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Full Length Research Paper

Genotoxic activity and toxicity of *Baccharis trimera* Less. regarding the bioaccumulation of heavy metals

Regildo M. G. Silva*, Vanessa M. Oliveira, Fillipi Valadares, Gustavo F. Mecina and Luciana P. Silva

Universidade Estadual Paulista (UNESP), School of Science and Letters of Assisi, Department of Biological Sciences, Laboratory of Herbal Medicines (FITOLAB), Avenida Dom Antonio 2100, CEP: 19806-900, Assis, São Paulo, Brazil.

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***Baccharis trimera* Less. ("carqueja") is a native plant from Brazil, used in folk medicine preparations such as infusions and/or decoctions. The objective of this study was to evaluate the toxicity and genotoxicity of aqueous and ethanolic extracts of *B. trimera* and quantify heavy metals bioaccumulated in this specie. The extracts were prepared with ultra pure water. The *Allium cepa* test was conducted to examine the genotoxic activity of the extracts. The toxicological activity was used as bioassay of *Artemia salina*. Heavy metals were analyzed by atomic absorption spectrophotometery. The ethanolic extract showed the greatest effect on the mitotic index and chromosomal aberrations when treated with aqueous extract. The extracts showed activity in the toxicological concentrations. Heavy metals analyzed (Cd = 0.013, Cu = 0.089, Pb = 0.097 and Zn = 1.918 µg/ml) were measured at levels above those recommended. Therefore, *B. trimera* is a heavy metal bioacumulator specie, being able to promote genotoxicity activity in meristematic cells of *A. cepa* and toxicological activity on larvae of *A. salina*.**

Key words: *Allium cepa*, *Artemia salina*, atomic absorption, chromosome aberration, genotoxicity, micronucleus.

INTRODUCTION

The *Baccharis* gender (Asteraceae) is represented by more than 500 species distributed mostly in tropic areas of South America. Many of this species are widely utilized in popular medicine in the treatment and prevention of diseases (Torres et al., 2000; Verdi et al., 2005; Borella et al., 2006; Fukuda et al., 2006; Pádua et al., 2010). Fitochemical study about the species belonging to the *Baccharis* gender demonstrated the presence of flavonoids (flavone and flavonols), diterpenes (labdane and clerodane) and triterpenes (Verdi et al., 2005; Biondo et al., 2011).

Baccharis trimera Less. is popularly known as "carqueja". Preparations like infusions and/or decocts of this plant are used in popular medicine to treat liver diseases, rheumatism, diabetes, just as digestive system diseases, hepatic and renal (Januário et al., 2004), especially by the low socioeconomic status population (Grance et al., 2008). Although investigations with *B. trimera* have demonstrated its efficiency, there is little available study about its toxic effects. Recently, histopathological alterations were found in the liver of pregnant rats treated with a hydroethanolic extract of *B. trimera* (Grance et al., 2008). Furthermore, this study demonstrates genotoxic and mutagenic effects over liver cells of mice, but in the same experiment a reduction of genotoxicity induced by H_2O_2 was found because of its antioxidant properties (Rodrigues et al., 2009; Nogueira et al., 2011).

However, this specie has demonstrated metal accumulation capacity, as Mn and Zn, which are found in higher concentration in the leaves, suggesting a higher attention on the consumption of these plants, even when utilized in infusions (Souza et al., 2007). Heavy metals are harmful because of their non-biodegradable nature, their long biological half-life and are easily accumulated

*Corresponding author. E-mail: regildos@yahoo.com.br. Tel: +55 18 33025848. Fax: +55 18 33025802.

in plants (Silva et al., 2007; Arora et al., 2008). The toxicity produced by metals usually involves neurotoxicity, nephrotoxicity and hepatotoxicity (Benavides et al., 2005). Heavy metals are potential mutagenics (Divan et al., 2009).

Considering the lack of studies over the toxic effects of this plant correlated with capacity of heavy metal accumulation, the objective of this study was to evaluate the genotoxicity and toxicity of aqueous and ethanolic extracts of *B. trimera*, by mitotic index assay and chromosomal aberrations in meristematic roots of *Allium cepa* Linn (*A. cepa* assay), evaluation of lethal concentration in cultures of *Artemia salina* Leach (*A. salina* assay) and heavy metals quantification by spectrophotometer analysis of atomic absorption.

