage induced by cold aqueous extract of *Baccharis articulata* on normal cells

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ABSTRACT

Species of Genus *Baccharis* (Asteraceae) possess various bioactivities *in vitro*. The aim was to determine the cytogenotoxic activity of the cold aqueous extract (CAE) of *Baccharis articulata.*
Peripheral blood lymphocytes from healthy individuals were *in vitro* faced to CAE (40, 78, 156, 313, 625 and 1250 μg/mL) for 18-24 h. Toxicity was evaluated by staining of trypan blue exclusion and MTT reduction.
The genotoxicity was evaluated by the Micronucleus Test. Balb/c mice were injected with CAE (1800, 900 and 450 mg/kg), saline solution and cyclophosphamide as negative and positive controls respectively. Animals were sacrificed at 6 h post-injection. Bone marrow samples were fixed and stained with May-Grünwald and Giemsa. Two thousand polychromatic erythrocytes (PCE) were counted to determine number of micronuclei (MN) and normochromatic erythrocytes (NCE)/250 PCE to calculate toxicity index (TI).
CAE toxicity on human lymphocytes was dose-dependent (Cytotoxic concentration 50% = 150μg/mL). The number of MNPCE for negative control was: 5 (±1), positive control: 372 (±23) and CAE in three doses: 26 (±8), 16 (±5) and 8 (±3). TI for negative control: 1.38 (±0.35) positive control: 3.1 (±1.06) and CAE in three doses: 0.78 (±0.05), 0.82 (±0.08) and 1.21 (±0.2). CAE treatment showed no statistical difference respect to negative control. CAE of *B articulata* was not cytogenotoxic.

Keywords: *Baccharis articulata*, Asteraceae, cytotoxicity, genotoxicity, normal cells

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Received: February 22, 2010. Accepted: March 10, 210

Molecular Medicinal Chemistry
IDECEFYN
vol 21 January-April 2010, 4-7 http://www.idecefyn.com.ar ISSN 1666-888X

Introduction

Herbs have different chemical compounds, many of which may be bioactive against various diseases, but also can be toxic. Plant extracts or isolated substances from them may interact directly or indirectly with DNA, causing changes that affect cell function and long-term cause malignant transformation. It is extremely important to detect the action of drugs on genetic material using different biomarkers of effect (Carballo *et al*., 2005).

Furthermore, it is necessary to evaluate the cytotoxicity of plant products on normal cells, to ensure whether they are able to act as bioactive agents without causing adverse effects (Inagaki *et al*., 2007).

It has been shown that extracts from various species of the genus *Baccharis* (Asteraceae), have in vitro antioxidant, antimicrobial, antitumor and antiviral activity (De Oliveira *et al*., 2003, Morales *et al*., 2008).

However, there are few studies assessing its cytogenotoxic action on normal cells. Therefore, the aim of this study was to determine the cytotoxic activity *in vitro* on lymphocytes obtained from healthy humans induced by the cold water extract obtained from *Baccharis articulata* (carqueja). In addition, was evaluated the genotoxic damage induced by the extract on erythrocytes of mouse bone marrow.

Experimental

Plant material

Leaves and stems from *B. articulata* Lam Pers (Asteraceae) collected in Alpa Corral, Córdoba province in April 2008, were used. The vegetal was classified taxonomically by members of the Department of Natural Sciences area Systematic Botany from Universidad Nacional de Río Cuarto, and herborized by the same professionals in the Herbarium of the Universidad Nacional de Río Cuarto Nº RCV 1810. The plant material was kept at -20°C until use.

Preparation of plant extract

Twenty grams of dried and ground plant material were extracted with 1 L of doubly distilled water for 48 hours at 4 ºC. The leaching was called Cold Aqueous Extract (CAE) which was lyophilized and stored at -20 °C. At the time of use was dissolved in RPMI-1640 medium (Sigma Aldrich St. Louis, US) supplemented with 10% fetal calf serum and antibiotics (penicillin, streptomycin and neomycin) to give an initial concentration of 10 mg/mL of extract.

