Advanced Studies in Biology, Vol. 7, 2015, no. 8, 351 – 364
HIKARI Ltd, www.m-hikari.com
http://dx.doi.org/10.12988/asb.2015.5420

Study of Acute Genotoxic Potential of an Aqueous Extract of *Schinus terebinthifolius* Raddi: an *in vivo* Micronucleus Assay

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Abstract

The *in vivo* Micronucleus Assay is a genotoxicity assessment system used for testing chemicals and herbal medicines that can induce chromosomal damage, acting as a biomarker. *Schinus terebinthifolius* Raddi (Anacardiaceae) is an evergreen tree that occurs on the Brazilian coast and several papers have shown the use of this plant in therapeutic approaches. The aim of this study is to analyze the genotoxic potential of *Schinus terebinthifolius* Raddi extract in Wistar rats bone marrow through the frequency of micronuclei in polychromatic erythrocytes. The results indicate that there was no genotoxic effects in all tested concentration, showing only an increase in the ratio of polychromatic and normochromatic erythrocytes.

Keywords: micronucleus assay, *Schinus terebinthifolius* Raddi, genotoxicity

Introduction

The *in vivo* Micronucleus Assay is used on rats (*Wistar*) to evaluate the genotoxicity that is used to identify alterations in the frequency of chromosomal damage in cells, through its exposure to certain agents that eventually might lead them [1, 2]. This test assesses the aneugenic and clastogenic abilities of these agents [3, 4].

The micronucleus is comprised of a small nuclear mass bounded by a membrane and separated from the main core. Micronuclei are developed during telophase of mitosis or meiosis, when the nuclear envelope is reconstituted to the contour of the chromosomes of the daughter cells. Derived from acentric chromosome fragments or whole chromosomes that are not incorporated in the main core, micronucleus represents the chromatin loss as a result of chromosomal damage in its structure (fragment) or damage to the mitotic apparatus [5–9].

Micronucleus are formed regardless of the type of injury during the cell cycle. Thus, the DNA damage caused, for example, by exposure to a genotoxic agent given, only manifest as a micronucleus after a cell division cycle, being dependent on the proportion of cells that are on division [7, 10, 11]. However, the micronucleus formation can occur spontaneously in the body. The frequency level of micronuclei and the area of the DNA removed in the main core are elements which can be significant in the development of a pathological process [3, 10, 12].

The *in vivo* Micronucleus Assay is indicated by the Organization for Economic Co-operation and Development (OECD) for risk evaluation to xenobiotics exposure [13]. Among the main advantages of the micronucleus assay for monitoring exposure to genotoxic substances and environmental contaminants, could be mentioned: its simplistic analysis, high sensitivity of detection and accuracy of chromosomal losses, non-disjunction events, besides its capacity to measure the length and the progression of nuclear division and its ability to detect events of repairing and excision [11].

Due to these characteristics the micronucleus assay in rodent bone marrow has long been used in the analysis of genotoxicity for many herbal medicines, especially those of pharmaceutical interest. Highlights are the analysis of Boriollo *et al* [14] with *Ziziphus joazeiro* Martius; Mahon *et al* [15] with *Dilodendron bipinnatum* Radlk; Oliveira *et al* [16] with *Calophyllum brasiliense*; Shin *et al* [17] with *Polygala tenuifolia* and Tolentino *et al* [18] with *Rubus niveus*.

Schinus terebinthifolius Raddi (Anacardiaceae) is an evergreen tree that occurs on the Brazilian coast, popularly known as "aroeira-vermelha" and "aroeira pimenteira", among other names [19]. The medicinal use of *Schinus terebinthifolius* Raddi is reported for years and is mentioned in Brazil since the first edition of the Brazilian Pharmacopoeia [20]. The main parts of the plant that are mentioned into articles are: peeling [21] bast [22] and leaves [23].

