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In vitro study of mutagenic potential of *Bidens pilosa* Linné and *Mikania glomerata* Sprengel using the comet and micronucleus assays

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ABSTRACT

Teas of *Bidens pilosa* and *Mikania glomerata* are popularly consumed to medicinal ends. The capacity to induce DNA damages and mutagenic effects of these teas were evaluated, *in vitro*, on HTC cells, with comet assay and micronucleus test. The teas tested at various doses were prepared differently: infusion of *Mikania glomerata* (IM) and *Bidens pilosa* (IB), macerate of *Mikania glomerata* in 80% ethanol (MM80) and decoction of *Bidens pilosa* (DB). In IM and MM80, the quantity of coumarin was determined by high-performance liquid chromatography (HPLC) with UV detection. Methylmethanesulfonate was utilized as positive control, phosphate-buffered saline as negative control, 80% ethanol as solvent control and 2-aminoanthracene as drug metabolism control. The comet assay demonstrated genotoxic effects for both plants. The genotoxic potential of IB was upper than DB, showing dose-response. In the MN test, excepting IM 40 μL/mL, all treatments was not mutagenic. The effects did not show direct relation with cumarin quantity present in IM and MM80. The results demonstrated DNA damages at the highest concentrations of alcoholic macerate (10 and 20 μL/mL) and infusion of *Mikania glomerata* (20 and 40 μL/mL) and of *Bidens pilosa* infusion (40 μL/mL). Thus, both dose and preparation-form suggest caution in the phytotherapeutic use of these plants.

1. Introduction

The utilization of certain plants in combating diseases is common practice among the people. However, in the majority of cases, there is no proof of efficacy of treatment in popular use, or there has not been an adequate evaluation of medicinal plants for possible adverse effects (Silveira e Sá et al., 2003).

Bidens pilosa Linné var. (Asteraceae), whose common name in Brazil is "picão-preto", has a height of 30–100 cm and yellow flowers (Abajo et al., 2004) and it is widely distributed in the tropics and sub-tropics, from sea level to up to about 3000 m in altitude (Ballard, 1986). In places where it occurs, it is utilized as a phytotherapeutic product, especially for hepatic disorders (hepatitis and jaundice) (Vasques et al., 1986). According to Brandão et al. (1998), the tea is used in popular medicine as an anti-inflammatory, diuretic, anti-rheumatic and antidiabetic. It has also shown high bactericidal activity (Rabe and Van Staden, 1997), as well as antioxidant and immunomodulatory effects (Abajo et al., 2004), in addition to antimalarial activity (Brandão et al., 1997; Oliveira et al., 2004). The plant is rich in quercetin (Hoffmann and Hölzl, 1989; Lastra Valdes, 2001) and other polyphenolic compounds (Rice-Evans et al., 1996), which can be responsible for the antioxidant activity observed (Van Acker et al., 1996). The antimalarial activity is correlated with polyacetylene and flavonoids, compounds corresponding to quercetin (Oliveira et al., 2004).

Mikania glomerata Sprengel (Asteraceae), an important medicinal species, popularly known as "guaco", is a vine-like plant which has leaves that are opposite, simple, oval and acuminate and white flowers. A native of South America, it occurs throughout the continent and reaches some countries in Central America. It is a plant popularly used as an expectorant to treat respiratory diseases, promoting the flow of tracheobronchial exudate (Teske and Trentini, 1997). It is reported to have antifungal, antimicrobial, anti-allergy and anti-inflammatory activities (Fierro et al., 1999; Holetz et al., 2002), in addition to potent anti-snake venom effect (Maiorano et al., 2005). Studies have pointed to coumarin, one of its active principles (Oliveira et al., 1993; Cabral et al., 2001), as potential substance for the treatment of cancer (Lin et al., 1996), where they have shown growth inhibition and cell kill in various tumor cell lines, which reinforces the need for toxicological studies of these plants (Fátima et al., 2005).

Both plants, *Bidens pilosa* and *Mikania glomerata* are used in several countries in the word and are some of the most utilized herbal

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remedy in brazilian folk medicine (Lorenzi and Matos, 2002). In this folk medicine, differences in the use forms can be observed. *Bidens pilosa*, for example, generally is used, in folk medicine, in decoction of all parts of the plant except roots, but infusion is used also (Lorenzi and Matos, 2002; Abajo et al., 2004). In other hand, *Mikania glomerata* is utilized in infusion and ethanol extract forms. This last is the principal form of use in manufactured production (Teske and Trentini, 1997).

However, as observed with *Bidens pilosa*, while *Mikania glomerata* is a widely popular and well known herbal remedy with research validating much of its traditional use, there are no reports on the possible genotoxic or mutagenic effects of this plant with regard to the forms in which it is most often consumed by the public, that is, teas.

Therefore, the aim of this study was to determine the potential of these two plants, *Bidens pilosa* and *Mikania glomerata*, to induce genetic damage *in vitro*, using teas prepared by infusion, decoction or maceration in 80% ethanol, taking into account the dosages recommended for human daily consumption.

