

In vivo and *in vitro* genotoxicity studies of aqueous extract of *Xanthium spinosum*

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The use of plants as a source of palliative or cure for pathological conditions is quite common worldwide. *Xanthium spinosum* (Asteraceae), popularly known in Brazil as ‘espinho de carneiro’, is an annual weed from South America, which has been used by empiric medicine to treat neoplasias. Owing to the extensive use of the above-mentioned plant and to the lack of reports about the real effects of its infusion, current study evaluated the genotoxic potential of its aqueous extract at concentrations 0.02 g L⁻¹, 0.1 g L⁻¹ and 0.2 g L⁻¹ by fish micronucleus test and by comet human leukocytes assay. The micronucleus test featured at least 50 cells with micronuclei to every 2,000 cells scored, as a mutagenic parameter. The comet assay was used as a parameter for assessing the level of cell damage and the damage index. Since no significant changes in strain cells exposed to the aqueous extract in the comet and micronucleus assays were reported, it seems that no genotoxicity evidence is extant at the concentrations and in the assays performed.

Uniterms: *Xanthium spinosum*/pharmacognosy. *Xanthium spinosum*/aqueous extract/genotoxicity. Fish micronucleus assay. Comet assay.

Em diversos lugares do mundo a utilização de plantas como fonte paliativa ou de cura para determinadas condições patológicas é bastante comum. No Brasil, essa prática não se torna diferente devido à ampla biodiversidade da fauna e flora presentes no País. Nesse contexto, surge a *Xanthium spinosum* (Asteraceae), conhecida popularmente como “espinho-de-carneiro”, um arbusto anual introduzido na América do Sul, o qual tem sido utilizado empiricamente no tratamento de neoplasias. Sabendo do extensivo uso dessa planta em contrapartida com nenhum estudo reportando os reais efeitos de sua infusão, o objetivo do estudo foi avaliar a genotoxicidade do extrato aquoso nas concentrações de 0,02 g L⁻¹, 0,1 g L⁻¹ e 0,2 g L⁻¹, através do ensaio do micronúcleo písceo e do ensaio cometa em leucócitos de sangue humano. O ensaio do micronúcleo tem como parâmetro mutagênico a presença de no mínimo 50 células com micronúcleo em uma contagem de 2.000 células por amostra, enquanto o ensaio cometa utiliza como parâmetro o nível de dano e o índice de dano. Os resultados mostram que não foi possível observar mudanças significativas nas células expostas ao extrato aquoso, em ambos os testes, o que sugere não existir evidência de genotoxicidade nas concentrações utilizadas no ensaio.

Unitermos: *Xanthium spinosum*/farmacognosia. *Xanthium spinosum*/extrato aquoso/genotoxicidade. Ensaio do micronúcleo písceo. Ensaio cometa.

INTRODUCTION

The empirical knowledge on plants is a reflection of popular wisdom, with its ethnic references, throughout the history of civilization. In Brazil, up to the first half of

the 20th century, medicinal flora has been used on different occasions to treat several diseases (Lorenzi, Matos, 2002). This practice is foregrounded on the expensive prices of several medicines, which make them practically unavailable for the people (Simões *et al.*, 2010).

Although the use of infusions is a common practice to treat or cure diseases, it is important to note that medicines manufactured from most plants in self-medication or through prescriptions fail to exhibit information on

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the toxicological profile or their safety in use (Bresolin, Vargas, 1993; Fernandes, Vargas, 2003, Capasso, 2000, Veiga Júnior, 2008). This is a fact of great concern since several studies show mutagenic (Cardoso *et al.*, 2006; Déciga-Campos *et al.*, 2007; Mohd-Fuat *et al.*, 2007) and carcinogenic properties proper to some plants employed in empiric medicine (Sá Ferreira, Vargas, 1999).

On the other hand, according to the National Institute of Cancer (INCA), the state of Rio Grande do Sul has the highest incidence levels of cancer for 2010-2011. Skin cancer is top-ranked, followed by lung and prostate cancer for men, and breast and cervical cancer for women. Since a similar profile is observed for all Latin America (Ministério da Saúde, 2009), such data express clearly a public health problem.