MATERIALS AND METHODS

Collection and preparation of plant

The leaves of *B. trimera* were collected from specimens found in gantry located in the Park Recycling and Solid Waste Composting "José Santilli Sobrinho" (22° 40' 58.60" S, 50° 24' 51.00" W, altitude 560 m) in the city of Assis (São Paulo - Brazil). The leaves were brought to the Laboratory of Herbal Medicines Universidade Estadual Paulista (UNESP), College of Assis, São Paulo, Brazil, selected, dried in a forced air oven for 24 h at an average temperature of 40°C and pulverized in a grinder of knives.

Preparation of extracts

Aqueous extract (BtA)

The dust from the leaves of *B. trimera* was heavy to carry decoction of different concentrations (0.1, 5 and 10 mg/ml), after it was placed in ultra pure water at a temperature of 97 ± 2°C. The decoction was left to stand until it reached room temperature, after that filtering was done through a funnel lined with filter paper. The resulting decoction was stored in dark bottles and subsequently used in the tests.

Ethanolic extract (BtE)

The powdered leaves were extracted in a shaker, using 95% ethanol at a ratio of 1:10 (w/v) at room temperature for 24 h. After this period, the extract was filtered under low pressure in filter paper. The extraction was repeated three times, the extracts were gathered and dried at 60°C using a rotavapor and the yield was about 8%. The dry extract was stored in dark glass for the experiments, a procedure performed according to Almeida et al. (1998).

Allium cepa test

Onion bulbs (*A. cepa* L., 2n = 16) were obtained commercially in Assis, São Paulo, Brazil. They were cleaned and dried outer scales were removed, leaving the ring intact with primordial roots. The bulbs were used for the bioassay according to standard procedures (Rank and Nielsen, 1993; Chauhan et al., 1999; Babatunde and Bakare, 2006). The growth of roots was used a culture solution (Hoagland's solution). The bulbs were kept suspended in a 100 ml beaker leaving the ring of roots in contact with the solution, changed every 24 h for a period of 72 h, maintained at a photoperiod (18 h/6 h light/dark) and temperature (22 ± 2°C) in controlled chamber B.O.D. Bulbs with roots approximately 2 cm were used in the experiment.

To evaluate the mitotic index and induction of chromosomal aberrations (aberrant anaphase and telophase), six onion bulbs were exposed to each concentration of aqueous extract (0.1, 5 and 10 mg/ml) and ethanolic extract (1, 10, 50 and 100 µg/ml) of *B. trimera*. Mineral water and positive control solution of MMS (methyl methanesulfonate, Sigma-Aldrich ®) at 10 mg/L was used for the negative control, as described by Caritá and Marin-Morales (2008).

At the end of 48 h exposure and 24 h recovery in culture solution, the roots of treated and control bulbs were cut and fixed in ethanol: glacial acetic acid (3:1, v/v). These were hydrolyzed in 1 N HCl at 60°C for 8 min, after which they were rinsed in distilled water. The roots were stained with acetic carmine for 10 min, the tips were removed and the roots carefully crushed between slide and coverslip were sealed, as suggested by Grant (1982) and Akinboro and Bakare (2007). Five slides were prepared for each treatment and controls were analyzed at 1000× magnification. The mitotic index was calculated on the number of dividing cells per 1000 cells observed (Fiskesjö, 1985; Fiskesjö et al., 1997). The frequency was calculated based on the number of aberrant cells (telophases and anaphases) per total cells analyzed for each treatment and controls (Bakare et al., 2000).

Toxicity test in *Artemia salina*

Leach brine shrimp eggs were bought at a Pet Shop Assis (São Paulo, Brazil) and incubated in artificial seawater prepared from a saline solution NaCl (34.2%); MgH_2SO_4 (1.425%), $NaHCO_3$ (4.75%), distilled water and NaOH (solution to adjust pH to 9.0) with a temperature of 28 ± 2°C and light controlled artificially according to the methodology described by Meyer et al. (1982) and Nunes et al. (2008), with some modifications. After 48 h, nauplii were collected and distributed, 10 individuals in each culture plate were administered 0.1, 5 and 10 g/ml aqueous extract and 1, 10, 50 and 100 mg/ml ethanol extract of *B. trimera.* Number of dead at 24 and 48 h of exposure was counted, this number was used to calculate the LC_{50} using PROBIT analysis, with 95% confidence according to Sam et al. (1993), Pagliara and Caroppo (2011) and Nascimento et al. (2008). The tests were performed in triplicate according Mongelli et al. (1995), Moreira et al. (2003) and Luna et al. (2005).

