Rev. Biol. Neotrop. 2(1): 49-54. 2005

Investigation of boldus (*Vernonia condesata* Baker, Asteraceae) genotoxicity in *Drosophila melanogaster*

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Abstract: The objective of the present study is to determine the potential genotoxicity of the aqueous extract of boldus (the form commonly used in folk medicine treatment of digestive problems and liver ailments) using the rapid somatic mutation and recombination test (SMART/eye) and the Ring-X-loss test in *Drosophila melanogaster*. The chromosome loss test indicated that *boldus* does not cause partial or total chromosome loss or nondisjunction in post-meiotic *D. melanogaster* cells. The effects detected by the SMART/eye show that boldus is potentially genotoxic, inducing mutations and/or somatic recombinations in *D. melanogaster.*

Key words: Genotoxicity, boldus, *Vernonia condensata*, medicinal plants, Ring-X-loss test, SMART/eye test.

Resumo: Este trabalho tem como objetivo determinar o potencial genotóxico de um extrato aquoso do boldo (forma comumente usada na medicina popular para tratar problemas digestivos e do fígado), através dos testes rápidos "SMART/olho" (Somatic Mutation And Recombination Test) e Ring-X-loss em *Drosophila melanogaster*. Quanto às perdas cromossômicas que podem ser detectadas pelo Ring-X-Loss, os resultados obtidos demonstraram que o boldo não provoca perdas cromossômicas parciais, totais ou não-disjunção. Quanto aos efeitos recombinogênicos detectados através do SMART/olho, os resultados foram, em geral, positivos, indicando que o boldo apresenta atividade genotóxica potencial, sendo capaz de induzir mutações e/ou recombinações somáticas em *D. melanogaster*.

Palavras-chave: Genotoxicidade, boldo, *Vernonia condensata,* plantas medicinais, teste Ring-X-Loss, teste SMART/olho.

Introdução

There is a growing interest in the investigation of potential genotoxic activity of medicinal drugs and of medicinal plants. In Brazil, many plants are used for medicinal purposes (e.g., Brito & Brito, 1993; Di Stasi et al., 2002; Caldas & Machado, 2004; Gonçalves et al., in press; Duarte et al., 2005). Among the number of genotoxicity tests available, particularly important are the rapid Drosophila tests whose results, with some reservations, may be extrapolated to man. Among the various *Drosophila* tests, the Ring-X-loss test and use post-meiotic cells of treated males, and the SMART/eye test allows the detection of the potential genotoxicity of the tested agent in terms of mutations and/or somatic recombination based on the wing mosaic system or on the eyes of adult flies (white/white plus system). The SMART/eye test is highly sensitive (0.75-0.85) and accurate (0.83-8.86) and therefore tends to complete with germ line assays such as the sex linkage recessive lethal (SLRL) test (Vogel, 1987).

The objective of the present study was to determine the potential genotoxicity of boldus (*Vernonia condensata* Baker), a plant of the Asteraceae family widely used in Brazilian folk medicine and especially in the city of Goiânia (Rizzo et al., 1990, 1999; Tridente, 2002), state of Goiás, where the study was

conducted. Two tests were conducted the rapid Ring-X-loss test and the SMART/eye (more specifically the white/white plus system: w/w^+) and applied to *Drosophila*. Boldus leaves contain approximately 0.1% of the alkaloid boldine, which may be responsible for the therapeutic properties of this plant.

Material and Méthodos

The material used for this study was colleted on the grounds of the Federal University of Goiás, Campus II, city of Goiânia, Goiás, Brazil. The voucher specimen is *V. L. Gomes-Klein 2033A*, collected 22 March 1993, and is deposited at the herbarium of the same university (UFG).