Xanthium spinosum (Asteraceae), popularly known as 'espinho de carneiro', is an annual weed from South America, with an erect stalk, one meter high, densely branched, and featuring a branched taproot. The leaves' upper surface is shiny dark green, while the underside of the leaves is pale green and downy (Loretti, 1999; Miguel, 2004). This weed has been used by popular medicine in the form of infusions to treat several neoplasias in the region of the Brazilian pampas. However, as far as it is known, no document exists which indicate the plant's pharmacological or toxicological effects, although some reports on the genus *Xanthium* are extant which describe its pharmacological and toxicological properties, namely, cholinergic effects, kidney failure and acute hepatic necrosis (Matos *et al.*, 2011).

Since it is widely used in the Brazilian pampas, current analysis tests the safety of *Xanthium spinosum* infusion by *in vivo* and *in vitro* genotoxicity studies.

MATERIAL AND METHODS

Plant

Samples of *Xanthium spinosum* were collected in April in the rural area of the Federal University of Pampa, Uruguaiana (RS), 29° 45' 23" S; 57° 05' 37" W., and identified by Dr. Angelo Schneider, from Department of Botany, Federal University of Rio Grande do Sul, Porto Alegre RS Brazil, registered ICN 169161.

Extract preparation

The plant was initially dried in an incubator (Biopar S20D model) for a few days until the root was completely dry, at a controlled temperature of 35 °C, and kept in a desiccator (Brasil, Ministério da Saúde, 2010).

The infusions were performed in three different concentrations (0.02 g L⁻¹, 0.1 g L⁻¹, and 0.2 g L⁻¹) using hot water (70 °C) that was poured into a flask containing root samples previously triturated and weighted and then kept closed for 15 minutes. The determination of the final concentration was carried out by drying 1mL of the infusion and left to dry in a controlled temperature of 30 °C until constant weight. The final concentration was then measured.

Comet assay samples

Protocol 0089.0.243.000-07 was approved by the committee of UFSM for the undertaking of current assay.

Samples of venous blood were obtained from male adult volunteers (18-30 years old) attended to in the hospital of the Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil. Two milliliters of dextran 5% were used to separate leukocytes of the heparinized blood sample (8mL). The samples were then mixed and kept at room temperature for 45 minutes. The supernatant was centrifuged at 3,500 rpm for 10 min and the bottom layer formed was washed with a lysis solution when the layer began to appear yellow. The bottom layer was suspended in 2 mL Hank's buffer solution saline and adjusted to 2×10^3 leukocytes/mL with HBSS/heparin.

The blood samples were assayed by the comet assay under alkaline conditions, following procedures by Santos *et al.* (2009). Peripheral leukocytes were incubated during 3 hours with the extract at three different concentrations, 0.02 g L⁻¹, 0.1 g L⁻¹ and 0.2 g L⁻¹, the latter two being five and ten times more concentrated than normal when the plant is used. Whereas distilled H₂O was used as negative control, H₂O₂ 4 mM was the positive control. After incubation, leukocytes were mixed with low-melting point agarose, of which 90µL were added to 15 µL of the leukocyte solution and placed on a microscope slide pre-coated with normal melting point agarose 1.0%. A coverslip was added and the slides were placed on ice during 5 minutes. After solidification, the coverslips were removed and the slides were immersed in a lysis solution. The slides were kept frozen in the lysis solution (4 °C) and protected from light for approximately 14 hours. They were subsequently incubated in freshly prepared alkaline buffer for 20 minutes for DNA unwinding. Electrophoresis (20 min at 300 mA and 25 V) was performed in the same buffer. Each stage was performed under indirect yellow light. After electrophoresis, the slides were neutralized in Tris 400 mM (pH 7.5), rinsed three times in distilled water, and left to dry overnight at room temperature. The dry slides were re-hydrated for 3 min in distilled water and then fixed

for 10 min, rinsed three times in distilled water, and dried for at least 5 h. The dry slides were re-hydrated for 3 min in distilled water, stained, and constantly shaken for 25 min. The slides submerged in the stop solution were rinsed again and immediately tagged for analysis. The slides were analyzed under blind conditions by at least two individuals. DNA damage is given as DNA damage index (DI). The DNA damage was calculated from cells in different damage classes (completely undamaged: $100 \text{ cells} \times 0$ to maximum damaged – $100 \text{ cells} \times 4$).

