Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **29** (7): 1088-92 (2010)

Original Article
Received: November 16, 2009
Revised version: February 8, 2010
Accepted: February 9, 2010

Genotoxic Effect of Aqueous Extracts from South American *Achyrocline* and *Gnaphalium* Species (Asteraceae: Gnaphalieae) on Human Lymphocytes

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SUMMARY. Crude drug and different extracts of *Achyrocline satureioides* (Lam.) DC., A. tomentosa Rusby, *Gnaphalium cheiranthifolium* Lam. and *G. gaudichaudianum* DC. (Gnaphalieae: Asteraceae) are widely used in South America mainly as digestives and hepatics. These are raw material for phytotherapics preparations and the manufacture of traditional bitter drinks. In order to establish some aspects on their safety, we have evaluated four different concentrations (1, 10, 100, and 1000 μL.mL^{-1}) of the aqueous extracts of these plants against Single Cell Gel Electrophoresis Assay (SCGEA) in human peripheral blood lymphocytes. Results show a significant increase in damage index ($p < 0.001$) for all aqueous extracts concentrations assayed of the four plant species, in relation to negative control values. This is a contribution to the development of screenings related to the potential health risk associated with the consumption of South American medicinal plants, especially taking in mind that these plants are widely used as over-the-counter herbs.

INTRODUCTION

The use of some medicinal plants has become more popular worldwide, despite its few regulations [1,2]. In addition, some plants are important for the pharmaceutical and even for food industries as raw material. Consumers self-medicate with herbs for preventive and/or therapeutic purposes assuming that these products are safe because they are "natural". Nevertheless some of them can cause adverse effects or interact strongly with other medications [3,4].

Much attention of preventive medicine research is focused in the isolation and identification of new biologically active molecules by the pharmaceutical industry, but also because of the emergent public interest in using plant crude extracts. Thus, the use of what we know as traditional medicine has generated a demand for therapeutic alternatives in Latin American, Caribbean and industrialized countries [5]. Within this context, considerable interest has arisen in the possibility that several medicinal plants extracts can develop a negative impact by indiscriminate consumption.

Four medicinal plants native from South America, *Achyrocline satureioides* (Lam.) DC., *Achyrocline tomentosa* Rusby, *Gnaphalium cheiranthifolium* Lam. and *Gnaphalium gaudichaudianum* DC. (Asteraceae, Tribe Gnaphalieae) are known under their vernacular names "marcelas" (in Spanish) or "macelas" (in Portuguese). These plants are widely used in folk medicine especially for their digestives, carminatives, and antiespasmodics properties, as well as choleretics and for liver protection, and to obtain herbal remedies, phytotherapy products and cosmetic preparations (infusions, decoctions, tinctures and glycolic extracts) [6-8]. From the point of view of their nourishing value, some of these especies (*A. satureioides* and *G. gaudichaudianum)* are widely used for the preparation of bitter traditional beverages, called "amargos" (in Spanish) [7].

It has been suggested that *A. satureioides,*

KEY WORDS: Achyrocline, Comet assay, DNA damage, Genotoxicity, Gnaphalium.

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ISSN 0326-2383

which is the most widely spread species, could be used for primary health care because their therapeutic properties [9]. It is an official drug in Brazil [10], and was included among the herbs approved for human use in Argentina, both as herbal drug [11] and for bitter drinks [12]. On the other hand, it has been included in non-prescription herbal list in Uruguay [13]. Moreover, must be taken in account that *A. satureioides* is frequently misidentified with the other above mentioned species, that belongs to the same Tribe Gnaphalieae. These species show similar morphological characters [14] and share a lot of secondary metabolites. The phytochemical studies on *A. satureioides* has been proven that it is rich in polyphenols and flavonoids [15], lactones and polysaccharides [16], coumarine [17], dibenzofurans [18], and essential oils [19]; on the other hand, it has been studied the mineral composition of *A. satureioides* and *A. tomentosa* [20]. Some of its popular uses have been validated by *in vivo* and *in vitro* pharmacological studies [21]: cytoprotection against the oxidative stress [22-24]; hepatoprotection [25] increasing the bile flow; anti-inflammatory and analgesic activities [26]; molluscicide [27], antimicrobial [28], antitumor [29] and antiviral actions [30], including HIV-1 virus [31], and also a strong immunostimulant activity [32,33]. But some extracts of this species have shown both cytotoxicity, mutagenicity [29,34-36], and other forms of toxicity, for instance genotoxic effects on prokaryotic cells [36]. However, has not been demonstrated genotoxicity on eukaryotic cells by extracts of any of the species involved in this study, until today.

