



## Antigenotoxic effects of *Mandevilla velutina* (Gentianales, Apocynaceae) crude extract on cyclophosphamide-induced micronuclei in Swiss mice and urethane-induced somatic mutation and recombination in *Drosophila melanogaster*

Regildo Márcio Gonçalves da Silva<sup>1</sup>, Neila Coelho de Sousa<sup>1</sup>, Ulrich Graf<sup>2</sup> and Mário Antônio Spanó<sup>1</sup>

<sup>1</sup>Laboratório de Mutagênese, Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil.

<sup>2</sup>Institute of Animal Sciences, Swiss Federal Institute of Technology Zurich, Schwerzenbach, Switzerland.

### Abstract

A *Mandevilla velutina* crude extract was investigated using the mouse micronucleus test (MNT) and the *Drosophila melanogaster* somatic mutation and recombination test (SMART) using standard (ST) and high bioactivation (HB) crosses. The MNT used 10 mg, 20 mg or 40 mg per 100 g of body weight (bw) of extract with and without 0.2 mg per 100 g bw peritoneal cyclophosphamide. There was no genotoxicity in the negative control or extract only groups and, compared to the cyclophosphamide control, there was a significant reduction in micronucleated polychromatic erythrocytes in all the groups given extract plus cyclophosphamide. For SMART larvae were fed 5 or 10 mg mL<sup>-1</sup> of extract for seven days with and without 0.89 mg mL<sup>-1</sup> of urethane given on day seven. The ST and HB flies showed no significant differences in spots between the negative control and the extract only groups. The number of urethane-induced spots was reduced by the highest concentration of extract for the ST flies and by both concentrations of extract for the HB flies. The results suggest that *M. velutina* extract is not genotoxic but is antigenotoxic.

**Key words:** antigenotoxicity, genotoxicity tests, micronucleus assay, SMART, wing spot test.

Received: September 20, 2007; Accepted: November 8, 2007.

### Introduction

The herbaceous plant *Mandevilla velutina* (Mart. Ex Stadelm.) Woodson (Gentianales, Apocynaceae), a member of the dogbane family (Apocynaceae) which also includes the periwinkle genera *Catharanthus* and *Vinca*, is found in the southern Atlantic Forrest (Mata Atlântica in Portuguese) and southeastern Brazil. An infusion of the rhizomes of this plant is employed in folk medicine as a popular remedy to treat snakebites, possibly by inhibition of phospholipases A2 (PLA2), and as a general anti-inflammatory agent (Hirschmann and Dearias, 1990; Mors, 1991; Bento *et al.*, 2003; Biondo *et al.*, 2003).

The selective antagonism of *M. velutina* crude extract against bradykinin and its derivatives has been studied by various authors (Calixto *et al.*, 1985; Calixto and Yunes, 1986; Calixto *et al.*, 1987; Calixto and Yunes, 1990) and some active compounds in the extracts have been chemically characterized. The pregnane glycoside compounds

isolated from *M. velutina* have been shown to be effective in antagonizing bradykinin (BK) responses in a variety of preparations and also exhibit potent and long-lasting analgesic, anti-inflammatory and anti-oedematogenic activities against a variety of inflammatory and fever-inducing phlogistic agents but was more effective in inhibiting processes involving kinins (Calixto and Yunes, 1991; Henriques *et al.*, 1991; Maraschin *et al.*, 2000).

Yunes *et al.* (1993) investigated *M. velutina* and isolated and purified the pregnanic steroid velutinol A, a potent anti-inflammatory agent and bradykinin antagonist which has also been detected in *M. velutina* cell cultures (Maraschin *et al.*, 2001). Velutinol A has also been shown to selectively block edema responses (Mattos *et al.*, 2006a) and produces peripheral antinociceptive action in some models of acute and persistent inflammatory pain by interacting with kinin B-1-receptor mediated effects (Mattos *et al.*, 2006b). A review summarizing recent advances in the identification and the potential therapeutic properties of non-peptide antagonists for kinin B-1 receptors is presented by Campos *et al.* (2006). Bento *et al.* (1996) used quantitative-<sup>1</sup>H-<sup>1</sup>H nuclear Overhauser enhancement (NOE) data

and molecular dynamics/energy minimization calculations to show that velutinol A has the structure (15*R*,16*R*,20*S*)-14,16:15,20:16,21-triepoxy-15,16-seco-14 $\beta$ ,17 $\alpha$ -pregn-5-ene-3 $\beta$ ,15-diol. More recently, detailed spectroscopic analysis has shown the structure of the potent bradykinin antagonist velutinoside A to be a pentasaccharide derivative of velutinol A, with the unusual sugars oleandrose and digitalose (Bento *et al.*, 2003).

Bioactive triterpenoids which have been isolated from *M. velutina* include the bradykinin antagonist MV 8613, the anti-inflammatory agent and bradykinin antagonist MV 8612 and the lipoxygenase inhibitor MV 8608 (Brito and Brito, 1993), with the latter two compounds also having exhibited anti-inflammatory and anti-oedematogenic effects in mice (Calixto *et al.*, 1991; Zanini *et al.*, 1992) and inhibited PLA2-induced paw edema in rats (Neves *et al.*, 1993). Santos *et al.* (2003) reported that MV8612 caused graded and complete inhibition of bradykinin-induced thermal hyperalgesia (*i.e.* an increased sensitivity to pain) in mice and inhibited both the neurogenic and inflammatory pain responses to formalin. In addition, Silva and Hamaguchi (1997) demonstrated that different concentrations of *M. velutina* crude extract are capable of reducing *Trypanosoma cruzi* infection in mice. These results provide strong experimental support for the beneficial use of this plant extract in folk medicine.