2. Materials and methods

2.1. Materials

Bidens pilosa was collected in Londrina (PR), South Brazil, in June 2004. A voucher of the specimen was deposited in Department of Animal and Vegetal Biology, State University of Londrina (UEL) under number FUEL 39281.

Mikania glomerata was also collected in Londrina (PR), in June 2004, and a voucher was deposited in the same herbarium, under number FUEL 35507.

2.2. Cell culture

In the present study, rat hepatoma cells (*Rattus norvegicus*) or HTC, acquired from Cell Bank of Rio de Janeiro at Federal University of Rio de Janeiro, Brazil, were grown as adherent monolayers in sterile 25 cm^2 culture flasks for the micronucleus (MN) test and in 2.5 mL culture tubes for the comet assay (*single-cell gel electrophoresis*—SCGE *assay*). The cells were cultivated in D-MEM-F12 (GibcoBRL) culture medium supplemented with 10% fetal bovine serum (FBS, Gibco). They were grown at 37 °C in a BOD type incubator and, under these conditions, the duration of the cell cycle was 24 h. The cells were sub-cultured for about 3–4 days for stabilization in culture medium. A fixed number of cells was then distributed in culture flasks for each type of assay, where they were grown for at least one complete cell cycle before receiving experimental treatments.

2.3. Test substances

Four types of extracts of the two plants were tested, prepared according to the Farmacopéia Portuguesa, 6th ed. (1997): (a) infusion of *Bidens pilosa* (IB) and (b) decoction of *Bidens pilosa* (DB), both using aerial parts without roots; (c) infusion of *Mikania glomerata* (IM), using the leaves and (d) alcoholic extract of *Mikania glomerata*, prepared by simple maceration of leaves in 80% ethanol (M80).

For the solutions tested, the infusion was obtained by adding 1000 mL of ultra pure water (MilliQ), at a temperature of 96 °C, to 50 g of slightly minced leaves and, after 45 min, the separation was made by simple filtration. The decoction, in turn, was prepared from the boiling of 100 g of plant in 1500 mL of ultra pure water (MilliQ), for about 1 h, until the volume was reduced to 1000 mL, followed by simple filtration. The ethanolic extract was obtained after 24 h of simple maceration of the leaves in 80% ethanol (dilution that showed the most effective extraction of coumarins), at a plant:solvent proportion of 1:5 (m/V). This was provided by Dr. Andréa Diniz of the Department of Technology of Foods and Medications (TAM) at the Center for Agricultural Sciences (CCA), State University of Londrina (UEL). These test solutions were stored at −20 °C.

The concentrations of infusion and decoction used in the MN test were 20 and 40 μL/mL of culture medium, while in the comet assay these were 10, 20 and 40 μL/mL. If they were converted as "mg plant/mL" the last concentrations would be like 0.5, 1 and 2 mg/mL in the infusion and 1, 2 and 4 mg/mL in the decoction. All concentrations were determined taking into account the indices of 50, 100 and 200% of the daily recommended intake (DRI) for the infusion and decoction (RDI = 250 mL, four times a day, for a person of 70 kg).

However, the macerate in 80% ethanol, because being prepared at a plant:solvent proportion four times greater than that in teas, was tested in the comet assay at final concentrations four times less: 2.5, 5 and 10 μL/mL of culture medium, plus a dose of 20 μL/mL used for comparative purposes. In the MN test, were utilized only the concentrations of 5 and 10 μL/mL of culture medium.

The alkylating and clastogenic agent methylmethane sulfonate (MMS) was used to induce DNA damage (positive control), at different concentrations adjusted to the biological assay. In the MN test, the indirect-acting mutagenic agent 2-aminoanthracene (2aa) was also used, at a final concentration of 5 μL/mL of culture medium, as the control for drug-metabolizing capacity. It is an aromatic amine that is highly mutagenic after being metabolized by hepatic enzymes of the cytochrome P450 group (CYPs) (Cancino-Badías et al., 2003). Phosphate-buffered saline (PBS), Ca^{++}- and Mg^{++}-free, pH 7.4, was used as the negative control, at 30 μL/mL of culture medium, and 80% ethanol was used as the solvent control, at final concentrations of 10 and 20 μL/mL, to determine any possible solvent interference with the results of the *Mikania glomerata* macerate in 80% ethanol.

2.4. Quantification of coumarin

Coumarin concentration in the infusion and the ethanolic extract of *Mikania glomerata* was determined by HPLC with UV detection, according to the method described by Celeghini et al. (2001). The analyses were carried out with a Shimadzu liquid chromatograph, pump model LC6A, with UV detector, C-R6A integrator, manual 7125 Rheodyne injector (20 μL), Shim-Pack ODC-18 analytical column (4.6 mm × 250 mm, 5 μm), and RP-18 pre-column (Lichrospher® 100, 5 μm, 4 mm × 4 mm, Merck). Acetonitrile and water were used as the mobile phase solvents. The solvents were filtered through an HVHP membrane and deaerated in an ultrasound bath. The column was eluted at a flow rate of 0.7 mL/min, using an isocratic system of acetonitrile:water (40:60, v/v). Detection was at 274 nm.