Obtaining of human lymphocytes

Peripheral blood from healthy volunteers (18 to 25 years old) was drawn. The lymphocytes were separated by density gradient with Ficoll-Hypaque (Sigma Aldrich, St. Louis, US). From an optimal suspension of $1x10^6$ cells/mL, cell viability was determined by staining of trypan blue exclusion (Mongini and Waldner, 1996).

Cytotoxicity assays

Mononuclear cells ($2x10^5$ células/mL) were placed in a sterile 96-well microplate (NUNCLO®) and were exposed to different concentrations of CAE (40, 78, 156, 313, 625 y 1250 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, the toxicity of CAE was evaluated by two methods, conducted independently, where each determination was performed in triplicate: a) staining of trypan blue exclusion using Neubauer chamber for counting of viable cells (Militão *et al.*, 2006). b) Colorimetric method of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide] reduction (Mossman *et al*., 1983). Briefly, 10 µL of MTT solution (1 mg/mL MTT in PBS 0.01 M pH 7.2) was added in each well and the plate was incubated at 37 °C with 5% CO_2 for 4 h. Then, 50 µL of dimethylsulfoxide per well were added to dissolve the formazan crystals resulting from the conversion of MTT. Finally, the result was interpreted by reading spectrophotometer (Labsystems Multiskan MS) at 560 nm.

Genotoxicity assay

This trial was carried out using the Micronucleus Test in mouse bone marrow. Balb/c mice, separated into groups of 6 (3 males and 3 females), injected intraperitoneally, in independent trials, with a single dose (1ml) of 3 concentrations of 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 6 h post-injection. The bone marrow samples of femoral bone obtained with fetal calf serum were fixed with ethanol and stained with May-Grünwald and Giemsa. To evaluate the mutagenic properties induced by the plant extract 2000 polychromatic erythrocytes (PCE) per treatment were counted. The presence of erythrocytes with micronuclei (MN) was

Molecular Medicinal Chemistry
IDECEFYN
vol 21 January-April 2010, 4-7 http://www.idecefyn.com.ar ISSN 1666-888X

considered. Furthermore, to detect possible cytotoxic effects of CAE, we calculated the toxicity index (TI) by count of normochromatic erythrocytes (NCE)/250 PCE (Castro *et al*., 2009).

Results and Discussion

Citotoxicity assays

By staining of trypan blue exclusion, could be demonstrated that the toxicity exerted by CAE on lymphocytes was dose-dependent manner **(Figure 1).** By MTT assay could not quantify the cytotoxic effect of CAE, since it was able to reduce tetrazolium salt in the absence of living cells.

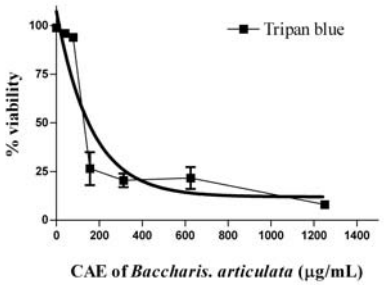


Figure 1: Percentage of viability of lymphocytes from healthy humans in terms of *in vitro* treatment with different concentrations of cold aqueous extract (CAE) of *Baccharis articulata*

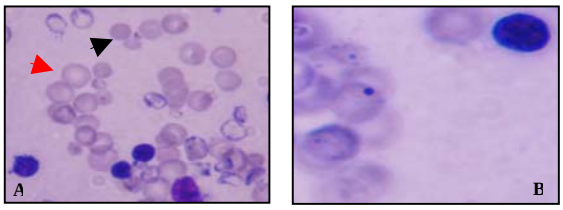
Shoemaker *et al.,* 2004 showed that different extracts can reduce MTT in the absence of cells, being free thiol groups (SH) or other antioxidants responsible for this effect. Based on the results obtained with CAE of *B. articulata* might think that such molecules would be present and would be responsible for the reduction of salt without the involvement of mitochondrial enzymes of the cell.

These findings suggest that to determine the cytotoxicity of various extracts of *B. articulata* be included controls without cells to observe any possible direct reaction between these vegetal fractions with MTT. Furthermore, it should be used alongside another test to quantify cell viability. In our case, the test of viability by exclusion of trypan blue was chosen and was able to calculate the cytotoxic concentration 50% (CC_{50}) of CAE in 150 µg/mL. Several authors have performed antitumor studies with vegetal derivatives employees at these concentrations (Uddin *et al.,* 2009, Valko *et al.,* 2006).