The wide distribution of *Mandevilla* and its use in folk medicine plus the lack of information on its genotoxicity or antigenotoxicity prompted us to assess the genotoxicity of *M. velutina* crude extract and investigate possible antigenotoxic effects of the extract on the induction of micronuclei by cyclophosphamide using the *in vivo* mouse bone marrow cell micronucleus test and somatic mutation and recombination induced by urethane using the *Drosophila melanogaster* wing spot somatic mutation and recombination test (SMART).

The micronucleus assay serves to detect the genotoxicity of chemicals through their ability to induce the formation of small membrane-bound DNA fragments (Ouanes *et al.*, 2003), while SMART is a short-term *in vivo* assay for the detection of mutation, recombination, chromosome damage and/or aneugenic effects in somatic cells of *D. melanogaster* (Graf *et al.*, 1984).

## Materials and Methods

### Crude chemical compounds and media

The subterranean system of *Mandevilla velutina* (Mart. Ex Stadelm.) Woodson consists of a xylopodium, the basal region of which is joined to a tuberous root (Apezzato-da-Glória and Estelita, 2000). We collected *M. velutina* tuberous roots at a site (19°09'20" S, 48°23'20" W) in the Brazilian cerrado (savanna) near the town of Uberlândia in the Brazilian state of Minas Gerais. The plants were identified by an expert plant collection. The roots

were minced and extracted using an aqueous ethanol (70% w/v) solvent in the proportion of 1:10 (w:v) minced root:solvent and mechanical stirring at room temperature (24 ± 1 °C) for 24 h, the crude extract thus obtained being filtered and lyophilized.

### Mouse bone marrow micronucleus test

Antigenotoxic effects of *M. velutina* extract on cyclophosphamide-induced micronuclei in mice was tested using seven-week to twelve-week old male Swiss albino mice (*Mus musculus* Rodentia, Muridae) weighing 25-35 g purchased from an animal breeding center (Vallé, Uberlândia, MG, Brazil) and acclimatized in cages at 24 ± 1 °C under 12 h light period for one week. During acclimatization and throughout the experiments the mice had free access to standard granulated chow and drinking water. Each cage contained five mice, which were randomly assigned to one of the four following groups: the negative control group, given distilled water by oral gavage; the positive control group, given a single intraperitoneal injection of the equivalent of 0.2 mg per 100 g of body weight (bw) of cyclophosphamide (CAS n. 50-18-0; Endoxan, Baxter Oncology Gmb, Germany), obtained from the Hospital Pharmacy of the University of Uberlândia, dissolved in distilled water; the extract group, given the equivalent of 10 mg, 20 mg or 40 mg per 100 g bw of extract each day for 7 days by oral gavage; and the experimental group, given the same treatment as for the extract group except that on the seventh day the mice also received the same treatment as the positive control group. All the mice were sacrificed by cervical dislocation on day eight. This study conforms to the relevant Brazilian guidelines regarding the ethical use of live animals. Genotoxic effects were evaluated in the mouse bone marrow by the micronucleus test (Schmid 1975). Immediately after sacrificing the mice both femurs were removed from each mouse and the bone marrow flushed out into centrifuge tubes containing 2 mL of fetal calf serum and 1000 revs min<sup>-1</sup> for 10 min, after which the supernatant was discarded and the pellet resuspended in a drop of serum and a smear made on a clean slide. The smear was air-dried, fixed with absolute methanol for 5 min then air-dried and either stored at room temperature or directly stained for 5 min with a freshly prepared working solution of Giemsa stain diluted 1:1 v/v in 0.06 M sodium phosphate buffer and 0.06 M potassium phosphate buffer (both at pH 6.8). After staining the slides were rinsed in distilled water, dried at room temperature and scored for micronuclei according to the criteria of Krishna and Hayashi (2000) using 100X magnification and a Carl Zeiss optical microscope. We scanned 2000 polychromatic erythrocytes (PCE) per mouse and recorded the number of micronucleated PCE (MNPCE). To compare the frequencies of MNPCE and normal PCE between treated and control groups the results were expressed as mean ± standard deviation and analyzed statistically using the nonparametric Mann-Whitney U-test

with the significance level set at  $\alpha = 0.05$ . The statistical analyses was carried out using the SPSS 12.0 statistical package for PCs (SPSS, Chicago, IL).

### The *D. melanogaster* SMART procedure

The antigenotoxic effects of *M. velutina* extract on urethane-induced somatic mutations in *D. melanogaster* were assessed by SMART for the multiple wing hairs (*mwh*) and flare-3 (*flr*<sup>3</sup>) recessive mutations. The *D. melanogaster* crosses used were the standard cross (ST cross) in which *flr*<sup>3</sup>/*In(3LR)TM3, ri p<sup>p</sup> sep l(3)89Aa bx<sup>34e</sup> e Bd<sup>S</sup>* females were mated with *mwh* males (Graf *et al.*, 1989) and the high bioactivation cross (HB cross) in which *ORR; flr*<sup>3</sup>/*In(3LR)TM3, ri p<sup>p</sup> sep l(3)89Aa bx<sup>34e</sup> e Bd<sup>S</sup>* females were mated with *mwh* males (Graf and Singer, 1992; Graf and van Schaik, 1992). The HB cross is characterized by a high sensitivity to promutagens and procarcinogens because the *ORR; flr*<sup>3</sup>/*TM3, Bd<sup>S</sup>* strain carries chromosomes 1 and 2 from a DDT-resistant Oregon R(R) line (Dapkus and Merrell, 1977) which is characterized by an increased level of cytochrome P-450 (Hällström and Blanck, 1985; Saner *et al.*, 1996).