Boldus (*Vernonia condensata* Baker) was tested in the decoction form, as normally used by the local population. The present tests were conducted in 1994. A toxicity test was first carried out to select the boldus doses that would allow greater survival of *Drosophila* flies (LD_{50}). Previous to genotoxicity test was performed a sterility and survival test of treated males were determined using the Ring-X-loss test, and a fecundity test was performed to determine the mean number of descendents per pair in the SMART/eye test. Eight leaves (100 g) were placed in 1250 mL of distilled water boiled for 5 minutes. The amount of tea obtained was divided into two portions: one was diluted with an equal amount of 0.05 mol/L phosphate buffer, pH 6.8, and called dose 2 (D_2 = 0.08g/mL), and the other more concentrated, undiluted portion was called dose 1 (D_1= 0.04g/mL). Mitomycin C at 0.15 mM concentration was used as positive control, and a 0.05 mol/L phosphate buffer solution, pH 6.8, was used as negative control.

In the Ring-X-loss test, a genomic test that detects the loss of the X chromosome in post-meiotic cells of treated *Drosophila* males, was used in approximately 500 males of the ring-X strain having a ring X chromosome. The flies were starved for 6 hours before being treated with the boldus tea. For this treatment it was placed 1 mL of the substance plus 5% sucrose in flat-bottomed glass tubes containing no culture medium, but lined with absorbent paper on the bottom where the males were added. After a 24 hours treatment, the males were mated with virgin $ywsn^3$/$ywsn^3$ females carrying the y (yellow) body marker, the w (white) eye marker and sn^3 (signed 3) short hair marker (Zijlstra, 1987). The crosses were performed by the brooding technique (brood 1, brood 2 and brood 3). Approximately 10 days after the crosses (at 25°C and 60% relative humidity), the F_1 was analyzed by observation of fly phenotype for the calculation of chromosome loss (Zijlstra, 1987).

For the SMART/eye test(white/white plus system), which detects mutation and/or recombination in the eye cells of *Drosophila*, yellow virgin females were mated with white males, both with markers on chromosome X, at the proportion of 1 male to 1 female. The females were left on standard medium for 48 hours for oviposition. Parent flies were then removed from the tubes and the larvae were treated with the boldus tea by the surface treatment, in which the substance is placed on the surface of the culture medium containing the larvae. This method is used for substances with a half-life of one hour or more (Vogel & Zijlstra, 1987). For larval treatment was used 0.2 mL of the tea per dose ($dose_1$ and $dose_2$) supplemented with 5% sucrose. In a later step, it was analyzed the eyes of female flies (F_1) were analysed, immersed in a solution of 90% ethanol, 1% Tween 80 and 9% distilled water, with a light source added to the microscope for better inspection. The cells of the eye of adult flies originated from continuous divisions during the larval period and genetic changes in theses cells can be recognized by the presence of mutant clones in the adult eye when appropriate genetic markers are used. The white-white plus study is done on the imaginal disc of trans-heterozygous w/w^+ females. The clones observed were classified into 10 categories according to size, since this type of classification allows the determination of the larval stage during which the mutational event was induced (Vogel, 1989). The mutagenic effectiveness in the cells of the imaginal disc of the eye of *Drosophila* is

determined by calculating the frequency of clones per 10^4 cells using the following formula:

$$F = \frac{2n.m}{NC}$$

Where: f = frequency of clones per 10^4 cells; n = total number of clones; m = mean clone size; N = number of eyes analyzed and C = 800 (number of ommatidia per eye).

The data obtained with the Ring-X-loss test and with the SMART/eye test were analyzed statiscally by the X^2 tests according to Frei and Wurgler (1988) based on two simultaneous hypotheses, a null hypothesis (H_o) and an alternative hypothesis (H_a), which allow the distinction of the possibilities of positive, weak-positive, negative and inconclusive results. In the null hypothesis (H_o), the assumption is that there is no difference in the frequency of mutation between the negative control and the substances tested, whereas in the alternative hypothesis (H_a) the assumption is that the frequency of mutation is increased m times, where m is the number of times the frequency of mutations is increased.

Results and discussion

The sterility and fecundity tests carried out on *Drosophila* showed that boldus did not alter the fertility (frequency of lineages capable of producing offspring) or fecundity (mean number of descendents per female) of the flies, since the result obtained was not statistically significant. However, the concentrated dose of boldus (D_1) caused a larger mean number of descendants per female ('x = 25.36) than observed in the negative control ('x = 19.50), suggesting that boldus may have had a nutritive effect on the culture medium.