Several medicinal properties are described in the academic literature. In a study by Lucena and colleagues [22] the use of hydro-alcoholic extract of *Schinus terebinthifolius* Raddi showed a favorable effect in the healing cystotomies done in rats. Numerous other studies demonstrate the potential use of species as an antioxidant and for helping the healing of skin wounds, the healing of surgical wounds, treatment of cervicitis and genital discharge [24-27]. Matsuo *et al* [29] and Gautam *et al* [30] described antitumor properties to *Schinus terebinthifolius* Raddi, as well as other studies also attribute the antimicrobial effect to *Schinus terebinthifolius* Raddi [19, 31-33]. Phytochemical studies conducted in leaves of species of the genus *Schinus* reported the presence of tannins, saponins, flavonoids, triterpenes and steroids [19, 34-36].

The aim of this study was to analyze the acute genotoxic potential of the aqueous extract of *Schinus terebinthifolius* Raddi in rats bone marrow through the micronucleus frequencies in polychromatic erythrocytes and check potential bone marrow suppression by varying the ratio between polychromatic and normochromatic erythrocytes in Wistar rats bone marrow.

Materials and Methods

The fresh leaves of *Schinus terebinthifolius* Raddi were collected, cleaned, dried and stored in the freezer for 16 hours at -28° C. A voucher specimen was deposited in the National Museum of the Federal University of Rio de Janeiro (code: R 210.885).

The aqueous extract was processed by the infusion of fresh leaves (leaf 1g / 150 ml water) as recommended by the Brazilian Pharmacopoeia (1st edition) [20] with some modifications. The extract was stored in amber vials, labeled and kept in a freezer at -28º C until the lyophilization. Then the aqueous extract was lyophilized (Lyophilizer Liotop. Model: L202 - Liobras) and stored at -28° C in the freezer to be used on the experiment.

The *Schinus terebinthifolius* Raddi aqueous extract was resuspended from a vial containing 1000 mg lyophilized extract diluted in 10 ml of NaCl sterile solution at 0.9%. Five concentrations of the extract were administered: 25, 50, 100, 150 and 200 mg.kg (milligrams per kilo of the animal).

Thirty-five healthy adult male Wistar rats were used (7 to 12 weeks). Rats were divided into 7 groups of 5 animals, each group corresponds to treatments, negative (0.9% NaCl solution) and positive (Cyclophosphamide 50 mg.kg) controls.

This study was submitted to the Ethics Committee for the Care and Use of Experimental Animals (CEUA number of protocol: 046/2011).

The animals were randomly selected in a way that the average weight of the treatment groups were not statistically significant difference. At the end of this step, each animal has been identified according to the weight, receiving adjusted dosages given orally by tube (gavage). After administration of *Schinus terebinthifolius* Raddi aqueous extract, the animals resumed their initial conditions, where they remained for 24 hours.

The positive control for the micronucleus assay was used with the substance cyclophosphamide according to the 474 OECD protocol [13, 39–41]. Cyclophosphamide (Genuxal® - Baxter 1000 mg vial) used in this test was diluted in sterile 0.9% NaCl and administered at a concentration of 50 mg.kg and; as a negative control was used the 0.9% NaCl solution (Baxter®) because of its neutrality of genotoxicity and cytotoxicity.

After 24 hours, the animals were subjected to a CO_2 chamber. After their death, the femurs were removed, epiphysis cut off and the bone marrow withdrawal, washing the spinal canal with 0.9% NaCl sterile solution.

The bone marrow was homogenized in 2 ml of 0.9% NaCl solution and centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded and the materials resuspended in 0.5 ml of 0.9% NaCl solution to obtain a homogeneous suspension.

Four microscope slides were prepared from each animal by stretching technique. These slides were air dried and afterwards fixed in methanol PA (Vetec®) for 10 minutes and the cells were stained 24 hours after the fixation by Giemsa method at 5% for 5 minutes.

The criteria for micronucleus identification are its size, its shape and its color. In size, they must have 1/10 to 1/20 of the polychromatic erythrocytes size. The micronucleus should be round or oval, with smooth and defined contour and darkblue color, presenting, generally, less evidence of internal structure that the core of nucleated cells, but are similar in appearance of these nuclei (Figure 1) [37].

For the quantification of cells with micronucleus, 1000 polychromatic erythrocytes were analyzed in a systematic count into different fields of the blade, randomly selected. The polychromatic cells are well rounded, defined and presents polychromatophilia.