Both crosses produce two types of progeny phenotypically distinguished by the *Bd<sup>S</sup>* marker, *i.e.*, marker-heterozygous (MH) flies (*mwh +/+ flr*<sup>3</sup>) with phenotypically wild type wings or (*ii*) balancer-heterozygous (BH) flies (*mwh/TM3, Bd<sup>S</sup>*) with phenotypically serrate wings. A detailed analysis of genetic markers is given by Lindsley and Zimm (1992).

Eggs were collected from both crosses during 8 h in culture bottles with an agar-agar base (4% w/v) and a thick layer of live yeast supplemented with sucrose. Third instar (72 ± 4 h) larvae were washed out of these bottles with tap water and collected in a stainless steel strainer. Batches of equal numbers of larvae were placed in glass vials containing 1.5 g of drosophila instant medium formula 4-24 (Carolina Biological Supply, Burlington, NC, USA) rehydrated with 5 mL of a solution containing a final concentration of 5 mg mL<sup>-1</sup> or 10 mg mL<sup>-1</sup> of *M. velutina* extract alone or combined with urethane (CAS N° 51-79-6; Fluka AG, Switzerland) at a final concentration of 0.89 mg mL<sup>-1</sup>, this chemical being a well-known promutagen which is metabolically activated by the cytochrome P-450 enzyme system. Negative (distilled water) and positive (0.89 mg mL<sup>-1</sup> urethane) controls were included in both experiments. Larvae were allowed to feed on the medium for the remainder of their larval life (~ 48 h). The experiments were carried out at 25 °C and 65% relative humidity.

The hatched adult flies were killed by placing in 70% (v/v) aqueous ethanol, in which they remained until needed for analysis. For analysis, wings of the MH flies were removed, mounted on glass slides with Faure's solution (30 g gum Arabic, 20 mL glycerol, 50 g chloral hydrate and 50 mL water) and examined for spots using a compound microscope at 400X magnification. The data were evalu-

ated according to the procedure described by Frei and Würgler (1988, 1995) for the occurrence of single spots (*mwh* or *flr*) and twin spots (*mwh* clone adjacent to *flr* clone). The different types of spots are due to different genotoxic mechanisms, *i.e.*, mutational events (deletions, point mutation, specific types of chromosome aberrations, etc.), mitotic recombination or, sometimes, monosomy (Graf *et al.*, 1984; Guzmán-Rincón and Graf, 1995).

The frequencies of spots per fly were compared to the concurrent control series according to Frei and Würgler (1988, 1995). Statistical comparisons were made using the Kastenbaum and Bowman (1970) test for proportions and followed a multiple-decision procedure according to Frei and Würgler (1988). For the final statistical analysis of all positive outcomes, the non-parametric Mann-Whitney *U*-test with significance levels  $\alpha = \beta = 0.05$  was used in order to exclude false positives (Frei and Würgler, 1995).

## Results

### Micronucleus test

The frequency of MNPCE ± the standard deviation (SD), for male Swiss albino mice in the distilled water negative control group was 4 ± 0.71, while for three groups of mice treated with the equivalent of 10 mg, 20 mg or 40 mg per 100 g bw of extract the frequency of MNPCE was 5.4 ± 2.68 for the 10 mg group, 0.7 ± 1.14 for the 20 mg group and 2.3 ± 2.51 for the 40 mg group. The negative control group and the extract groups were not significantly different by the Mann-Whitney *U*-test at  $p > 0.4$  (Table 1).

For the cyclophosphamide positive control group the frequency of MNPCE was 25 ± 6.96, which was significantly higher (*U*-test,  $p < 0.05$ ) when compared with the negative control group or the extract groups (Table 1). For the experimental groups pre-treated with the equivalent of 10 mg, 20 mg or 40 mg per 100 g bw of extract and then given cyclophosphamide we found that all the groups showed significantly lower frequencies of MNPCE as compared to the cyclophosphamide positive control group, the frequencies of MNPCE were 6.7 ± 5.32 for the 10 mg group, 2.6 ± 2.77 for the 20 mg group and 5.1 ± 1.64 for the 40 mg group (Table 1).

### Somatic Mutation And Recombination Test (SMART)

In the wing spot test, no statistically significant differences were found between the results of the two repeated experiments. The pooled data recorded in the marker-heterozygous (MH) flies are shown in Table 2 and demonstrate that no statistically significant differences in spot frequencies were observed for either the standard (ST) or the high bioactivation (HB) cross after chronic treatment of larvae with 5 mg mL<sup>-1</sup> or 10 mg mL<sup>-1</sup> of extract, indicating that under these experimental conditions the extract showed no

**Table 1** - Mouse bone marrow micronucleus test results showing the frequency of micronucleated (MN) polychromatic erythrocytes (PCE) in male Swiss albino mice treated with *Mandevilla velutina* crude extract (CE) with and without cyclophosphamide. There were five mice ( $n = 5$ ;  $M_1$  to  $M_5$ ) in each treatment and 2000 PCE were counted for each mouse. Since each mouse weighed 25 g to 35 g the extract doses for the treatments are 'equivalent to'. The negative control was distilled water, shown by zero in the first column under the heading 'Without cyclophosphamide'. The positive control was cyclophosphamide only at the equivalent of 0.2 mg per 100 g body weight (bw), shown by zero in the first column under the heading 'With cyclophosphamide'. Statistical analysis was by pair-wise comparisons using the nonparametric Mann-Whitney U test with the level of significance set at  $\alpha = 0.05$ .