For the Ring-X-loss test we first calculated the percentage of chromosome loss by the following formula:

$$\% \text{ loss} = \frac{\text{no. of mutants}}{\text{targets}}$$

Where the number of mutants represents the phenotypes observed in F_1 obtained from the crosses between treated ring-X males and virgin $ywsn^3/ywsn^3$ females that presented the loss of the ring X chromosome, and the targets represent the phenotypes that presented the ring X chromosome plus the phenotypes that lost (Zijlstra & Vogel, 1988). The mean results obtained in five experiments are presented in Table 1 and figure 1.

The frequency of spontaneous mutations in the Ring-X-loss test ranges from 2.0 to 4.3%, with a mean of 3% (Zijlstra & Vogel, 1988). The historical mean value for the University of Leiden (Netherlands) group is 2.5%. In our

Table 1 - Results of the Ring-X-loss test of *Vernonia condensata* Baker at two different concentrations in germ cells.

	Treatment	Progeny	No. of mutants	Targets	% Loss
Brood 1	Negative control	443	6	224	2.68
	Positive control	1558	40	839	4.77
	Dose-1	1060	14	527	2.66
	Dose-2	844	10	422	2.37
Brood 2	Negative control	829	14	409	3.43
	Positive control	300	7	191	3.66
	Dose-1	883	14	475	2.95
	Dose-2	429	5	233	2.14
Brood 3	Negative control	539	5	295	1.70
	Positive control	448	21	232	9.05
	Dose-1	374	5	205	2.44
	Dose-2	696	12	359	3.34

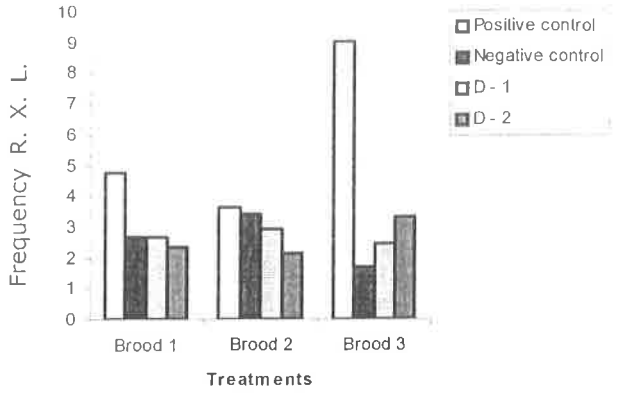

Figure 1 – Results of the Ring-X-loss test on male *Drosophila melanogaster* fed different concentrations of *Vernonia condensata* Baker.

experiments the mean percentages of chromosome loss obtained for the negative control were 2.68% for brood 1, 3.43% for brood 2, and 1.70% for brood 3, indicating that, in general, the negative control functioned well(Zijlstra & Vogel, 1988).

For the positive control (0.15 mM mitomycin C) we found a mean percentage of chromosome loss of 4.77% for brood 1, 3.66% for brood 2, and 9.05% for brood 3. Probably the percentage of chromosome loss was higher for brood 3 because the boldus tea acted more on this specific stage of spermatogenesis in treated *D. melanogaster* males, inducing chromosomes losses.

For the concentration tested (D_1 and D_2) of *Vernonia condensata*, the mean percentage of chromosome loss was approximately 2.6% for three broods.

The statistical analysis of the Ring-X-loss data using the method of Frei and Wurgler (1988) yielded inconclusive results for most experiments when compared with negative control. However, when statistical significance was detected, this was always in the negative sense, indicating that boldus did not cause partial or total chromosome loss or nondisjunction in post-meiotic *D. melanogaster* cells. The results obtained by the genomic tests for boldus agree with those reported by Moreno et al. (1991) who observed that boldine, present in *Peumus boldus* Molina (Monimiaceae) was not genotoxic to bacteria (with or without metabolic activation) and did not induce point mutations or frame shift mutations. Our results also agree with those reported by Tavares and Takahashi (1991), who observed no clastogenic effect of boldine on Balb/c rats or human lymphocytes.