The method was validated for linearity, recovery, reproducibility and intermediate precision.

2.5. Comet assay

Approximately 0.5×10^5 HTC cells, previously stabilized, were distributed and incubated for 24 h in culture tubes containing 2.5 mL of culture medium supplemented with FBS. Cells were treated with teas and with ethanolic extract at all previously defined concentrations. MMS was used at a final concentration of 90 μM as positive control. PBS (pH 7.4) served as negative control and 80% ethanol as solvent control. After 2 h of treatments, the cells were harvested using trypsin (0.025%), centrifuged and resuspended in culture medium.

Table 1
Mean frequency of cells with comet and of cells with micronuclei obtained in control treatments and in treatments with infusion and ethanolic macerate of *Mikania glomerata* and with infusion and decoction of *Bidens pilosa* in HTC cells

Treatment (μL/mL)	Mean number of positive cells in comet assay ± S.D.	Comet class				Mean score	Mean number of HTC cells with MN ± S.D.
		0	1	2	3		
PBS	7.7 ± 1.5	92.7	5.7	1.7	–	9.0	8.3 ± 1.2
MMS	99.0 ± 1.2	0.7	50.7	41.0	8.3	158.3	42.7 ± 3.1*
EtOH 80 10	11.6 ± 2.5	88.3	9.7	2.0	–	13.7	–
EtOH 80 20	11.7 ± 0.0	88.3	9.7	1.7	0.3	14.0	–
IM10	11.3 ± 4.6	89.3	11.0	–	–	11.7	–
IM20	17.0 ± 5.0*	83.0	14.7	2.0	0.3	19.7	11.0 ± 1.7
IM40	31.7 ± 6.5*	68.3	29.7	2.0	–	33.7	14.7 ± 1.2*
MM80 2.5	8.0 ± 2.0	92.0	6.0	2.0	–	10.0	–
MM80 05	9.7 ± 1.5	90.3	7.7	2.0	–	11.7	11.7 ± 2.1
MM80 10	35.3 ± 1.5*	64.7	30.3	4.7	0.3	40.3	11.7 ± 1.5
MM80 20	99.3 ± 0.6*	0.3	18.3	76.0	5.3	186.3	–
IB10	18.3 ± 5.5*	81.7	17.3	1.0	–	19.3	–
IB20	39.3 ± 6.7*	60.7	32.3	5.0	2.0	48.3	11.7 ± 1.5
IB40	98.7 ± 2.3*	1.3	27.3	48.3	23.0	193.0	11.7 ± 0.6
DB10	19.3 ± 2.5*	80.7	19.0	0.3	–	19.7	–
DB20	17.0 ± 2.6*	83.0	16.0	1.0	–	17.7	9.0 ± 1.0
DB40	23.7 ± 3.0*	76.0	23.3	0.7	–	24.7	8.7 ± 0.6

PBS: phosphate-buffered saline; MMS: methylmethane sulfonate; EtOH80: 80% ethanol; IM: infusion of *Mikania glomerata*; MM80: macerate of *Mikania glomerata* in 80% ethanol; IB: infusion of *Bidens pilosa*; DB: decoction of *Bidens pilosa*; S.D.: standard deviation; MN: micronucleus. *Statistically significant difference, $p < 0.05$.

Afterward, the cells were prepared for the comet assay according to the protocol described by Speit and Hartmann (1999). After cells were harvested, 20 μL of cell suspension were mixed with 120 μL of low melting point agarose (0.5%). The cell suspension was then placed on microscope slides that were pre-gelatinized with normal melting point agarose (1.5%). The slides were then coverslipped and kept refrigerated at 4 °C to solidify the gel. After 20 min, the slides were immersed in chilled lysis solution [89 mL of stock solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–EDTA, NaOH to yield pH 10, 1% sodium laurylsarcosinate) plus 1 mL Triton X-100 and 10 mL DMSO] for 24 h, protected from light and refrigerated. The slides were then placed in an electrophoresis chamber, covered with electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH > 13), maintained in the dark at 4 °C, for 20 min. The electrophoresis ran at 300 mA and 1.6 V/cm for 20 min. The slides were then neutralized with 0.4 M Tris–HCl buffer, pH 7.5, three times for periods of about 5 min each, air-dried and fixed in absolute ethanol for 10 min. Just before being examined, the slides were stained with 100 μL ethidium bromide (2 μg/mL). The cells were then viewed with a fluorescence microscope (excitation filter of 420–490 nm and barrier filter of 590 nm) at 400×. Based on the criteria established by Kobayashi et al. (1995), 100 nuclei were examined per slide and classified as follows: class 0 (absence of tail); class 1 (tail of up to 1× the diameter of the nucleus of negative control); class 2 (tail of up to 2× the diameter of the nucleus); class 3 (tail of more than 2× the diameter of the nucleus). Apoptotic cells were not counted (Speit et al., 1996). A total of 300 cells were analyzed per treatment, and the mean score of the damage was calculated by multiplying the number of cells showing damage in each class (n) by the value of the class, according to following formula:

$$\text{mean score} = \frac{(1n_1) + (2n_2) + (3n_3)}{3}.$$

2.6. Micronucleus test

In the MN test, cells were incubated in complete culture medium for 1.5 cell cycle (36 h), then washed with 5 mL PBS (pH 7.4) and treated with the test substances: teas (infusion and decoction) of the two plants and alcoholic extract of *Mikania glomerata*, each one at two earlier defined concentrations. Cells were also treated with PBS as negative control, MMS (0.3 mM) as positive control and 2-aminoanthracene as cellular drug metabolism control, for exposure times of 2 and 4 h. Afterward, cells were washed again twice with PBS, placed in fresh culture medium with 10 μL/mL cytochalasin-B (300 μL/mL) and incubated for another 20 h to obtain binucleated cells as described by Fenech (1997). Cells were then harvested, slides were prepared according to Salvadori et al. (1993) and stained with Rosenfeld's solution (0.053% May Grunwald and 0.097% Giemsa in methanol). The criteria utilized for analyses were established by Fenech (2000). The treatments were carried out in triplicate in independent experiments, and 2000 binucleated cells were examined on each slide (Garriot et al., 2002).

2.7. Statistical analysis

Statistical analysis of the data was carried out using ANOVA for all the results of each test and Dunnet's test was applied to the means, comparing the mean number of cells with DNA damage in the comet assay and the micronucleus test, obtained from experiments performed in triplicate, with the mean values in PBS or MMS controls. $p < 0.05$ was accepted as statistically significant.

3. Results

The results obtained with all the forms of extracts of the two plants evaluated by the biological assays applied, comet assay and MN test, are shown in Tables 1 and 2.

Drug-metabolizing capacity was demonstrated in the HTC cells used in the two types of assays, where the positive results observed

Table 2
Mean number of micronucleated cells obtained in control treatments and with 2-aminoanthracene (2aa) at two different times of exposure of HTC cells

Treatments (μL/mL), time (h)	Mean frequency of cells with MN ± S.D.
PBS	5.67 ± 0.47
MMS	20.33 ± 0.47*
2aa 05, 2 h	9.00 ± 0.82*
2aa 05, 4 h	11.33 ± 0.47*

PBS: phosphate-buffered saline; MMS: methylmethane sulfonate; S.D.: standard deviation; MN: micronucleus; 2aa: 2-aminoanthracene. *Statistically significant difference, $p < 0.05$.

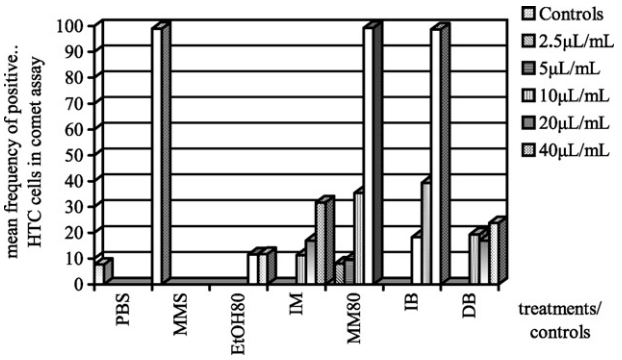

Fig. 1. Mean frequency of positive HTC cells in comet assay obtained in controls: phosphate-buffered saline (PBS), methylmethane sulfonate (MMS) and 80% ethanol (EtOH 80) and in treatments: infusion of *Mikania glomerata* (IM), macerate of *Mikania glomerata* in 80% ethanol (MM80), infusion of *Bidens pilosa* (IB) and decoction of *Bidens pilosa* (DB), all at different concentrations.

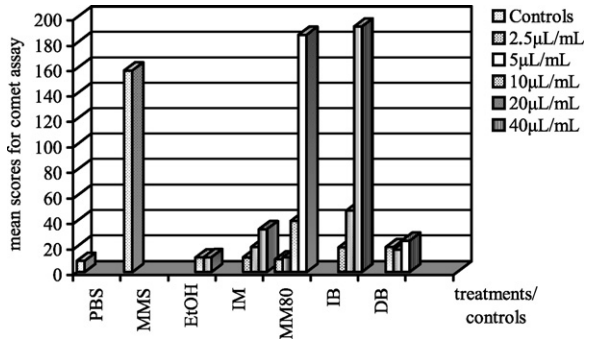

Fig. 3. Mean scores for comet assay obtained in controls: phosphate-buffered saline (PBS), methylmethane sulfonate (MMS) and 80% ethanol (EtOH) and in the treatments: infusion of *Mikania glomerata* (IM), macerate of *Mikania glomerata* in 80% ethanol (MM80), infusion of *Bidens pilosa* (IB) and decoction of *Bidens pilosa* (DB), all at different concentrations.

with 2aa differed from the PBS control after 2 h as well as after 4 h exposure (Table 2).