The technique for assessing the ratio of polychromatic and normochromatic erythrocytes is a systematic counting of these cells into different fields of the blade. These fields are chosen randomly without overlapping. The normochromatics cells are slightly smaller than the polychromatics, presenting pink color (acidophilus). Only intact cells are considered in the count. In each slide were analyzed simultaneously polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs). Based on a total cell count of 200, it was generated a ratio of polychromatic on all normochromatic.

Statistical Analysis

Data were analyzed for normality distribution by the Shapiro-Wilk test. After this analysis was carried out a comparison of groups by the nonparametric statistical model of Kruskal-Wallis [37]. The data are presented in the table with mean and standard deviation (Table 1).

Results

The frequency of Micronucleus in Polychromatic Erythrocytes (MNPCE) and the proportion of Polychromatic and Normochromatic Erythrocytes (PCE / NCE) after treatment at different doses of freeze-dried aqueous extract of *Schinus terebinthifolius* Raddi (STR) are shown in Table 1.

Treatment	Time (h)	Total MNPCE per 10.000 analyzed cells	MNPCE Mean ± SD	(PCE / NCE) Mean ± SD
Negative Control (NaCl 0,9%)	24	10	2.00 ± 1.73	2.79 ± 2.18
Positive Control (CPM50mg.kg)	24	20*	4.00 ± 2.12	0.95 ± 0.90
25 mg.kg STR	24	14	2.80 ± 1.92	4.74 ±2.65[b]
50 mg.kg STR	24	11	2.20 ± 1.30	6.76 ± 3.81[ab]
100 mg.kg STR	24	7	1.40 ± 0.54	5.29 ± 2.86[b]
150 mg.kg STR	24	9	1.80 ± 0.44	5.74 ± 2.25[ab]
200 mg.kg STR	24	14	2.80 ± 1.09	5.22 ± 2.15[b]

Table 1. Comparing to the positive control group ($p > 0.05$) *, tested doses (25, 50, 100, 150 e 200 mg.kg) indicated no statistically significant increase in the MNPCE frequency. 50 e 150 mg.kg doses showed an increase in the PCE/NCE ratio when compared to the negative control group ($p < 0.05$)[a]. Comparing the positive control group ($p < 0.05$)[b], all the doses showed an increase in the PCE/NCE ratio. **CPM**. Cyclophosphamide; **STR**. *Schinus terebinthifolius* Raddi.

Discussion

Table 1 shows that the negative control group had a total of 10 micronuclei, in the ratio of 1 for every 1,000 polychromatic erythrocytes analyzed the PCE / NCE intercourse was 2.79. This result reiterates the neutrality of 0.9% NaCl solution about its genotoxicity and cytotoxicity. The presence of micronuclei in the negative control group occurs spontaneously in the process of cell division and, what distinguishes a spontaneous process at random from the micronucleus formation and a cellular process that was influenced by a genotoxic substance is the total frequency of generated micronuclei [3, 38].

The data in table 1 reinforce the statements of Melo *et al* [39], Misik *et al* [40] and Singh *et al* [41] in relation to the cytotoxic and genotoxic potential of cyclophosphamide [42], an alkylating agent used for the treatment of neoplasias, which inhibits DNA replication and RNA transcription by crossing inter and intra chains [42]. There was an increase in the frequency of micronuclei in polychromatic erythrocytes caused by cyclophosphamide in the concentration of 50mg.kg, this effect is related to damage caused to the cell nucleus [43]. On the other hand, there was a decrease in PCE / NCE relation, indicating cytotoxic effect of cyclophosphamide. The frequency of micronuclei found approximates to that described by Melo *et al* [39].

The results obtained in the treatments with aqueous extract of *Schinus terebinthifolius* Raddi indicate that there was no genotoxic effects when compared

to the positive control ($p > 0.05$). Carvalho *et al* [44] evaluated the genotoxic effect of an aqueous extract of the fruit of *Schinus Terebinthifolius* Raddi against plasmid DNA, indicating that the extract has not been capable of inducing double strand breaks of plasmid DNA, confirming the results of the present study. These data also confirmed previous findings such as Lemos *et al* (2011) and Arnobio *et al* (2012), which the aqueous extract of *Schinus terebinthifolius* Raddi showed no cytotoxicity effects in *Escherichia coli* [45] , *Trypanosoma cruzi* and *Leishmania amazonensis* by minimum inhibitory concentration [46].