Treatment Extract (mg per 100 g bw)	MNPCE per mouse					Total number of MNPCE	$^0/_{00}$ MNPCE $\pm$ SD
	$M_1$	$M_2$	$M_3$	$M_4$	$M_5$		
Without cyclophosphamide							
0	08	08	08	07	09	40	$4.0 \pm 0.71$
10	11	15	08	09	11	54	$5.4 \pm 2.68$
20	01	00	03	02	01	07	$0.7 \pm 1.14$
40	03	09	04	03	04	23	$2.3 \pm 2.51$
With cyclophosphamide <sup>†</sup>							
0	46	59	45	44	56	250	$25.0 \pm 6.96^*$
10	15	11	22	10	09	67	$6.7 \pm 5.32^{**}$
20	08	02	08	03	05	26	$2.6 \pm 2.77^{**}$
40	09	12	11	11	08	51	$5.1 \pm 1.64^{**}$

<sup>†</sup>Equivalent to 0.2 mg per 100 g bw.

\*Significantly different from negative control ( $p = 0.05$ ).

genotoxic or antigenotoxic effects on spontaneous DNA lesions.

The mean total spot frequencies per fly was 0.60 in the HB cross negative control group and 0.67 in the ST cross negative control group, both values being within the normally observed range. However, the frequencies of spontaneous spots normally observed in HB crosses are usually slightly higher than those observed in the ST cross (Graf and van Schaik, 1992; Lehmann *et al.*, 2000; Cunha *et al.*, 2001). Compared to the negative control group, urethane induced a statistically significant number of small single spots and large single spots in the ST cross and in all three categories of spots with the HB cross and demonstrated a high bioactivation effect, with 2.4 spots per fly in the ST cross vs. 10.2 spots per fly in the HB cross. After chronic treatment of the ST cross larvae with different concentrations of extract only there were no statistically significant differences in any of the three categories of spot frequencies in the adult flies (Table 2). However, for ST cross larvae grown on media supplemented with 10 mg mL<sup>-1</sup> of extract plus 0.89 mg mL<sup>-1</sup> of urethane there was a statistically significant reduction in the total spots recorded on adult flies as compared to the number of spots seen on adult flies the larvae of which had been exposed to urethane only. For the HB cross, for both concentrations of *M velutina* extract there was a significant reduction in all three categories of urethane-induced spots and in the total urethane-induced spots seen on adult flies.

The spot size distributions for the negative control group, the extract only group, the urethane only group and the extract plus urethane group are presented in Figures 1A

and 1B, which clearly show that the extract was not genotoxic under our experimental conditions. In contrast, after chronic treatment with urethane alone or urethane plus extract there was a predominance of small single spots, with the frequency of larger spots decreasing with increasing spot size. These data are in line with those reported with bleomycin, diethylnitrosamine and procarbazine (Graf *et al.*, 1984), cyclophosphamide (Spanó *et al.*, 2001) and a phytotherapeutic extract from *Stryphnodendron adstringes* (Mart.) Coville (Sousa *et al.*, 2003).

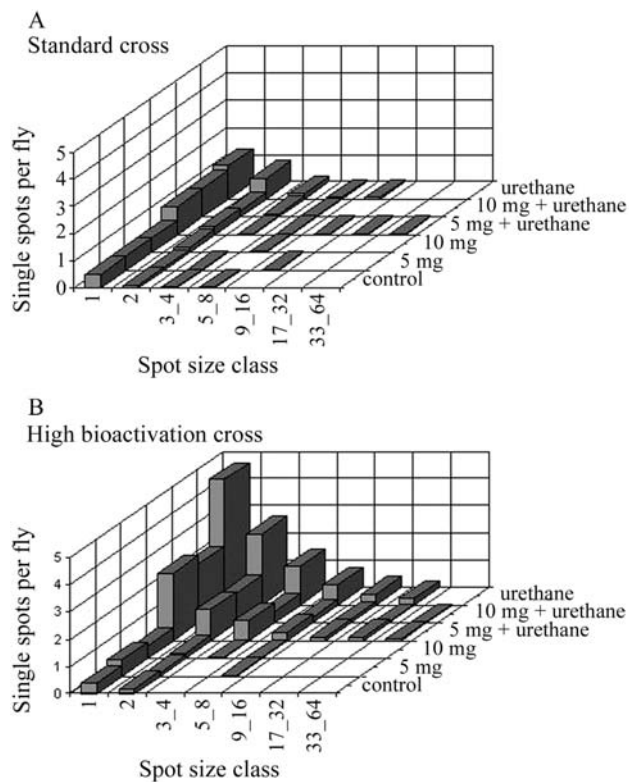
## Discussion

Evaluation of micronucleus induction is the primary *in vivo* test in a battery of genotoxicity tests and is recommended by regulatory agencies around the globe as part of product safety assessment. The assay, when performed correctly, detects both clastogenic and aneugenic effects (Krishna and Hayashi, 2000). Micronuclei in young erythrocytes arise primarily from acentric fragments or chromosomes that are unable to migrate and follow the mitotic spindle during cell division in erythropoietic blast cells (Salamone and Heddle, 1983; Ouanes *et al.*, 2003), an increase in the MNPCE frequency being an indication of induced chromosome damage (Krishna and Hayashi, 2000). In our experiments, we found that the MNPCE frequency in the groups treated with the three different extract concentrations was not significantly different from the frequency seen in the negative control group. Cyclophosphamide has been widely used as a positive control in rodent micronucleus assay (Krishna and Hayashi, 2000) and, in our experiments, showed a statistically significant induction of

**Table 2** - The *D. melanogaster* wing spot somatic mutation and recombination test (SMART) results showing the number of flies and frequency of spots observed in the marker-heterozygous (MH) progeny of the standard (ST) and the high bioactivation (HB) *D. melanogaster* crosses after chronic treatment of larvae (n = 30 per treatment) with different concentrations of *Mandevilla velutina* crude extract (CE) either alone or supplemented with 0.89 mg mL<sup>-1</sup> of urethane. Marker-heterozygous flies (*mwh/flr<sup>3</sup>*) were evaluated.

