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In vivo Clastogenicity Assessment of the *Austroplenckia populnea* (Celastraceae) Leaves Extract using Micronucleus and Chromosome Aberration Assay

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Summary The clastogenic effect of the *A. populnea* leaves extract was tested *in vivo* on bone marrow cells of Wistar rats by evaluating the induction of chromosome aberrations and micronuclei induction on polychromatic erythrocytes. The extract was administered by gavage at doses of 300, 600 and 900 mg/kg body weight. Experimental and control animals were submitted to euthanasia 24 h after the treatment. Under the conditions used, *A. populnea* leaves extract did not induce decrease in mitotic index and did not induce a statistically significant increase in the mean number of micronucleated polychromatic erythrocytes or chromosome aberrations in the bone marrow cells of Wistar rats.

Key words *Austroplenckia populnea*, Celastraceae, Micronucleus test, Chromosome aberrations.

Interest in the medicinal properties of natural products has increased due to their popular use in traditional medicine. *Austroplenckia populnea* (Reiss) Lundell is a Brazilian Cerrado plant from Celastraceae family. This botanical family includes several plant species that have been widely used in traditional medicine for their antiulcerogenic, analgesic, male antifertility, antiinflammatory and other activities (Corrêa 1985). *A. populnea* is commonly known as "marmelinho do campo, mangabeira-brava, mangabarana and vime" and it is a folk medicine used as an anti-dysenteric and anti-rheumatic (Corrêa 1985). Antiulcerogenic and analgesic effects of the leaves extracts were reported in mice (Seito *et al.* 2002) and Mazaro *et al.* (2000) related decrease in sperm number after treatment of rats with this extract.

Studies involving chemical aspects of the leaves preparations revealed the presence of friedelane and oleanane triterpenes and sesquiterpenes (Vieira-Filho *et al.* 2000, 2001), and Andrade (2005) reported quantitative differences between *A. populnea* bark wood and leaves crude extract. Zanoni *et al.* (2005) observed that high concentrations of *A. populnea* bark wood crude extract showed clastogenic effect in the bone marrow cells of *Rattus norvegicus*.

In view of the potential therapeutic use of the *A. populnea* extracts, to the observed mutagenic potential of the bark wood extract and of the absence of any data on its mutagenicity on the leaves extract, the objective of the study described in this paper was to investigate the clastogenic potential

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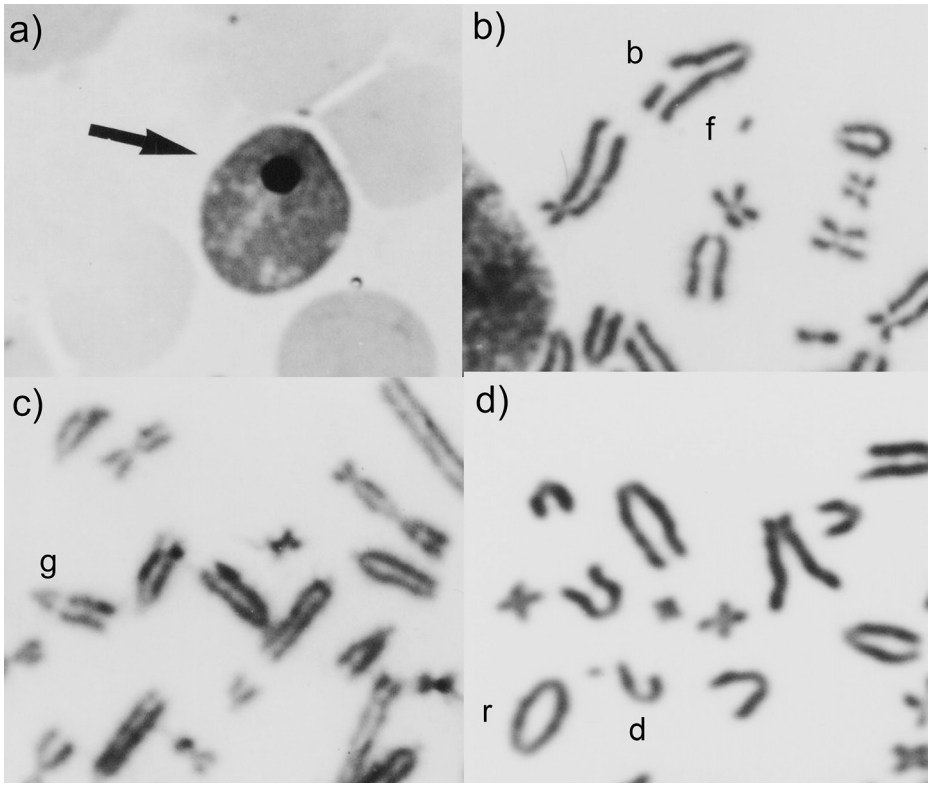