Cell viability

Cell viability assay was carried out by Mischell and Siingi method (1980), with slight modifications. Cells were exposed to the same concentration of both compounds for 3 hours, as in the comet test. After the incubation, 40 mL of a cell suspension were mixed with 30 mL of buffer HBBS and 50 mL of Trypan Blue solution 0.4% for 8 min. Cell viability was determined microscopically ($400 \times$ magnification) and two categories of cells were scored: (1) live cells, which appeared uncolored or light blue, (2) dead cells, with a blue color.

Micronuclei assay in fish erythrocytes

To evaluate genotoxicity using the micronucleus assay, specimens of *Astyanax bimaculatus*, popularly known as ‘lambari’, were collected from the breeding tanks of the Núcleo de Pesquisa Ictiológicas, Limnológicas e Aquicultura da Bacia do Rio Uruguai (NUPILABRU), in Uruguaiana, RS, Brazil. The fish ($n=32$) were randomly distributed in four different 50 L aquariums. Groups ($n=8$ for each) were divided into control group and test groups (EC1, EC2, and EC3) and treated with three different concentrations of an aqueous extract (0.02 g L^{-1} to EC1; 0.1 g L^{-1} to EC2; 0.2 g L^{-1} to EC3). The animals were kept in contact with the extract during 72 hours. Temperature, pH, resistivity, conductivity, salinity and oxygen levels were controlled throughout the experiment (Delincé, 1992).

After treatment, blood samples were collected from all fish and an extension in glass slide was made for each sample, according to technique by Heddle (1973).

After drying, the slide was fixed in ethanol PA (MERCK®) for 30 minutes. Slides were stained by Panótico Rápido® (Labor Clean) and then analyzed under optical microscopy (Olympus) in immersion lens. Further, 2000 cells were counted so that the slide would identify the micronucleus (Campana *et al.*, 1999; Grisolia, 2002). All particles clearly separated from the

principal core, not exceeding 1/3 of their real size, with visible borders, same color and refringent from core, were considered micronuclei. So that a substance or chemical may be mutagenic, it must cause the appearance of at least 50 cells with micronuclei to every 2000 cells scored (Carrasco *et al.*, 1990).

Statistical analysis

Data from damage levels were analyzed by Two-way ANOVA and index damage by One-way ANOVA; post-hoc were Bonferroni and Newman-Keuls Multiple Comparison Test, respectively ($p < 0.001$ was considered significantly different). Data from micronucleus assay were analyzed by One-way ANOVA; post-hoc Newman-Keuls Multiple Comparison Test ($p < 0.001$ was considered significantly different). All analyses were performed by BioEstat 5.0, at http://www.firstload.com/search.php?q=download_bioestat_5_0.

RESULTS AND DISCUSSION

Figure 1 shows the percentage of viable cells for both negative control group and for the three group tests (100%), each with different extract concentrations. This is not applicable to the positive control (85%) which, as

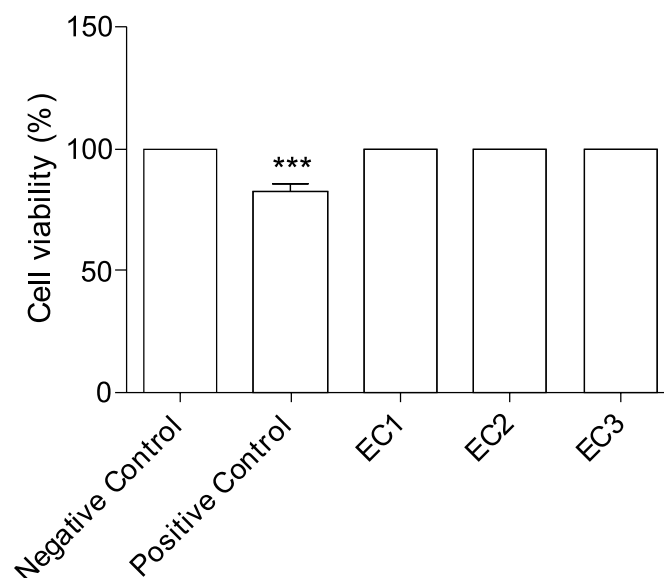


FIGURE 1 - Comet cell viability from the aqueous extract of *Xanthium spinosum* in three different concentrations in which positive control was different from the others, negative control and extract concentrations (***) ($p < 0.001$). One-way ANOVA and post-hoc Newman-Keuls Multiple Comparison Test). Data are expressed as percentage; $n = 4$. EC = extract concentration, EC1 = 0.02 g L^{-1} ; EC2 = 0.1 g L^{-1} ; EC3 = 0.2 g L^{-1} .

a whole, demonstrated the right conditions to perform the comet assay. According to Collins *et al.* (2008), high viability is required as a previous condition for the performance of the comet assay. It is highly relevant to note that the lowest concentration used (EC1) denotes the amount for empiric use, whereas the other extract concentrations, EC2 and EC3 were respectively five and ten times higher than the lowest concentration.