In examining the results of the comet assay, the infusion of *Mikania glomerata* did not induce DNA damage significantly at 10 μL/mL, but it showed dose-dependent genotoxicity at 20 and 40 μL/mL (correlated respectively to 100 and 200% of the recommended daily intake, RDI) (Fig. 1). Meanwhile, *Mikania glomerata* macerate in 80% ethanol (MM80) did not show a statistically significant difference in relation to the control at doses of 2.5 and 5 μL/mL, but it demonstrated genotoxicity with 10 and 20 μL/mL, that is, at the highest doses (200 and 400% of the RDI). The control treatments with 80% ethanol did not differ statistically from the PBS negative control.

In the case of *Bidens pilosa*, the frequency of damaged cells in the comet assay induced by the infusion differed statistically from that of the negative control for all three concentrations tested. This genotoxic potential of IB showed an evident dose-response effect, where the highest concentration of 40 μL/mL produced results very similar to those of MMS (Figs. 1 and 2). For the other form of extraction, decoction (DB), this plant also showed genotoxicity at the three concentrations tested. However, from the number of damaged cells and by the score of the damage (Figs. 2 and 3), it was evident that this effect was at levels lower than those for the infusion (IB).

Also by the comet assay, it was possible to see that the score of damages was elevated with the highest concentrations for both *Mikania glomerata* (IM, MM80) and *Bidens pilosa* (IB), given that MM80 at 20 μL/mL and IB at 40 μL/mL produced scores greater than that of MMS (Fig. 3). These high scores were due to the displacement of cells with damage to the greatest damage classes (Table 1).

In comparing the dose utilized and the effect elicited in the comet assay, it can be noted that, with the exception of DB, the higher the dose the greater the genotoxicity, either with regard to the number of damaged cells (Fig. 1) as to the mean score of damage caused (Fig. 3). This effect directly related to the dose tested (Fig. 2), in the case of IM, resulted in an approximately linear relationship, while with MM80 and IB, the relationship was almost exponential, since the double of dose caused an effect that was about five times greater.

In the case of the MN test, where no more than the highest concentrations were tested (20 and 40 μL/mL), of all the treatments carried out, IM at 40 μL/mL was the only one that induced a significant increase in the mean number of cells with MN (Figs. 4 and 5). All the other treatments did not show any mutagenic potential.

In comparing the results obtained between the two assays (Fig. 5), is evident that the positive responses of the comet assay were much more accentuated that those obtained in the MN test. Except for treatment with IM 40 μL/mL, in the MN test, all the treatments and concentrations showed a response that was well distinct from the positive control (MMS), differently of which was observed with the comet assay.

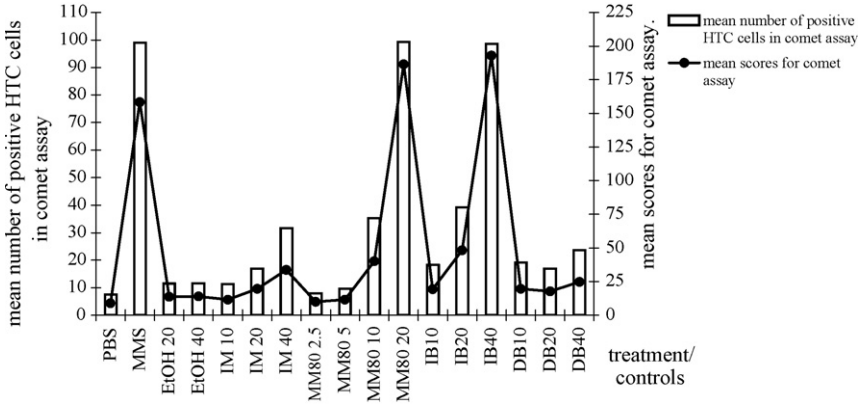

Fig. 2. Mean number of positive HTC cells in comet assay and respective mean scores obtained in the controls: phosphate-buffered saline (PBS), methylmethane sulfonate (MMS) and 80% ethanol (EtOH) and in the treatments: infusion of *Mikania glomerata* (IM), macerate of *Mikania glomerata* in 80% ethanol (MM80), infusion of *Bidens pilosa* (IB) and decoction of *Bidens pilosa* (DB), all at different concentrations.