Fig. 1. Some typical chromosome aberrations on bone marrow cells of Wistar rats after Cyclophosphamide treatment. a) Micronucleus on polychromatic erythrocyte (arrow); b) b=chromatidic break, f=fragment; c) g=isochromatidic gap; d) d=deletion, r=ring.

Table 1. Mean of polychromatic erythrocytes with micronuclei (MNPCE) observed in bone marrow cells of male (M) Wistar rats treated with a Austroplenckia populnea leaves extract, and respective controls

Treatments	Dose mg/kg	Number of MNPCE per animal						MNPCE (Mean±SD)
		M_1	M_2	M_3	M_4	M_5	M_6	
Negative control (Water)	0	2	0	0	1	2	2	1.16±0.98
A. populnea extract	300	1	2	0	1	4	1	1.50±1.37
A. populnea extract	600	2	0	3	1	3	2	1.83±1.16
A. populnea extract	900	1	1	2	1	1	1	1.16±0.40
Positive control (Cyclophosphamide)	30	6	11	5	9	7	3	6.83*±2.85

Two thousand cells were analyzed per animal, for a total of 12000 cells per group. SD=standard deviation of the mean.
* Significantly different from negative control (p<0.001).

of the *A. populnea* leaves extract in terms of induction of micronuclei and chromosome aberratios in bone marrow cells of Wistar rats treated *in vivo*.

Materials and methods

Plant material

The plant material of *Austroplenckia populnea* was collected in the Cerrado at Botucatu, in the state of São Paulo, Brazil. A voucher specimen (n° 20415) has been identified and deposited in the herbarium, BOTU of Bioscience Institute of State University of São Paulo, IBB, UNESP. The hidro-alcoholic extract was obtained from leaves (5155.55 g) that were dried and pulverized yielding 859.2 g, and cold macerated with 96% ethanol for 48 h (3 times). The final filtrate was concentrated (rota-evaporator) yielding an hidro-alcoholic extract.