Table I and Figure 2 show low-level damage by the plant extract in the three different concentrations used. As may be observed, the lowest concentration (0.02 g L⁻¹) was similar to that found in negative control (Table 1), whereas the other concentrations (0.1 g L⁻¹ and 0.2 g L⁻¹) were close to the above. Similarly, damage indexes showed low levels at the concentrations tested. Indeed, there was no significant statistical difference at concentration 0.02 g L⁻¹ when compared to that in negative control. Although the damage indexes presented at concentrations 0.1 g L⁻¹ and 0.2 g L⁻¹ were higher than that of negative control and the 0.02 g L⁻¹ concentration, they were significant and statistically lower than positive control.

It is well documented in the literature that genetic mutations act by different steps on the carcinogenesis process and some tests, such as the comet assay, identify genotoxic agents and substances with potential risks to the human health. Genotoxic substances have in common chemical and physical properties that allow the interaction of nucleic acids. The most relevant consequence of these alterations in somatic cells may be the presence of benign

or malignant tumors (De Flora *et al.*, 1996; Andreassi *et al.*, 2000; Aruoma, 2003; Ross, Margolis, 2005).

These findings suggest that the plant extract used does not provide significant injury to genetic cellular apparatus, especially at concentration 0.02 g L⁻¹, since no DNA fragments form the 'comet'. The above indicates low genotoxicity for the different concentrations on human leukocyte DNA, at least in the experimental conditions tested. Moreover, considering that the 0.02 g L⁻¹ concentration corresponds to that used by non-traditional medicine and does not reflect bioavailability concentration on individuals after the intake of the infusion, its safety becomes even more significant. Furthermore, even the other concentrations tested seem to provide relative safety in use, even though they are five and ten times higher than the concentration used by non-traditional medicine.

Figure 3 shows the number of micronucleus found at the three concentrations of plant extract under analysis. The micronucleus assay did not reveal any statistically significant difference between the groups. Moreover, data clearly demonstrated that there was no mutagenic effect in the tested extract concentrations since all the results revealed less than 50 micronuclei per sample analyzed.

According to Hoofman and Raat (1982), the micronuclei assay in fish erythrocytes may be an alternative for chromosomal aberrations and mutagenic damages that may be evaluated by several methods. Among the cytogenetic methods, the micronuclei assay in erythrocytes is a simple, reliable and sensitive technique (Klingerman,

TABLE I - Comet Assay: Frequency of damage levels and index damage from the aqueous extract of *Xanthium spinosum*, in three different concentrations

| Treatment | Concentration | Levels of damage | | | | | DI |
|----------------------------------|-----------------------|--------------------------|------------------------|-------------------------|-------------------------|-------------------------|--------------------------|
| | | 0 | 1 | 2 | 3 | 4 | |
| Negative Control | 0 | 91.25±1.25 | 9.25±2.39 | 1.25±0.69 | 0±0.0 | 0±0.0 | 11.75±2.68 |
| Positive Control | 4mM | 11.5±4.11 ^a | 7.75±1.79 | 17±2.5 ^a | 26±2.27 ^a | 37.75±7.88 ^a | 282.8±17.37 ^a |
| <i>Xanthium spinosum</i> extract | 0.02g.L ⁻¹ | 92.75±1.49 ^b | 6±1.08 | 1±0.4 ^b | 0.25±0.25 ^b | 0±0.0 ^b | 8.750±2.13 ^b |
| | 0.1 g.L ⁻¹ | 84.5±2.06 ^{ab} | 9.5±1.55 ^b | 3.75±1.10 ^{ab} | 0.75±0.25 ^{ab} | 0±0.0 ^b | 19.25±2.68 ^{ab} |
| | 0.2 g.L ⁻¹ | 87.75±1.93 ^{ab} | 8.75±1.93 ^b | 2.75±0.47 ^{ab} | 0.5±0.28 ^{ab} | 0±0.0 ^b | 16.75±2.83 ^b |

Damage levels from aqueous extract of *Xanthium spinosum* (Two-way ANOVA; post-hoc Bonferroni); DI = Damage Index (One-way ANOVA; post hoc Newman-Keuls Multiple Comparison Test). Data are expressed as mean ± SEM, n = 4; ^aStatistically significant difference when compared to negative control; ^bStatistically significant difference when compared to positive control.