Genotypes and treatments		Spots per fly (number of spots) statistical diagnosis <sup>a</sup>				
<i>M. velutina</i> extract (mg mL <sup>-1</sup> )	Urethane (mg mL <sup>-1</sup> )	Small single spots (1 to 2 cells) <sup>b</sup> m = 2	Large single spots (> 2 cells) <sup>b</sup> m = 5	Twin spots m = 5	Total spots m = 2	Spots with <i>mwh</i> clone <sup>c</sup> (n)
ST cross						
0	0	0.57 (17)	0.03 (01)	0.07 (02)	0.67 (20)	18
5	0	0.63 (19) i	0.03 (01) i	0.03 (01) i	0.70 (21) -	21
10	0	0.70 (21) i	0.00 (00) i	0.07 (02) i	0.77 (23) -	23
0	0.89	2.03 (61) +	0.23 (07) +	0.13 (04) -	2.40 (72) +	70
5	0.89	1.33 (40) -	0.13 (04) -	0.10 (03) -	1.57 (47) -	45
10	0.89	1.30 (39) -	0.13 (04) -	0.03 (01) -	1.47 (44) +	43
HB cross						
0	0	0.57 (17)	0.00 (00)	0.03 (01)	0.60 (18)	18
5	0	0.73 (22) i	0.00 (00) i	0.03 (01) i	0.77 (23) i	23
10	0	0.67 (20) i	0.03 (01) i	0.03 (01) i	0.73 (22) i	22
0	0.89	7.23 (217) +	1.93 (58) +	1.03 (31) +	10.20 (306) +	301
5	0.89	3.53 (106) +	1.03 (31) +	0.40 (12) +	4.97 (149) +	149
10	0.89	3.23 (97) +	0.57 (17) +	0.30 (09) +	4.10 (123) +	123

<sup>a</sup>Statistical diagnoses according to Frei and Würzler [1988]: +, positive; w+, weakly positive; -, negative; i, inconclusive; Multiplication factor: m. p < 0.05. <sup>b</sup>Including rare *flr<sup>3</sup>* single spots. <sup>c</sup>Considering *mwh* clones from *mwh* single and twin spots.



**Figure 1** - Size distributions for single spots after chronic treatments with 5 mg mL<sup>-1</sup> and 10 mg mL<sup>-1</sup> of *Mandevilla velutina* crude extract (CE) and 0.89 mg mL<sup>-1</sup> of urethane. (A): Standard (ST) cross. (B): High bioactivation (HB) cross.

MNPCE. However, we also found that all concentrations of the extract prevented significant induction of MNPCE by cyclophosphamide. Taken together, these results indicate that the extract was not genotoxic under the conditions of this assay but does contain components that exert an antigenotoxic effect on cyclophosphamide-induced DNA lesions.

In the SMART the genotoxicity and antigenotoxicity of the extract was investigated in somatic cells of the *D. melanogaster* imaginal disk by feeding larvae derived from both ST and HB crosses on media containing different concentrations of the extract. The HB cross results in constitutively increased cytochrome P-450-dependent enzyme activities and therefore make the wing spot test more sensitive for the detection of promutagens and procarcinogens (Graf and Singer, 1992; Graf and van Schaik, 1992). Urethane, a promutagen which can occur naturally in some fermented food (Ough, 1976), was used as positive control because it has a clear genotoxic potential in *Drosophila*, with a clear dose response and dependence on metabolic activation. The metabolic pathway most probably involves cytochrome P-450-dependent enzyme activities (Frölich and Würzler, 1990).

The SMART data showed that at the concentrations tested the extract did not induce somatic mutation and recombination in the *D. melanogaster* ST or HB crosses. As expected, when compared to the negative control group, urethane not only showed a statistically significant induc-

tion of small single spots and large single spots in the ST cross and in all three categories of spots for the HB cross but also showed a high bioactivation effect. When compared to the positive control, chronic co-treatment of ST larvae with different concentrations of extract plus urethane produced no statistically significant differences for any of the three spot frequency categories but, however, there was a statistically significant reduction in total spots for ST flies originating from larvae in the group fed on media supplemented with 10 mg mL<sup>-1</sup> of extract plus urethane. Furthermore, with the HB cross there was a statistically significant reduction in all three categories of spots and in total spots for flies emerging from larvae treated with both concentrations of extract plus urethane. These results indicate that the extract acts as an antigenotoxic on urethane-induced DNA lesions and suggests that components of the extract interact with cytochrome P-450 leading to a reduction in the formation of the active urethane metabolite that provides genomic instability.