Animals and assay procedures

Experiments were carried out on six-week-old Wistar rats (*Rattus norvegicus*), weighing approximately 100 g. The animals were acquired from the Animal House of University of Alfenas (UNIFENAS), kept in polyethylene boxes ($n=6$), in climate-controlled environment (25 ± 4°C, 55 ± 5% humidity), light/dark control each 12 h (7 a.m. to 7 p.m.). Food and water were available *ad libitum*. Male rats were divided into experimental groups of 6 animals each. The *Austroplenckia populnea* leaves extract was administered at a single dose of 0.5 ml by gavage at concentrations of 300, 600 and 900 mg/kg body weight. The dose selected for these *in vivo* bone marrow tests were based on the acute toxicity studies in mice using *A. populnea* leaves extract (Seito *et al.* 2002). The negative control group received distilled water and the positive control group received 30 mg of cyclophosphamide/kg. Animals were injected intraperitoneally with 0.5 ml of 0.16% colchicine 90 min before euthanasia, which occurred 24 h after experimental treatment. Both femur bones were then excised and their bone marrow flushed into test tubes using a syringe. For the micronucleus (MN) assay the bone marrow cells were prepared as recommended by Schimid (1976). The slides were coded, fixed with methanol and stained by Giemsa solution. Two thousand polychromatic erythrocytes (PCE) from each animal were scored for MN presence (Fig. 1). Bone marrow preparations for the analysis of chromosome aberrations in metaphase cells were obtained by the technique of Ford and Hamerton (1956). One-hundred metaphases per animal (600 metaphases per group) were analyzed in order to determine the number of chromosomal aberrations in a blind test. The chromosomal aberrations analyzed were gaps, breaks, deletions, fragments, rings and dicentric chromosomes (Fig. 1). Gaps were not considered in statistical analysis. The mitotic index (MI), was obtained by counting the number of mitotic cells in 1000 cells analyzed per animal. The data obtained were submitted to the One-way analysis of variance test (ANOVA) and the Tukey-Kramer multiple comparison test using the GraphPad Instat® software (version 3.01). Results were considered statistically significant at $p<0.05$. These experimental protocol was approved by the Ethical Committee for Animal Research of the UNIFENAS, Alfenas, MG, Brazil.

Results

Tables 1 and 2 summarize the results of the analysis of micronucleus and chromosome aberrations respectively, in bone marrow cells of Wistar rats following treatment with different concentrations of the *A. populnea* leaves extract and controls.

Administration of *A. populnea* extract not induced a significant increase in the average number of micronucleated polychromatic erythrocytes (MNPCE) for all tested doses and as was expected, cyclophosphamide induced a clear increase in the MNPCE mean (Table 1).

Table 2 presents the results obtained in the *in vivo* test system using bone marrow cells from Wistar male rats treated by gavage with the different *A. populnea* leaves extract doses. The MI values obtained from the analysis of 1000 cells/animal for a sample of 30 animals (6/treatment) ranged from 1.68% to positive control to 3.51% to 600 mg/kg extract dose (means) and statistical analysis

Table 2. Mitotic Index (MI) and distribution of the different types of chromosomal aberrations (CA) observed in male (M) Wistar rat bone marrow cells treated with a *Austroplenckia populnea* leaves extract, and respective controls

Treatments	Animals	MI (%)	Chromosomal aberrations					
			Gaps		Breaks		OA	Total (CA) without gaps
			C	IC	C	IC		
Negative control	M_1	2.5	0	0	0	0	1 del	1
(Water)	M_2	2.5	0	0	0	0	0	0
	M_3	3.2	0	0	1	0	0	1
	M_4	3.1	0	0	0	1	0	1
	M_5	3.5	0	0	0	0	0	0
	M_6	2.9	0	0	0	0	0	0
	Mean±SD	2.95±0.39						0.50±0.54
A. populnea extract	M_1	2.4	1	0	1	0	0	1
(300 mg/kg)	M_2	3.4	0	0	0	0	0	0
	M_3	2.9	0	1	1	1	1 del	3
	M_4	3.0	0	0	1	0	0	1
	M_5	6.1	0	0	2	0	0	2
	M_6	3.1	0	0	2	0	0	2
	Mean±SD	3.48±1.32						1.50±1.04
A. populnea extract	M_1	2.0	0	0	0	0	0	0
(600 mg/kg)	M_2	3.4	0	1	1	1	0	2
	M_3	4.5	1	0	2	0	0	2
	M_4	4.2	0	0	0	0	0	0
	M_5	3.5	0	0	0	0	0	0
	M_6	3.5	0	0	0	0	0	0
	Mean±SD	3.51±0.86						0.66±1.03
A. populnea extract	M_1	2.7	0	0	1	1	0	2
(900 mg/kg)	M_2	1.6	0	1	1	0	0	1
	M_3	2.8	1	0	1	0	0	1
	M_4	3.2	0	0	0	0	0	0
	M_5	2.0	0	0	0	0	0	0
	M_6	2.3	0	0	0	0	0	0
	Mean±SD	2.43±0.58						0.66±0.81
Positive control	M_1	1.5	2	0	2	0	2 del	4
(Cyclophosphamide)	M_2	0.9	0	2	2	0	1 del	3
(30 mg/kg)	M_3	1.3	5	0	6	3	0	9
	M_4	1.7	1	2	3	2	2 del	7
	M_5	2.1	2	1	5	1	0	6
	M_6	2.6	0	0	1	1	1 del	3
	Mean±SD	1.68±0.60						5.30*±2.42