A wide range of methods are used for detection of early biological effects induced by DNA-damaging agents in environmental and occupational settings. Besides traditional cytogenetic methods, the single cell gel electrophoresis assay (SCGEA), or comet assay, allows evaluation of DNA fragmentation resulting from a variety of DNA damages (single and double strand breaks and alkali labile sites, including abasic sites). The comet assay has become increasingly popular in the last 10 years because it is fast, inexpensive, and requires little biological material. It has been developed in an empirical way, with two basically different protocols [37,38]. As the comet assay has gained in popularity as a standard laboratory technique for evaluating DNA damage and/or repair, the question of how it can be applied within the current regulatory strategy of genotoxicity testing has become a matter of debate [39]. This is especially important now that acceptance of the *in vivo* Comet assay by regulatory agencies in a number of countries is increasing, with some already citing it as an acceptable second test [40,41].

The aim of this study was to determine the genotoxic effects of the four mentioned herb decoctions on isolated human cells using the alkaline version of the Comet assay to investigate their potential to induce *in vitro* genetic damage in human peripheral blood samples.

MATERIALS AND METHODS

Plant material

Samples of plant species were collected in the mountains near San Luis city, Argentina, at 850-1,200 m above sea level, in late summer, 2007. The species were authenticated by means of herbarium samples, preserved at UNSL Herbarium, and identified as follows: *Achyrocline satureioides,* L.A. Del Vitto # 8603; *A. tomentosa,* L.A. Del Vitto et al. # 6765; *Gnaphalium cheiranthifolium,* L.A. Del Vitto *& al.* # 9195; *G. gaudichaudianum,* L.A. Del Vitto & al. # 8604 (UNSL).

Plant decoction preparations

Aerial parts of authenticated samples of the four studied species were dried in forced air heater at 40 °C to hygroscopic moisture, and then milled. Decoctions were prepared by adding 100 mL of water on 5 g of plant material and boiling during 10 min [42]; then were left at rest for 15 min, sterilized through a 0.22 ?m filter, and stored at -20 °C.

Blood sampling and treatment

Peripheral blood samples obtained from 3 healthy volunteers with no history of exposure to any potential genotoxic agent were heparinized. Cell suspension containing 50 ?L of blood in 950 µL of RPMI 1640 medium was dispensed into Eppendorf tubes. The decoctions were added in four different concentrations (1, 10, 100 and 1,000 µL/mL^{-1}). Negative control was developed by adding distilled water (100 µL), while positive control was prepared by adding H_2O_2 50 µM (50 µL) to each donor blood sample. Viability of the cell suspension was evaluated by mean of fluorescent DNA-binding dyes. Recovery of cells was also measured. The remaining cell suspension was used for the preparation of slides.

Cell Viability using Fluorescent Dyes

A cell suspension was mixed with fluores-

cent DNA-binding dyes and examined by fluorescent microscopy to visualize and count cells with aberrant chromatin organization. A dye-mix working solution of 100 µg/mL acridine orange (Sigma) and 100 µg/mL ethidium bromide (Sigma) was prepared in Ca^{2+} and Mg^{2+} free PBS. A volume of 4 µL of this mixture was added to 100 µL of cell suspension. This mixture was examined with a 40x objective using a fluorescent microscope. A minimum of 200 total cells was counted, recording the number of each of the following cellular states: viable cells (V) and nonviable cells (NV) [43]. The percentages of each of these cellular states in relation to the total cells were obtained.

Alkaline comet assay

The standard procedure originally described by Singh *et al.* [37] with modifications was used. Two slides were processed for each sample, including negative and positive controls. A freshly prepared suspension of 50 µL blood in 950 µL of RPMI 1640 at 37 °C was centrifuged at 1,000 g for 4 min. Cell pellets were mixed with 200 µL of a 0.1 % low-melting point agarose solution (Sigma) at 43 °C and were spread onto two frosted slides precoated with 0.1 % NMP agarose. To lyse cellular and nuclear membranes of the embedded cells and to allow for DNA unwinding in alkaline conditions, the key-coded slides were immersed in ice-cold, freshly prepared, pH 10 lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM trizma base, 1 % Triton X-100 and 10 % DMSO) and left at 4 °C overnight. The slides were then placed in an electrophoresis alkaline buffer (300 mM NaOH and 1 mM Na_2EDTA, pH > 13) and the embedded cells were exposed to this alkaline solution for 20 min to allow DNA unwinding. Electrophoresis was performed in the same alkaline buffer at 0.75 V/cm (25 V, 300 mA) for 20 min at 4 °C (the temperature of running buffer did not exceed 12 °C). After electrophoresis, the slides were neutralized with 0.4 M Tris, pH 7.5 and the DNA was stained with 50 µL of ethidium bromide (2 µg/mL) and covered with cover slips. All steps were conducted in darkness to prevent additional DNA damage.