Our results are in line with those of Idaomar *et al.* (2002), who reported the results of a *D. melanogaster* wing spot test which showed that essential oils extracted from the medicinal plants *Helichrysum italicum*, *Ledum groenlandicum* and *Ravensara aromatica* produced an antimutagenic effect against urethane and that this could be explained by the interaction of constituents of these plants with the cytochrome P-450 activation system leading to a reduction of the formation of the active urethane metabolite. The effect could also be attributed to certain molecules that are involved in these oils. El Hamss *et al.* (2003) described the modulating action of bell pepper (*Capsicum annuum*) and black pepper (*Piper nigrum*) on the effects of the alkylating agent methyl methanesulfonate and the promutagen urethane when investigated using a *D. melanogaster* wing spot test. These authors concluding that suppression of metabolic activation or interaction with the active groups of mutagens could be mechanisms by which these spices exert their antimutagenic activity. Kuroda *et al.* (1992) has suggested that in co-treatments an antimutagen can act as a desmutagen which can chemically or enzymatically inactivate a mutagen or inhibit the metabolic activation of promutagen.

It has been reported that *M. velutina* contains bioactive triterpenoids (Calixto and Yunes, 1991; Maraschin *et al.*, 2000) which include velutinol A (Yunes *et al.*, 1993) and the active terpenes MV 8608, MV 8612 and MV 8613 (Brito and Brito, 1993), while Bento *et al.* (2003) has also reported a pentasaccharide derivative of velutinol A called velutinoside A. Nevertheless, there is still a lack of information on the genotoxic and antigenotoxic properties of compounds extracted from *M. velutina*.

The *in vivo* rodent micronucleus assay has been widely used to detect genotoxicity (Krishna and Hayashi, 2000; Villani *et al.*, 2007; Doppalapudi *et al.*, 2007) and the somatic mutation and recombination tests in *D.*

*melanogaster* are versatile and sensitive *in vivo* eukaryotic systems for the determination of genotoxic and antigenotoxic activity of chemical compounds and complex mixtures (Graf *et al.*, 1998; Idaomar *et al.*, 2002; Silva *et al.*, 2006; Fragiorge *et al.*, 2007; Pantaleão *et al.*, 2007; Téllez *et al.*, 2007).

Our study shows that in the mouse micronucleus test *M. velutina* crude extract was non-clastogenic because it did not induce chromosome breakage and was non-aneugenic since it did not affect spindle fiber function, while the *D. melanogaster* SMART data showed that the extract did not induce somatic mutation or recombination. Furthermore, the results show that the extract has antigenotoxic effects on cyclophosphamide-induced lesions in mice and urethane-induced DNA lesions in *D. melanogaster*. In addition, the SMART results indicate that the action of the extract may involve the interaction of constituents of the extract with cytochrome P-450, but, however, the exact mechanisms are not well understood due to the limited data reported in the literature with respect to the inhibitory effects of these constituents. The results of our study show that the *in vivo* rodent micronucleus test and the *D. melanogaster* SMART are versatile and sensitive *in vivo* eukaryotic systems for the determination of non-genotoxic and antigenotoxic activity of *M. velutina* extract. However, further studies are needed to elucidate the precise mechanisms involved in the antigenotoxic activity of *M. velutina* extract, with such studies including not only the investigation of different concentrations of *M. velutina* crude extract but also the main constituents of the extract.

## Acknowledgments

This work was partially supported by the Brazilian agencies CNPq, CAPES, FAPEMIG and UFU.

## References

- Appezato-da-Glória B and Estelita MEM (2000) The developmental anatomy of the subterranean system in *Mandevilla illustris* (Vell.) Woodson and *M. velutina* (Mart. ex Stadelm.) Woodson (Apocynaceae). *Rev Brasil Bot* 23:27-35.
- Bento ES, Calixto JB, Hawkes GE, Pizzolatti MG, Sant'Ana AEG and Yunes RA (1996) The structure of velutinol A is (15R,16R,20S)-14,16:15,20:16,21-triepoxy-15,16-seco-14 $\beta$ ,17 $\alpha$ -pregn-5-ene-3 $\beta$ ,15-diol. A combined quantitative Overhauser effect and molecular modelling study. *J Chem Soc, Perkin Trans 2*:1359-1366.
- Bento ES, Sant'Ana AEG, Hawkes GE, Calixto JB and Yunes RA (2003) The structure of velutinoside A: A pregnane pentasaccharide from *Mandevilla velutina*. *Tetrahedron Lett* 44:8335-8337.
- Biondo R, Pereira AMS, Marcussi S, Pereira PS, França SC and Soares AM (2003) Inhibition of enzymatic and pharmacological activities of some snake venoms and toxins by *Mandevilla velutina* (Apocynaceae) aqueous extract. *Biochimie* 85:1017-1025.