Quantification of heavy metals in *B. trimera*

The ethanol extract was diluted in 100 ml of ultra pure water (30 g/L). For the release of metals, 10 ml mixture of nitric acid (HNO_3) and perchloric acid ($HClO_4$) was added to the solution of the plant in proportion of 6:1. The solutions were heated to obtain a white precipitate, which was separated and dissolved in a flask with 10 ml HNO_3 (0.1 N) according to methodology described by Barthwal et al. (2008). The reading of the heavy metals present in the extracts was performed in triplicate and obtained by means of atomic absorption spectrophotometer (Perkin, 3300). The present study examined the following metals: cadmium, copper, lead and zinc. The gases were used for reading the compressed air and acetylene flame analysis with hollow cathode lamps, a procedure performed according to Santos et al. (2006) and Barthwal et al. (2008).

Statistical analysis

The mitotic index and frequencies of chromosomal aberrations obtained for each treatment during the period between exposure

Table 1. Mitotic Index and Chromosomal for the aqueous extract of *Baccharis trimera* (BtA) at concentrations 0.1, 5 and 10 g/ml. Negative control (NC) treated with mineral water and a positive control (PC) treated with methyl methanesulfonate (MMS) to 10 mg/L.

Treatment	Mitotic index%	Aberrations		Total aberrant cells%	Average aberrant cells%
		Anaphase	Telophase		
NC	13.92 ± 1.90[a]	19	19	38	0.76 ± 0.19[a]
BtA (µg/ml)					
0.1	10.78 ± 0.99[b]	109	40	149	2.98 ± 0.23[b]
5	12.66 ± 2.96[a]	140	69	209	4.18 ± 0.82[c]
10	11.14 ± 2.25[b]	90	46	136	2.72 ± 0.86[b]
PC	06.14 ± 0.48[c]	62	43	105	2.10 ± 0.12[b]

5000 cells analyzed. Mean ± standard deviation. Same letters in columns do not differ statistically average assessed using the Kruskal-Wallis ($p < 0.05$).

Table 2. Mitotic Index and Chromosomal for ethanolic extract of *Baccharis trimera* (BtE) in concentrations 1.0, 10, 50 and 100 µg/ml. Negative control (NC) treated with mineral water and a positive control (PC) treated with methyl methane sulfonate (MMS) to 10 mg/L.

Treatment	Mitotic index%	Aberrations		Total aberrant cells%	Average aberrant cells%
		Anaphase	Telophase		
NC	13.92 ± 1.90[a]	19	19	39	0.76 ± 0.19[a]
BtE (µg/ml)					
1	13.90 ± 3.96[a]	162	55	217	4.34 ± 1.12[b]
10	17.82 ± 2.08[b]	181	54	235	4.70 ± 3.14[b]
50	18.54 ± 0.76[b]	230	72	302	6.06 ± 1.06[c]
100	24.02 ± 3.15[c]	130	114	244	4.88 ± 2.27[b]
PC	6.14 ± 0.48[d]	62	43	105	2.10 ± 0.12[d]

5000 cells analyzed. Mean ± standard deviation. Same letters in columns do not differ statistically average assessed using the Kruskal-Wallis ($p < 0.05$).

and the samples were compared with controls and statistically analyzed using the Kruskal-Wallis ($p < 0.05$), as described by Grisolia et al. (2005) and Leme and Marin-Morales (2008).

To test *A. saline* to obtain the LC_{50} values, PROBIT analysis was used through the software StatPlus® 2009, with 95% confidence. For comparison of LC_{50} values between the different concentrations of ethanolic and aqueous extracts, confidence intervals obtained by PROBIT analysis were used.

RESULTS

Allium cepa test

Table 1 shows the results of the effect of aqueous extract of *B. trimera* (BtA) on the mitotic index and the frequency of chromosomal aberrations (aberrant anaphase and telophase) in root cells of *A. cepa*, where the mitotic index in the group treated with 5 µg/ml was not significantly different when compared with the negative control (NC) while the treatments with 0.1 and 10 g/ml did not differ, but showed significant difference compared with NC. The experimental groups treated with BtA showed significant difference when compared with the positive control (PC) and had the lowest mitotic index in the analysis of total cells. As for the results of chromosomal aberrations promoted by treatment with BtA, the concentrations used showed a significant difference when compared with NC, but the concentrations of 0.1 and 10 g/ml did not differ significantly among themselves and in comparison to PC, but significant differences in comparison treatment with 5 µg/ml.

Table 2 presents the results of mitotic index and chromosomal aberrations in the groups treated with ethanol extract of *B. trimera* (BtE). As for the mitotic index, the concentrations of 10 and 50 µg/ml did not differ significantly, but differed in comparison with those treated with 1 and 100 µg/ml. The group treated with 1 µg/ml showed no significant difference compared with NC. However, all results obtained with the treated groups showed significant difference when compared with PC; these results correspond to a dose-effect of each concentration of BtE on the mitotic index of each treatment. The results showed that chromosomal aberrations

Table 3. Test for toxicity to *Artemia saline* of aqueous (BtA) and ethanolic (BtE) of *Baccharis trimera* (death rate per observation time, LC_{50} and confidence interval of 95%).