However, the National Cancer Institute indicates that substances with CC_{50} <20 µg/mL should be considered cytotoxic against cells treated and should only be studied as potential antitumor (Geran *et al.,* 1972). So, Banskota *et al.,* 1998 showed that pure compounds isolated from different species of the genus *Baccharis* exerted strong cytotoxic activity on HT-1080 cells of human fibrosarcoma and 26-L5 cells of colon carcinoma of mice, with values of CC_{50} less or equal to 10 µg/mL. The value of the CC_{50} obtained in our investigation indicates that CAE of *B. articulata* not be toxic to human lymphocytes.

Genotoxicity assay

The count of normochromatic erythrocyte (NCE)/250 PCE showed a TI of 1.38 (± 0.35) for the negative control and of 3.1 (± 1.06) for the positive control. The TI for CAE, in three doses (1800, 900 and 450 mg/kg) was 0.78 (± 0.05), 0.82 (± 0.08) and 1.21 (± 0.2), respectively **(Table 1).** These results suggest that CAE would have no cytotoxic effects on the erythrocytes of mouse bone marrow at 6 h post-injection**.** The number of micronuclei in PCE (MNPCE) was 5 (±1) for the negative control, 372 (±23) for the positive control and 26 (±8), 16 (±5) and 8 (±3) for CAE in three doses (1800, 900 and 450 mg/kg) respectively **(Table 1, Photograph 1).**



Photograph 1: Erythrocytes from mouse bone marrow dyed with May Grünwald and Giemsa (100X). **A)** Normal erythrocytes (black arrow indicates PCE and red arrow NCE). **B)** Micronuclei PCE.

In those mice treated with CAE, was observed a dose-dependent increase in the frequency of MNPCE. Concentrations of 1800 and 900 mg/kg showed statistically significant difference with the negative control ($p<0.02$ and $p<0.05$, respectively). In contrast, the CAE at concentration of 450 mg/kg showed no difference with the negative control. However, treatment with CAE in three concentrations showed statistical difference with respect to the positive control ($p<0.0001$), indicating that the extract does not possess mutagenic action. Our results agree with those found by Andrade *et al.,* 2008 who evaluated the genotoxic effects of ethanol extract of *B. dracunculifolia* (1000, 1500 and 2000 mg/kg) through the micronucleus test in mouse bone marrow. The authors found that this extract showed no genotoxic effects at 48 or 72 h post-injection

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vol 21 January-April 2010, 4-7 http://www.idecefyn.com.ar ISSN 1666-888X

Table 1: Results of Micronucleus Test in mouse bone marrow

Treatment	Animals	TI (± SD)	Analyzed PCE	MNPCE
Negative control (saline solution)	6	1.38 ±0.35	2000	5(±1)
CAE *B. articulata* (mg/kg)				
1800	6	0.78 ± 0.05	2000	26(±8)
900	6	0.82 ± 0.08	2000	16(±5)
450	6	1.21 ± 0.2	2000	8(±3)
Positive control (cyclo-phosphamide)	6	3.1 ±1.06	2000	372(±23)

TI: Toxicity Index, **PCE**: polychromatic erythrocytes, **MNPCE**: micronucleus in polychromatic erythrocytes

Conclusions

The CAE did not exert cytotoxic activity on human lymphocytes, nor mutagenic in erythrocytes of mouse bone marrow at 6 h post-injection, which encourages the continuation of this study at 24 and 48 h post-injection. The results obtained with this plant fraction are promising and suggest their research in other fields.

Acknowledgements

This work was supported by grants from CONICET, PICTOR 8/20325 BID 1728/OC-AR, Agencia Córdoba Ciencia-Nación, Resolution #222, years 2007/09, and SeCyT, Resolution #425, years 2007/09, from Universidad Nacional de Río Cuarto

Note: Part of this study was presented at the 'II Reunión de Biotecnología aplicada a plantas medicinales y aromáticas' (Second Biotechnology Meeting on Medicinal and Aromatic Plants), Córdoba, Argentina, 2009.

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