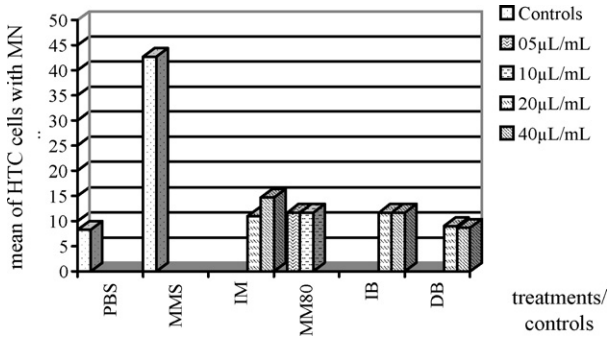

Fig. 4. Mean number of HTC cells with MN obtained in controls: phosphate-buffered saline (PBS) and methylmethane sulfonate (MMS) and in the treatments: infusion of *Mikania glomerata* (IM), macerate of *Mikania glomerata* in 80% ethanol (MM80), infusion of *Bidens pilosa* (IB) and decoction of *Bidens pilosa* (DB), all at different concentrations.

With regard to the quantification of coumarin, HPLC with UV detection proved to be adequate, with sufficient sensitivity and reproducibility for the quality control of *Mikania glomerata* (Celeghini et al., 2001). In this system, infusion (IM) and macerate in 80% ethanol of *Mikania glomerata* (MM80) showed a concentration of coumarin of 27.26 μg/mL and 1500 μg/μL, respectively. These results were used to determine the quantity of coumarin present in each extract concentration tested (Table 3). The chromatograms (Fig. 6) revealed various divergent peaks between the two solutions. The macerate showed a larger number of peaks compared to the infusion. The peak of coumarin was at 9.33 min for IM and 8.68 min for MM80.

Table 3
Final concentration of coumarin present in the test solutions used in the treatments *in vitro* with infusion and macerate in 80% ethanol of *Mikania glomerata*

Treatment (μL/mL)	Final concentration of coumarin (μg/mL)
IM10	0.2726
IM20	0.5452
IM40	1.0904
MM80 2.5	3.78
MM80 5	7.5
MM80 10	15.0
MM80 20	30.0

IM: infusion of *Mikania glomerata*; MM80: macerate in 80% ethanol of *Mikania glomerata*.

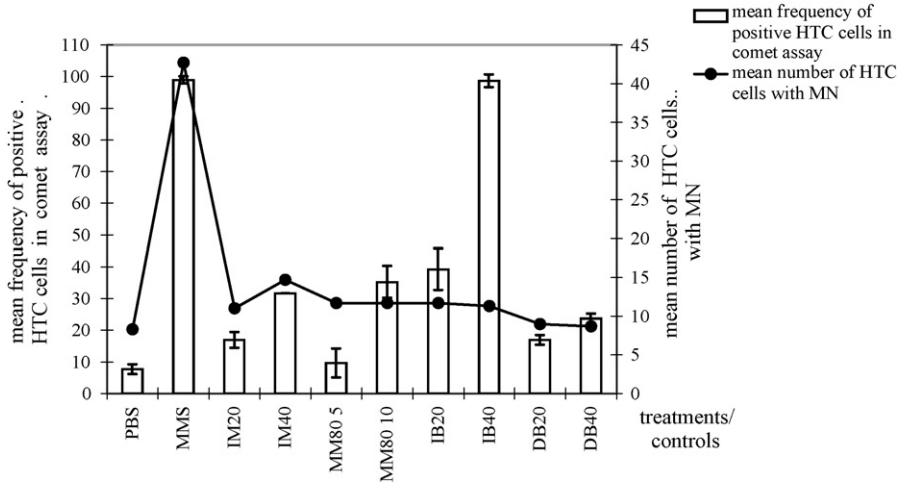

Fig. 5. Comparison among means of results obtained in the comet assay and micronucleus test for the controls: phosphate-buffered saline (PBS) and methylmethane sulfonate (MMS) and for the treatments: infusion of *Mikania glomerata* (IM), macerate of *Mikania glomerata* in 80% ethanol (MM80), infusion of *Bidens pilosa* (IB) and decoction of *Bidens pilosa* (DB), all at different concentrations.