Treatment	Concentration (µg/ml)	Mortality (%/h)		Values LC_{50}[a] (µg/ml)	Confidence interval 95% (µg/ml)[b]
		24	48		
BtA	0.1	03	10	53.14	51.23 - 136.5
	5	00	17		
	10	33	50		
BtE	1	30	100	27.98	16.34 - 98.76
	10	46	97		
	50	100	100		
	100	83	100		

[a]LC_{50} calculated after 48 h of exposure; [B]Sam et al. (1993) and Nascimento et al. (2008).

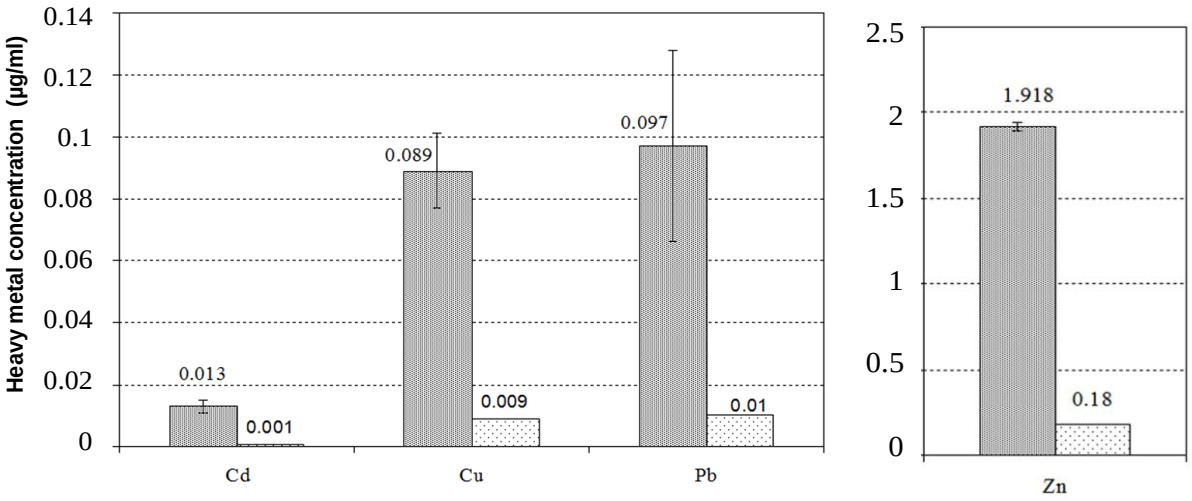

Figure 1. Concentration of heavy metals (Cadmium, Copper, Lead and Zinc) found in the solution of ethanolic extract of leaves of *Baccharis trimera* and maximum permitted under the Regulatory Determination CONAMA (2005) and recommended by WHO (1998).

of the treated groups showed significant difference when compared with NC and PC. However the concentrations of 1, 10 and 100 µg/ml not differ significantly, but differed when compared with 50 µg/ml.

Toxicity test in *Artemia salina*

The percentage mortality after 24 and 48 h of observation for the different concentrations of aqueous and ethanolic extracts of leaves of *B. trimera* and their LC_{50} are presented in Table 3. For both extracts evaluated, it was observed that mortality is proportional to the concentration increase, which corresponds to linearity in the dose-effect of each extract. However, concentrations of 0.1 and 5 µg/ml aqueous extract showed no activity on the larvae of *A. saline* according to LC_{50} presented for this statement.

Quantification of heavy metals in *B. trimera*

Figure 1 shows the accumulation of Cd, Cu, Pb and Zn in the solution of ethanol extract of leaf *B. trimera*. All metals analyzed showed a concentration (mg/ml) higher than the maximum allowed in accordance with the recommendation of WHO (1998) and Normative deliberation CONAMA (2005). Among the metals assessed, values found for Cu (0.089 µg/ml) and Pb (0.097 µg/ml) exceeded the allowable limit (Cu and Pb = 0009 = 0.01 µg/ml) about 100 times. The values of Cd (0.013 µg/ml) and Zn (1.918 µg/ml) exceeded the allowed limits

(Cd and Zn 0.18 µg/ml = 0.001) at about 10 times.