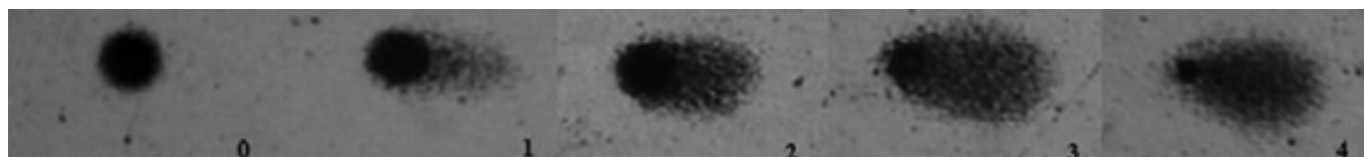


FIGURE 2 - Damage class levels of H₂O₂ from 0 to 4 in comet assay.

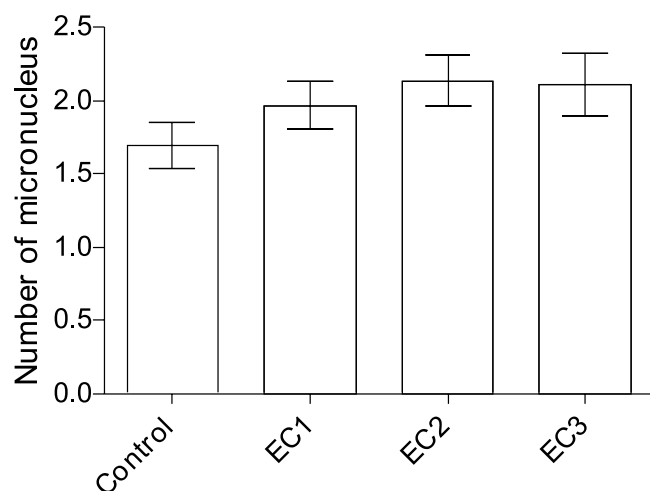


FIGURE 3 - Number of fish micronucleus after exposition to three different concentrations of the aqueous extract of *Xanthium spinosum* (One-way ANOVA; post-hoc Newman-Keuls Multiple Comparison Test, $p < 0.001$). Data are expressed as mean \pm SEM of 8 animals per group. EC = extract concentration, EC1 = 0.02 g L⁻¹; EC2 = 0.1 g L⁻¹; EC3 = 0.2 g L⁻¹.

1982), applied to any fish species regardless of karyotype characteristics (Hose *et al.*, 1987).

The genotoxic potential of several substances may be detected and quantified using fish as the experimental model, since these animals, similar to mammals, may activate the enzymatic system from cytochrome P450 (Goksoyr *et al.*, 1991; Andrade *et al.*, 2004). Furthermore, spontaneous formation of micronuclei is normally low and nearly uniform among species. Thus, the action of any genotoxic agent may give rise to an increase in micronucleus frequency (Siu *et al.*, 2004).

Another interesting point is that in the literature the toxicity attributed to some plants of the Asteraceae family, such as *Atractylis gummifera* L., *Xanthium cavanillesii* Schouw and *Xanthium strumarium* L., is connected to carboxyatractyloside found only in fruits and grains, and absent in other parts of the plant, such as roots and leaves (Obatomi, Bach, 1998; Driemeier *et al.*, 1999; Hamouda *et al.*, 2004; Daniele *et al.*, 2005; Turgut *et al.*, 2005). These data might be partially connected to the low toxicity shown by the extract concentration of *X. spinosum* when the comet and the micronucleus assays were employed since the root was the part of plant used.

CONCLUSIONS

Results by micronucleus assay suggest that the different concentrations of the aqueous extract of *X. spinosum* tested showed no mutagenic effect for fish erythrocytes. Furthermore, the comet assay in human

leukocyte cells showed that there was no DNA damage in the lowest concentration of the extract tested. However, low damage level was detected in the other two concentrations tested.

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