- Brito ARMS and Brito AAS (1993) Forty years of Brazilian medicinal plant research. *J Ethnopharmacol* 39:53-67.
- Calixto JB and Yunes RA (1986) Effect of a crude extract of *Mandevilla velutina* on contractions induced by bradykinin and des-Arg-9-bradykinin in isolated vessels of the rabbit. *Br J Pharmacol* 88:937-941.
- Calixto JB and Yunes RA (1990) Blockade of kinin-induced responses of the guinea-pig isolated urinary bladder by the extract of *Mandevilla velutina*. *Gen Pharmacol* 21:285-290.
- Calixto JB and Yunes RA (1991) Natural bradykinin antagonists. *Mem Inst Oswaldo Cruz* 86:195-202.
- Calixto JB, Nicolau M and Yunes RA (1985) A selective antagonist of bradykinin action from a crude extract of *Mandevilla velutina*. Effect on isolated rat uterine smooth-muscle. *Braz J Med Biol Res* 18:A728-A728.
- Calixto JB, Nicolau M, Pizzolatti MG and Yunes RA (1987) Kinin antagonist activity of compounds from *Mandevilla velutina* in the rat isolated uterus. *Br J Pharmacol* 91:199-204.
- Calixto JB, Zanini JC, Cruz AB, Yunes RA and Medeiros YS (1991) Extract and compounds obtained from *Mandevilla velutina* inhibit arachidonic acid-induced ear edema in mice, but not rat stomach contraction. *Prostaglandins* 41:515-526.
- Campos MM, Leal PC, Yunes RA and Calixto JB (2006) Non-peptide antagonists for kinin B-1 receptors: Never insights into their therapeutic potential for the management of inflammation and pain. *Trends Pharmacol Sci* 27:646-651.
- Cunha KS, Reguly ML, Graf U and Andrade HHR (2001) Taxanes: The genetic toxicity of paclitaxel and docetaxel in somatic cells of *Drosophila melanogaster*. *Mutagenesis* 16:79-84.
- Dapkus D and Merrell DJ (1977) Chromosomal analysis of DDT-resistance in a long-term selected population of *Drosophila melanogaster*. *Genetics* 87:685-697.
- Doppalapudi RS, Riccio ES, Rausch LL, Shimon JA, Lee PS, Mortelmans KE, Kapetanovic IM, Crowell JA and Mirsalis JC (2007) Evaluation of chemopreventive agents for genotoxic activity. *Mutat Res* 629:148-160.
- El Hamss R, Idaomar M, Alonso-Moraga A and Muñoz-Serrano A (2003) Antimutagenic properties of bell and black peppers. *Food Chem Toxicol* 41:41-47.
- Fragiorgis EJ, Spanó MA and Antunes LMG (2007) Modulatory effects of the antioxidant ascorbic acid on the direct genotoxicity of doxorubicin in somatic cells of *Drosophila melanogaster*. *Genet Mol Biol* 30:449-455.
- Frei H and Würigler FE (1988) Statistical methods to decide whether mutagenicity test data from *Drosophila* assays indicate a positive, negative or inconclusive result. *Mutat Res* 203:297-308.
- Frei H and Würigler FE (1995) Optimal experimental design and sample size for the statistical evaluation of data from somatic mutation and recombination tests (SMART) in *Drosophila*. *Mutat Res* 334:247-258.
- Frölich A and Würigler FE (1990) Genotoxicity of ethyl carbamate in the *Drosophila* wing spot test: Dependence on genotype-controlled metabolic capacity. *Mutat Res* 244:201-208.
- Graf U and Singer D (1992) Genotoxicity testing of promutagens in the wing somatic mutation and recombination test in *Drosophila melanogaster*. *Rev Int Contam Ambient* 8:15-27.
- Graf U and van Schaik N (1992) Improved high bioactivation cross for the wing somatic mutation and recombination test in *Drosophila melanogaster*. *Mutat Res* 271:59-67.
- Graf U, Abraham SK, Guzman-Rincón J and Würigler FE (1998) Antigenotoxicity studies in *Drosophila melanogaster*. *Mutat Res* 402:203-209.
- Graf U, Frei H, Kägi A, Katz AJ and Würigler FE (1989) Thirty compounds tested in the *Drosophila* wing spot test. *Mutat Res* 222:359-373.
- Graf U, Würigler FE, Katz AJ, Frei H, Juon H, Hall CB and Kale PG (1984) Somatic mutation and recombination test in *Drosophila melanogaster*. *Environ Mutagen* 6:153-188.
- Guzmán-Rincón J and Graf U (1995) *Drosophila melanogaster* somatic mutation and recombination test as a biomonitor. In: Butterworth FM, Corkum LD and Guzmán-Rincón J (eds) *Biomonitoring and Biomarkers as Indicators of Environmental Change*. Plenum Press, New York, pp 169-181.
- Hällström I and Blanck A (1985) Genetic regulation of the cytochrome P-450 system in *Drosophila melanogaster*. I Chromosomal determination of some cytochrome P-450-dependent reactions. *Chem Biol Interact* 56:157-171.
- Henriques MGMO, Fernandes PD, Weg VB, Yunes RA, Cordeiro RSB and Calixto JB (1991) Inhibition of rat paw edema and pleurisy by the extract from *Mandevilla velutina*. *Agents Actions* 33:272-278.
- Hirschmann GS and Dearias AR (1990) A Survey of medicinal-plants of Minas-Gerais, Brazil. *J Ethnopharmacol* 29:159-172.
- Idaomar M, El Hamss R, Bakkali F, Mezzouga N, Zhiri A, Baudoux D, Muñoz-Serrano A, Liemans V and Alonso-Moraga A (2002) Genotoxicity and antigenotoxicity of some essential oils evaluated by wing spot test of *Drosophila melanogaster*. *Mutat Res* 513:61-68.
- Kastenbaum MA and Bowman KO (1970) Tables for determining the statistical significance of mutation frequencies. *Mutat Res* 9:527-549.
- Krishna G and Hayashi M (2000) *In vivo* rodent micronucleus assay: Protocol, conduct and data interpretation. *Mutat Res* 455:155-166.
- Kuroda Y, Jain AK, Tezuka H and Kada T (1992) Antimutagenicity in cultured mammalian cells. *Mutat Res* 267:201-209.
- Lehmann M, Graf U, Reguly ML and Andrade HHR (2000) Interference of tannic acid on the genotoxicity of mitomycin C, methylmethanesulfonate, and nitrogen mustard in somatic cells of *Drosophila melanogaster*. *Environ Mol Mutagen* 36:195-200.
- Lindsley DL and Zimm GG (1992) *The Genome of Drosophila melanogaster*. Academic Press, San Diego, 1133 pp.
- Maraschin M, Carobrez SG, Persike D, Peixoto ML, Ferreira AG, Ferracin R, Verpoorte R and Fontana JD (2000) Cell wall polysaccharides from *Mandevilla velutina* (Apocynaceae) cultured cells: Extraction and chemical structure. *Carbohydr Polym* 41:55-60.
- Maraschin M, Sugui JA, Wood KV, Bonham C, Lancas FM, Araujo PS, Yunes RA, Verpoorte R and Fontana JD (2001) Supercritical CO<sub>2</sub> extraction of velutinol A from *Mandevilla velutina* (Apocynaceae) cultured cells and MALDI-TOF MS analysis. *Biotechnol Lett* 23:77-82.
- Mattos WM, Campos MM, Fernandes ES, Richetti GP, Niero R, Yunes RA and Calixto JB (2006a) Anti-edematogenic ef-