DNA strand breaks were measured with the Comet assay. One hundred randomly selected Comet assays from each of two duplicate gels were analyzed visually on a scale of 0–4 (categories depending on DNA damage level). The four categories were established according to the total comet length: I (<20 µm); II (20-40 µm), III (40-80 µm) y IV (>80 ?m). Damage Index (DI) was an established relation between damage categories and was calculated by means of the following formula [44]: *DI: Cel nº I + 2 x Cel nº II + 3 x Cel nº III + 4 x Cel nº IV*. The overall score, between 100 and 400 arbitrary units, was related to the DNA break frequency and a comet-like image indicated the presence of DNA breaks [45].

Statistical analysis

Data from experiments were presented as media ± standard deviation (SD). Media values were compared by ANOVA test. Due to the fact that ANOVA test showed significant differences the Holm-Sidak test for multiple comparisons was applied to know which media are statistically different between each (Sigma Stat 9).

RESULTS AND DISCUSSION

The determination of cell viability in the donor blood samples was always above 85 %. Results of the comet induction analysis in human peripheral blood lymphocytes are shown in Table 1.

	Average DI ± SD
Negative Control	108,66 ± 7,57
Positive Control	182 ± 31,04
Gnaphalium gaudichaudianum	163,10* ± 17,96
Gnaphalium cheiranthifolium	131,89* ± 8,94
Achyrocline satureioides	140,77* ± 16,75
Achyrocline tomentosa	153,44* ± 11,61

Table 1. DNA Damage Index (DI) induced by *Gnaphalium* and *Achycroline* species at 100 µL.mL^{-1} of plants extracts. * p<0,001.

Negative controls exhibited a high proportion of types I and II of comets and more than 80 % of the nuclei images presented no detectable damage, and 20% of them showed a low level of DNA damage. As expected, cells treated with a positive control (H_2O_2) showed a high percentage of type III comets.

To the various concentrations studied for the four entities the lowest concentration that showed significant differences was 100 µL.mL^{-1} compared with the negative control.

It is important to note the individual susceptibility which can be evidenced in Figure 1, where individual 1 shows a particular response in relation with the exposure to the decoctions, since in the case of *Gnaphalium gaudichadia-*

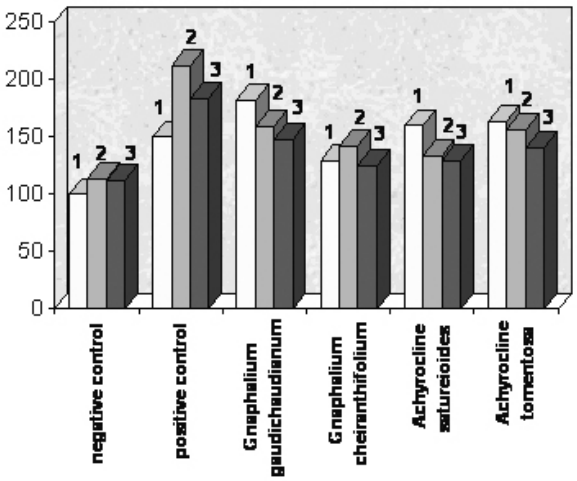

Figure 1. Individual susceptibility in DNA damage induced by the studied species of *Gnaphalium* and *Achyrocline*.

num its behavior is similar to positive control, while another susceptibility example is individual 3 who exhibits the slightest response against exposure to the decoctions.

The genotoxic action shown by the species of *Achyrocline* and *Gnaphalium* investigated in this case, could be due to bioactive compounds that have been found in some of these plants (especially *A. satureioides),* as is the case of some sesquiterpene lactones and flavonoids [15,16], the same compounds that have been identified as a cause of toxicity in related Asteraceae. On the other hand, although a study have demonstrated that the aqueous extracts of *A. satureioides* were devoid of acute toxicity in rat and mice at least in the case of 2 % infusions [35], has already been shown that these extracts are able to induce genotoxicity and/or mutagenicity in certain prokaryotes and *Trypanosoma* [34,36,46,47]. These results extend the knowledge of the genotoxic action of extracts of these plants to eukaryote cells of human tissues.

It should be noted that genotoxic substances leading to genetic damage in important regions of the DNA molecule, and these damages can affect the cell cycle control and apoptosis, giving rise to a neoplastic process [48-50]. This demonstrates once again that among the studies of toxicity, genotoxicity tests must be included because it is a chronic condition with lethal effects, which can impact on much of the world's population, especially those from low-income, that requires information and knowledge about the potential risk of consumption of these over-the-counter herbs.

Acknowledgements. This work was supported by Marta A. Carballo grants UBACyT B040 and PICT 38238, and PROIPRO 2-0207 SECyT-UNSL.

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