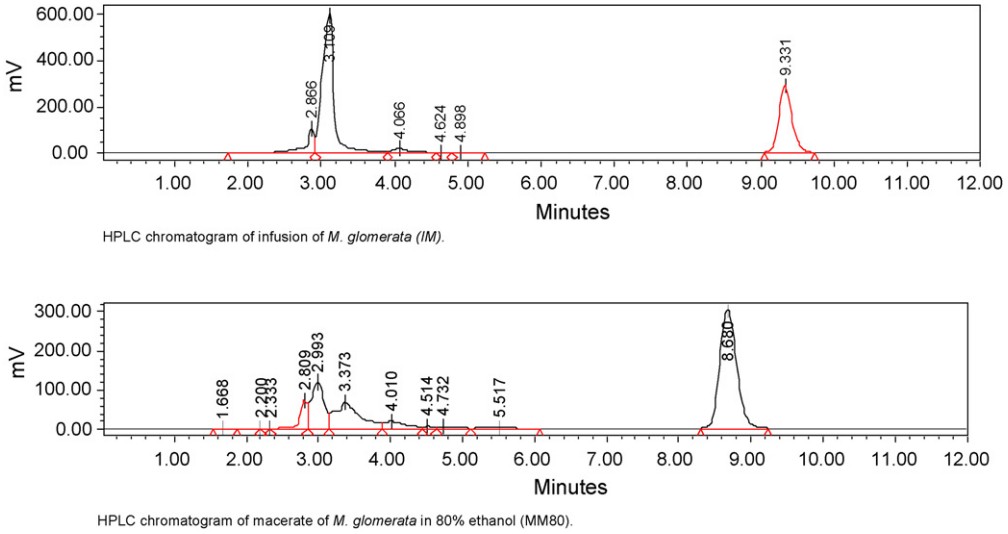

Fig. 6. HPLC chromatograms of infusion of *Mikania glomerata* (IM) and macerate of *Mikania glomerata* in 80% ethanol (MM80).

4. Discussion

The drug-metabolizing capacity of HTC cells employed in the present study was detected by the 2aa effects in the MN test. Also it was demonstrated that the exposure time utilized in each assay (2 h for comet assay and 4 h for the MN test) was adequate for the metabolic action. Therefore, it is possible to believe that, under the conditions used in the present study, the plants "guaco" (*Mikania glomerata*) and "picão-preto" (*Bidens pilosa*) showed genotoxicity.

In the case of *Mikania glomerata*, even though the increase in genotoxicity observed with the use of its infusion was not exactly proportional to the increase in dose, this response was clearly dose-dependent. The macerate in 80% ethanol, as the infusion, showed genotoxicity at the highest doses, which was certainly due only to the substances extracted from the *Mikania glomerata* leaves, since the solvent ethanol did not cause genotoxicity.

The infusion and the macerate of *Mikania glomerata*, at equivalent doses, that is, 20 μL/mL IM and 5 μL/mL MM80, both corresponding to 100% of RDI, produced distinct responses, being the former genotoxic and the latter not. These results suggest that there is a difference in chemical composition between the two forms of extracts studied. In fact, chromatographic analysis demonstrated that MM80 contained about 55 times more coumarin than did the IM. The coumarin peak in the two types of extract differed in elution time by 0.65 min, although this difference is acceptable for this type of analysis, which may have occurred due to temperature changes in the laboratory from one assay to another. Also, the difference in number of peaks in each solution tested provides evidence that the concentration of the component in the two solutions is truly distinct. Nonetheless, despite that chromatogram of the macerate showed a higher concentration of coumarin and a larger number of peaks, and consequently, a greater variety of constituents than the chromatogram of the infusion, IM showed genotoxicity at the dose corresponding to 100% of the RDI (IM 20 μL/mL), while MM80 only showed such effect at 200% of the RDI (MM80 10 μL/mL).

Due to the fact that this study used a drug-metabolizing cell line (HTC), it is plausible that this contradiction observed between dose and effect as well as differences observed between results, were caused not only by components detected in the teas but also by resultant products of cellular metabolism of the chemical substances present in these teas.

An example for this hypothesis is viewed by the results of IM at 10 μL/mL and MM80 at 2.5 and at 5 μL/mL, that were not statistically different, despite that these doses contained distinct levels of coumarin. MM80 at 20 μL/mL, in turn, also showed a level of coumarin 27.5 times greater than that in IM at 40 μL/mL. However, in comparing the respective mean scores, the extent of damage induced was not in this same proportion greater than that caused by IM at 40 μL/mL. Also it was observed that MM80 at 10 μL/mL caused DNA damage similar to that induced by IM at 40 μL/mL, even with coumarin level 13.8 times higher. The same difference of level was found between MM80 5 μL/mL and IM 20 μL/mL, but in this case, the greater genotoxicity was produced by even a lower quantity of coumarin.

On the other hand, in the MN test, the only result that differed statistically from the negative control was IM at 40 μL/mL. In this test, neither MM80 at 10 μL/mL, with 13.8 times more coumarin, nor IM at 20 μL/mL with two times less coumarin than IM at 40 μL/mL, showed significant damage.

Based on these results, it can be inferred that the amount of coumarin in the samples did not influence the extent of damage observed in the comet or micronucleus assays. It is thus believed that other substances could have been responsible for producing these effects. The infusion as well as the macerate in 80% ethanol could contain substances that were not detected by the chromatographic method employed, or that were not UV absorbing, or that absorbed UV light at a wavelength other than that used in the present study.

Despite the earlier observations that coumarins present in the raw leaves of *Mikania glomerata* are potentially protective against cancer (Lin et al., 1996), with reported antitumor activity (Fátima et al., 2005), here it was found that the coumarin present in the tea was not directly responsible for the genotoxic or mutagenic activity demonstrated in the test systems used. There was also no indication that coumarin had any protective property against DNA damage induced by MMS in HTC cells.