DISCUSSION

Genotoxic studies with medicinal plants are being conducted to assist in the evaluation of health risks due to ingestion of herbal teas and other forms of preparation (Akinboro and Bakare, 2007; Verschaevea and Van Staden, 2008; Akintonwa et al., 2009). This study evaluated the genotoxic activity and toxicity of aqueous and ethanolic extracts of *B. trimera* in relation to the accumulation of heavy metals in this specie.

The results of genotoxicity after bioassay in root meristematic cells of *A. cepa* demonstrated that both treatments (aqueous and ethanolic) altered the mitotic index and chromosomal aberrations in *B. trimera*, results, correspond to those found by Rodrigues et al. (2009) and Nogueira et al. (2011), which showed genotoxicity and mutagenicity effects caused by this plant extract on mouse cells, which were assessed by Comet and Micronucleus Tests and studies by Pinho et al. (2010), where it was demonstrated abnormalities in mitotic cells of *A. cepa* and in lymphocyte culture.

Comparatively, treatment with aqueous extract showed no effect on the mitotic index and chromosomal aberrations than treatment with ethanolic extract (Tables 1 and 2). This may be due in part to the extraction process because, although the aqueous extract was obtained by boiling water, extraction performed through the use of solvent (ethanol) results in greater achievement of the components present in the plant, resulting in concomitant a greater genotoxic effect (Verpoorte, 1989; Reid et al., 2006; Akinboro and Bakare, 2007; Maruška et al., 2010).

However, the changes found in the treatments (aqueous and ethanol extracts) can be directly correlated with the accumulation of heavy metals quantified in the leaves of *B. trimera* (Figure 1). Studies by Kumar et al. (1995) demonstrated that plants are a source of bioaccumulation of heavy metals. This property has been used for phytoremediation (Arora et al., 2008; Haque et al., 2008; Divan et al., 2009); on the other hand, it can be dangerous when plants are consumed as food or therapeutic agent in traditional medicine (Ernst, 2002; Barthwal et al., 2008; Zheljazkov et al., 2008). Studies by Souza et al. (2007) and Haque et al. (2008) demonstrated the ability of some species of *Baccharis* in accumulating different types of heavy metals when grown in soils from the mining area.

Chromosomal aberrations in the present study demonstrated increased frequency for both anaphase and telophase in both treatments (Tables 1 and 2). According to Rank and Nielsen (1993) and Leme and Marin-Morales (2008, 2009), in evaluation of the aberrant anaphases and telophases, *A. cepa* test is sensitive and suitable for analysis of chromosomal disorders promoted by excess of heavy metals. As to the genotoxic action promoted by heavy metals in plants, bio-accumulators are the most common elements, lead and cadmium (Glinska et al., 2007; Barthwal et al., 2008, Seth et al., 2008), but research has shown that copper and zinc can also participate and promote significant genotoxic effects (Arambaic et al., 1995; Kovalchuk et al., 1998; Youn-Joo, 2006; Glinska et al., 2007).

Results obtained on the genotoxicity of extracts from *B. trimera* evaluated the toxic activity of the same through the test of *A. salina*, where it was observed that the extracts evaluated showed activity at the concentrations used (Table 3). According to Nunes et al. (2006), the toxicity test with *A. salina* is characterized by sensitivity and activity in the chemicals within these heavy metals.

Toxicological evaluation of ethanol extract showed that all concentrations tested showed activity against *A. salina*, whereas the LC_{50} calculated for this statement, and this activity was dose dependent (Table 3). This result can be directly related to the genotoxic activity observed by test *A. salina* (Table 2) and quantification of heavy metals found in the leaves of *B. trimera* (Figure 1), because studies by Taylor et al. (2005) and Ferraz et al. (2009) demonstrate the relationship between the concentration of heavy metals and toxicity in *A. salina*, while other studies relate to the genotoxicity of this toxicity test organism (Isidori et al., 2005; Bortolotto et al., 2009).

The presence of elevated levels of heavy metals in leaves of *B. trimera* found in this study was possibly due to two factors, primarily soil and water used for cultivation and maintenance, and the ability of this specie to accumulate metals (Souza et al., 2007). As the place of cultivation, because it is a park for recycling and composting, studies have shown that these areas are prone to introduction of soil and water with high levels of waste, including heavy metals (Townsend et al., 2004; Jun-huia and Hang, 2009; Tang et al., 2010).

Conclusion

The present study showed that *B. trimera* is capable of bioaccumulating heavy metals, which can promote genotoxic activity in meristematic cells of *A. cepa* and toxicologic activity on larvae of *A. salina*, making it necessary to be cautious on the use of this species in traditional medicine, and attentive when considering the location and form of cultivation.

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cultivation and maintenance of plant species evaluated.

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