One hundred cells were analyzed per animal, for a total of 600 cells per treatment. C, Chromatid-type; IC, isochromatid-type; OA, other aberrations: del=deletion; SD=standard deviation of the mean. * Significantly different from negative control ($p<0.001$).

by the Tukey-Kramer test showed no significant differences ($p>0.05$) between the various treatments or between treatments and negative control group.

The analysis of chromosome aberrations obtained in 600 metaphases per treatment (100/cells/animal) in the different treatments showed that the most frequent types of aberrations were chromatid breaks, chromatid and isochromatid gaps, isochromatid breaks and deletions. The *A. populnea* extract dose of 300 mg/kg b.w. caused an increase in the mean number of chromosome aberrations than did the other 2 extract treatments and negative control group, but the difference was not statistically significant ($p>0.05$).

Discussion

Cytogenetic assays have been widely used in the genotoxicity assessment of test compounds under *in vitro* and *in vivo* conditions. Formation of micronuclei and chromosome aberrations are 2 important cytogenetic endpoints that are routinely used in genotoxicity evaluation (SanSebastian *et al.* 1990, Krishna *et al.* 1991, Chacon *et al.* 2002, Ferreira *et al.* 2003, Zanoni *et al.* 2005, among others). Micronuclei are thought to arise from both clastogenic (chromosome breakage) and aneugenic (chromosome lagging and effects on spindle) effects, while chromosome aberrations are thought to arise from chromosome breakage and exchange.

In the present study, a leaves extract from *A. populnea* was tested in the rat micronucleus and chromosome aberrations assay. After a single oral application, there was no evidence of an increase of micronucleated PCEs or chromosome aberrations in the bone marrow of Wistar rats when compared to controls, and the no observation of the decrease in the mitotic index of the bone marrow cells revealed no cytotoxic effects of the extract in the tested doses. These results are in disagreement with the findings of Zanoni *et al.* (2005) since the cytogenetic evaluation of the mutagenic potential of the *A. populnea* bark wood extract from Cerrado at Botucatu using *in vivo* micronucleus test in bone marrow cells of Wistar rats revealed a dose dependent clastogenic activity at 300, 600 and 900 mg/kg. The differences in the quantitative chemical composition between stalk and leaves extract obtained from *A. populnea* (bark wood extract is more rich in triterpenes than leaves extract, Andrade 2005) could to explain the differences in the clastogenic potential observed between these 2 extracts.

The hexanic extract obtained from the leaves of the *A. populnea* collected in Nova Lima, state of Minas Gerais, Brazil, caused decrease in cauda epididymis sperm number of Wistar rats; however, the hexanic extract obtained from the Cerrado at Botucatu, state of São Paulo, Brazil, did not produce this effect, with the same dose and periods of treatment (Mazaro *et al.* 2000). According to the authors, the different responses can be associated, at least in part, with soil and climatic differences between the regions of leaf collection. These parameters alter the chemical composition of the extract and, consequently, the biological activity.

The mutagenic potential of *A. populnea* leaves extract on bone marrow of Wistar rats was studied for the first time in the present work. The results indicated that this crude extract not presented clastonenic effect in bone marrow cells. Face the potential application of the *A. populnea* extracts how male contraceptive and how antiulcerogenic by humans, we intent to study others tree extract fractions obtained from crude bark wood extract from this plant to better to investigate the genotoxic risk of this matherial. Thus, is necessary be careful with the use of the different extracts from *A. populnea*.

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