- fects of velutinol A isolated from *Mandevilla velutina*: Evidence for a selective inhibition of kinin B-1 receptor-mediated responses. *Regul Pept* 136:98-104.
- Mattos WM, Ferreira J, Richetti GP, Niero R, Yunes RA and Calixto JB (2006b) Antinociceptive properties produced by the pregnane compound velutinol A isolated from *Mandevilla velutina*. *Neuropeptides* 40:125-132.
- Mors WB (1991) Plants against snake bites. *Mem Inst Oswaldo Cruz* 86:193-193.
- Neves PC, Neves MC, Cruz AB, Sant'ana AE, Yunes RA and Calixto JB (1993) Differential effects of *Mandevilla velutina* compounds on paw oedema induced by phospholipase-A2 and phospholipase-C. *Eur J Pharmacol* 243:213-219.
- Ouanes Z, Abid S, Ayed I, Anane R, Mobio T, Creppy EE and Bacha H (2003) Induction of micronuclei by Zearalenone in Vero monkey kidney cells and in bone marrow cells of mice: Protective effect of vitamin E. *Mutat Res* 538:63-70.
- Ough CS (1976) Ethylcarbamate in fermented beverages and foods. I. Naturally occurring ethylcarbamate. *J Agric Food Chem* 24:323-328.
- Pantaleão SM, Alcântara AV, Alves JPH, Pavanin LA, Graf U, Rezende AAA, Valadares BLB, Fragiorge EJ, Souza NC, Gutierrez ZR, *et al.* (2007) Assessing the impact of pollution on the Japaratuba River in Brazil using the *Drosophila* wing spot test. *Environ Mol Mutagen* 48:96-105.
- Salamone MF and Heddle JA (1983) The bone marrow micronucleus assay: Rationale for a revised protocol. In: de Serres FJ (ed) *Chemical Mutagens: Principles and Methods for their Detection*, v. 8. Plenum Press, New York, pp 111-149.
- Saner C, Weibel B, Würzler FE and Sengstag C (1996) Metabolism of promutagens catalyzed by *Drosophila melanogaster* CYP6A2 enzyme in *Saccharomyces cerevisiae*. *Environ Mol Mutagen* 27:46-58.
- Santos ARS, Trentin AP, Ferreira J, Yunes RA and Calixto JB (2003) Mechanisms involved in the antinociception caused by compound MV8612 isolated from *Mandevilla velutina* in mice. *Brain Res* 961:269-276.
- Schmid W (1975) The micronucleus test. *Mutat Res* 31:9-15.
- Silva LP, Costa-Cruz JM, Spanó MA and Graf U (2006) Genotoxicity of vesicular fluid and saline extract of *Taenia solium* metacestodes in somatic cells of *Drosophila melanogaster*. *Environ Mol Mutagen* 47:247-253.
- Silva RMG and Hamaguchi A (1997) Avaliação da atividade tripanomicida do extrato de *Mandevilla velutina* em tripomastigotas de *Trypanosoma cruzi*. *Rev Soc Bras Med Trop* 30:104.
- Sousa NC, Carvalho S, Spanó MA and Graf U (2003) Absence of genotoxicity of a phytotherapeutic extract from *Stryphnodendron adstringens* (Mart.) Coville in somatic and germ cells of *Drosophila melanogaster*. *Environ Mol Mutagen* 41:293-299.
- Spanó MA, Frei H, Würgler FE and Graf U (2001) Recombinogenic activity of four compounds in the standard and high bioactivation crosses of *Drosophila melanogaster* in the wing spot test. *Mutagenesis* 16:385-394.
- Téllez MGO, Rodríguez HB, Olivares GQ, Sortibrán ANC, Cetto AA and Rodríguez-Arnaiz R (2007) A Phytotherapeutic extract of *Equisetum myriochaetum* is not genotoxic either in the *in vivo* wing somatic test of *Drosophila* or in the *in vitro* human micronucleus test. *J Ethnopharmacol* 111:182-189.
- Villani P, Cordelli E, Leopardi P, Siniscalchi E, Veschetti E, Fresegna AM and Crebelli R (2007) Evaluation of genotoxicity of oral exposure to tetravalent vanadium *in vivo*. *Toxicol Lett* 170:11-18.
- Yunes RA, Pizzolatti MG, Santana AEG, Hawkes GE and Calixto JB (1993) The structure of velutinol-A, an anti-inflammatory compound with a novel pregnane skeleton. *Phytochem Anal* 4:76-81.
- Zanini JC, Medeiros YS, Cruz AB, Yunes RRA and Calixto JB (1992) Action of compounds from *Mandevilla velutina* on cróton oil-induced ear edema in mice - A comparative study with steroidal and nonsteroidal anti-inflammatory drugs. *Phytother Res* 6:1-5.

*Associate Editor: Carlos F.M. Menck*

License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.