In the seven experiments carried out here for the SMART/eye test, more than 250 eyes per dose were analyzed on average and clones per 100 eyes of varying sizes were observed, covering 1 to more than 8 s. The average clone size ranged from 1.57 to 5.22 for boldus (normal and diluted dose), from 1.60 to 5.75 for the negative control and from 2.11 to 9.50 for the positive control. The frequency of clones per 10,000 cells ranged from 2.21 to 9.54 for the negative control, from 5.46 to 19.08 for boldus (normal and diluted dose), and from 10.22 to 91.91 for the positive control. The mean results obtained in seven experiments are presented in Table 2 and Figure 2.

The statistical analysis of the data using the Frei and Wurgler (1988) method, showed that the results obtained with the use of boldus (normal and diluted dose) were usually positive, when compared with the negative control. These positive results were obtained for m^32, indicating that boldus at least doubled the frequency of spontaneous mutations in the SMART/eye. Thus, the results concerning the recombinogenic effects detected by the SMART/eye were positive, indicating that boldus was potentially genotoxic for *Drosophila* in terms of mutations and/or somatic recombinations. These results agree with those reported by Moreno et al. (1991) who detected slight recombinogenic effects for the alkaloid boldine present in *Peumus boldus* on diploid yeast cells, although the tests used by these investigators detected end points different from those detected by the SMART/eye. The results may suggest that the recombinogenic effects detected for boldus by the SMART/eye (w/ w^+) with *Drosophila* are due to the present of the alkaloid boldine in this plant, similar to *Peumus boldus*.

The results obtained in the present study can be summarized as follows:1) in the Ring-X-loss test, which detects chromosome losses, there was no statistical significance for the negative control, indicating that boldus did not provoke partial or total chromosome losses or nondisjunction in post-meiotic *D. melanogaster*

Table 2 - Results of the SMART/eye (white/white plus system) of *Vernonia condensata* Baker at two concentrations in somatic cells.

Treatment	No. of eyes	Clones/100 eyes			Mean size	Clones/10^4 cells
		Total	1-4	>4		
Negative control	352	7.59	6.26	1.33	3.02	5.69
Positive control	409	24.16	19.20	4.96	5.05	28.74
Dose -1	330	16.48	12.85	3.63	2.57	12.67
Dose -2	293	13.03	10.20	2.83	2.31	12.07

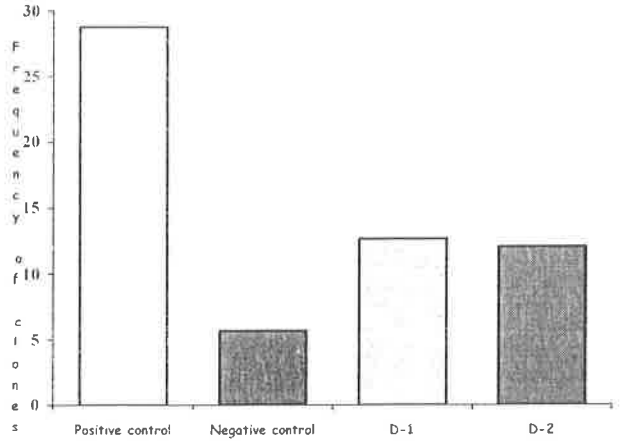

Figure 2 - Frequency of clones of the SMART/eye (white/white plus system) of Vernonia condensata *Baker at two concentrations in somatic cells of* D. melanogaster.

cells; 2) the results of the SMART/eye test, more specifically in the white/white plus system w/w$^+$ (which detects mutations and/or somatic recombinations) indicated that boldus is possibly genotoxic for mutations and/or recombinations in somatic cells of D. melanogaster.

Acknowledgments

This research was supported by Fundação de Apoio à Pesquisa, Universidade Federal de Goiás (FUNAPE/UFGO). We are grateful to Dr. Fabrizio D'Ayala Valva for critical comments, and to Kênia Silva Cunha, Piero G. Delprete and to Walter Alvarenga Rodrigues for reviewing the manuscript

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Recebido em 30.V.2005
Aceito em 19.VIII.2005