Treatments of 50 and 150 mg.kg of *Schinus Terebinthifolius* Raddi showed an increase in the ratio PCE / NCE when compared with the negative control group ($p<0.05$). The frequency of PCEs analyzed in bone marrow distension is used as an indicator of possible adverse effects of treatment on the function of the hematopoietic organ. Reducing the frequency reflects a decrease in the formation of new erythrocytes translating itself as a myelotoxic effect [41]. However, the increase in the ratio of PCEs may indicate a stimulating division and maturation of nucleated cells in erythropoiesis. This study can not relate the increase in this relation with a direct stimulus to erythropoiesis phenomenon, since the study was conducted in 24 hours – acute effect – and erythropoiesis phenomenon is related to a period of 48-72 hours [47]. There is speculation that this effect may be related to the presence of tannins in the *Schinus terebinthifolius* Raddi [48-49]. According to Olchowik and colleagues (2012) [50], the tannins are classified into two main groups, which structures are very different, although all of them have polyhydroxy phenols molecules or their derivatives. First group consists in hydrolysable tannins (such as tannic acid), commonly used for tanning leather and the second one contains other types of tannins, found in greater quantity and of greater importance in food, being called condensed tannins, which basic structure are related to the structure of catechin and 3', 4', 5, 7 - hydroxy-flavonoid [50].

Despite papers demonstrate that the tannins have antioxidant effects [51–54], other studies also show that the tannins hinder the absorption of iron, acting as chelating [50, 55–59]. Thus, the reduction of bioavailable iron will be translated into a low oxygen tension in the blood [18], and it is known that low oxygen tension signals to produce erythropoietin, stimulating erythropoiesis [60–61].

The comparison of the ratio of PCE / NCE between treatments at doses of 25, 50, 100, 150 and 200 mg.kg of Schinus terebinthifolius Raddi and the positive control (cyclophosphamide), indicate that there was not a cytotoxic effect on bone marrow [13]. These results are comparable to those reported by Silva *et al* [62], when evaluated the cytotoxic effects of the essential oil of leaves of *Schinus terebinthifolius* Raddi. Other studies reinforce this finding such as Carvalho *et al* [48] and Santana *et al* [63].

Conclusion

Based on these results it is possible to suggest that the lyophilized aqueous extract of *Schinus terebinthifolius* Raddi, showed no genotoxic effect through the micronucleus assay in Wistar rats bone marrow.

The extract used in the tested concentrations generated an increase of the ratio variation between polychromatic and normochromatic erythrocytes in Wistar rats bone marrow. However, more studies should be performed to better understand this phenomenon.

Conflict of Interests.
Authors have no conflict of interests.

Acknowledgements. The authors thank FAPERJ for research grants and for fellowships.

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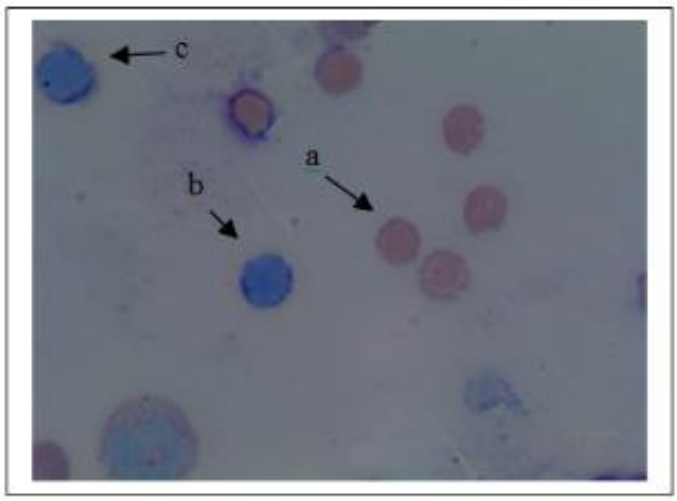

Figure 1. a) Normochromatic cell without micronucleus. b) Polychromatic cell without micronucleus. c) Polychromatic cell with micronucleus. Giemsa staining method, magnified 1000x (Optical Microscope Nikon E200).

Received: April 25, 2015; Published: June 25, 2015