With regard to *Bidens pilosa*, the genotoxic effect of the infusion at the three doses tested in the comet assay showed a dose-response relationship, in terms of number of cells damaged as well as total score, which demonstrated a marked increase in the extent of the damage induced. Although the decoction showed genotoxicity, its effect was less potent than that at respective concentrations of the infusion, and there was no evident dose-dependence. The lower genotoxicity of the decoction compared to the infusion, especially at the higher concentrations, is more intriguing still if concentrations of *Bidens pilosa* are converted as "mg plant/mL", two times more elevated in decoction, as given in Section 2 of this paper. This effect suggests a difference in chemical composition between the two forms of teas prepared with this plant. This notion is supported by Simões et al. (2001) who believes that the decoction is a technique that should be reserved for a limited number of medicinal plants, because many active principles in the plant may be altered by prolonged boiling.

Thus, it appears that the higher temperature employed in the preparation of the decoction could have caused destruction or inactivation of some active principle of *Bidens pilosa*, thereby resulting in a diminished damaging effect. On the other hand, the chromatograms of the extracts demonstrated that the concentration of substances extracted of *Mikania glomerata* was much less in IM than in MM80, showing that maceration in 80% ethanol is more efficient process for the extraction of UV-absorbing substances (at the wavelength used here).

Therefore, these findings suggest that the difference in genotoxicity observed with the comet assay, that is, between IM and MM80 or IB and DB, reflects the differential capacity of extraction of the techniques employed.

In the study of *Bidens pilosa* by the micronucleus test, for the solutions tested, there were no significant differences compared to the negative control. Even IB 40 μL/mL, which demonstrated high genotoxicity in the comet assay, did not produce a significant effect in this test. Considering that IM 40 μL/mL was the only treatment that caused positive response in this test, this suggests that the damage caused by the infusion of *Bidens pilosa* differs in nature from that caused by the infusion of *Mikania glomerata*.

Valentin-Severin et al. (2003) pointed out that the difference between the comet assay and MN test consists basically in the type of alteration detected in DNA: the comet assay detects primary lesions which are often reparable, while the MN test detects irreparable lesions. The damages detected by the comet assay, at alkaline pH, are mainly single-strand breaks, in addition to double-strand breaks, alkali-labile lesions and indirect excisions caused by repair enzymes (Singh et al., 1988; Vamvakas and Vock, 1997; Rojas et al., 1999). Thus, the infusion of *Mikania glomerata* could cause damage that is not reversible by the cell's DNA repair system (Simmons and Snustad, 2001), while the damage induced by the infusion of *Bidens pilosa* could be repaired by the cell, which would mean that this damage consists mainly of single-strand DNA breaks.

Phenolic compounds, which could be important in the prevention of various diseases, are known to be present in *Bidens pilosa* (Rose and Kasum, 2002), especially quercetin, a polyphenolic substance with demonstrated antioxidant activity *in vitro* and *in vivo* (Duthie et al., 1997; Wilms et al., 2005) and anticarcinogenic activity (Van Acker et al., 1996). In opposition, the teas of this plant studied here showed genotoxic effects. This observation suggests that the protective effects of quercetin could have been neutralized as a result of the form of extraction applied. Simões et al. (2001) postulated that the conditions of preparation can alter the concentration of active substances, and consequently the effect, efficacy and therapeutic safety of phytotherapeutic products. In addition, it is believed that during the preparation of the extracts tested new substances could have been produced that have damaging effects on genetic material. According to Jagerstad and Skog (2005), the processing of foods and cooking at high temperatures have been shown to generate various types of genotoxic substances.

In folk medicine, *Mikania glomerata* may be used as infusion or as ethanol extract (Teske and Trentini, 1997), although, in this study, the infusion showed lesser genotoxicity than ethanol extract. On the other hand, the ethanol extract showed a level of coumarin greater than that present in the infusion and, in the cases in what the coumarin levels have correlation with the required therapeutical effect, the use of ethanol extract could be recommended, since would necessary the use of smaller quantity of macerate than infusion for the same concentration of this specific component.

The utilization of *Bidens pilosa* in decoction showed less danger to DNA than its utilization in infusion. However, additional studies it would be necessary to verify if the therapeutical effect *in vivo* is the same.

Therefore, the present study demonstrates that although having various therapeutic properties, both *Mikania glomerata* and *Bidens pilosa* are not free of deleterious effects, which calls for precaution in their use as phytotherapeutic substances, with regard to both dose and form of preparation used. Thus, the utilization of doses near to the highest concentrations tested of alcoholic macerate and of infusion of *Mikania glomerata* (10 and 20 μL/mL and 20 and 40 μL/mL, respectively) and of infusion of *Bidens pilosa* (40 μL/mL) must be avoided.

Further studies are recommended to determine the conditions for the use of these plants *in vivo* that would offer the benefits of their therapeutic properties without putting at risk the human health.

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