Evaluation of the genotoxic potential of ethanolic extracts of stem bark and leaves of *Bathysa cuspidata* (A.St.-Hil.) Hook

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ABSTRACT

***Bathysa cuspidata* (A.St.-Hil.) Hook. is a native tree of the Brazilian Atlantic Forest biome, widely used in Brazilian herbal medicine. Despite its widespread use, there is no report as yet regarding the toxicology of this plant. In this study, the mutagenic and genotoxic effects of ethanolic extracts of *B. cuspidata* stem bark (SBC) and leaves (LBC) were assessed. The mutagenicity of the extracts was estimated by performing the Ames test on strains TA98 and TA100 of *Salmonella typhimurium*, in the absence and presence of metabolic activation. The direct action of the extracts on DNA was assessed through plasmid treatment. Phytochemical screening was conducted to compare the secondary metabolite composition of the extracts. No mutagenic activity was found in LBC when tested on strain TA98 or TA100, without or with metabolic activation. However, SBC did show mutagenic activity. Plasmid tests did not indicate genotoxic action for either SBC or LBC. Differences in the composition of secondary metabolites present in the bark and leaves, detected by phytochemical analysis, appear to be a deciding factor in differences in mutagenicity between SBC and LBC. The findings in this study suggest caution in the use of *B. cuspidata* bark.**

Keywords: *Bathysa cuspidata*. Genotoxicity. Mutagenesis. Ames test.

INTRODUCTION

In Brazil, a variety of native trees in the Atlantic Forest are claimed, in the local culture, to possess medicinal properties and are thus utilized especially in home remedies (Di Stasi et al., 2002). While many plants are widely used in popular medicine, a great number of them have not been studied for their toxicological potential. Research shows that some of the active substances present in medicinal plants, in addition to their pharmacological properties, may be potentially toxic, leading even to DNA alterations (Varanda, 2006; Akintonwa et al., 2009; Rodrigues et al., 2009). In the human body, mutagenic processes are involved in several degenerative diseases, such as cancer and arteriosclerosis (Santos et al., 2008).

One of the plants native to the Atlantic Forest that are widely used in herbal medicine, but have not been studied in terms of their phytochemical composition or genotoxic and mutagenic potential, is the tree *Bathysa cuspidata* (A.St.-Hil.) Hook., known commonly in Brazil as *quina-do-mato* (forest quinine) or *quina-cinzenta*. Its bark is used to prepare bitter tonics to treat anemia, cachexia, malaria and ancylostomiasis (Corrêa, 1984; Botsaris, 2007) and as an anti-inflammatory and pain killer (Vanderlinde et al., 2001). Belonging to the family Rubiaceae, which includes some 637 genera and around 10,700 species (Robbrecht, 1988), the genus *Bathysa* encompasses about 15 species distributed across several countries on the American continent. Of these, 7 species have been described as occurring in Brazil, being restricted almost exclusively to the Atlantic Forest biome (Barroso et al., 1986).

With a view to contributing to the assessment of potential health hazards arising from the consumption of remedies prepared from the stem bark and leaves of *B. cuspidata*, the phytochemical composition and mutagenic potential of ethanolic extracts of these plant parts were assessed in this study, as recommended for preclinical trials of plant medicines by the Brazilian Sanitary Surveillance Agency (Anvisa, 2004).

MATERIAL AND METHODS

Plant material

Samples of plant material of the species *B. cuspidata* were collected in the Atlantic Forest biome, in the village

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of Araponga, state of Minas Gerais, Brazil, (20°43' 00.0''S by 42°29'10.8''W, elevation 1,200 m above sea level). A voucher specimen was deposited in the herbarium of the Federal University of Viçosa (VIC 21.559). The stem bark and the leaves were separated and dried in a ventilated dark room at ambient temperature and were subsequently pulverized in a knife mill.

Extract preparation

The extracts of the stem bark (SBC) and leaves (LBC) were first prepared by maceration, in which each 100 g of the powdered plant material was left in contact with 500 mL of 95% ethyl alcohol for seven days and stirred twice daily. After this period, all the material was vacuum filtered on filter paper and the resulting extracts underwent drying in a rotary evaporator until the solvent had totally evaporated. Yields of SBC and LBC were 20.6 wt% and 13.8 wt%, respectively.

Phytochemical analysis

SBC and LBC phytochemical screening was conducted by chromatography on a thin layer of silica gel. The tests were aimed at detecting the presence of groups of secondary metabolites, namely: coumarins, anthraderivatives, triterpenes/steroids, saponins, flavonoids, cardiotonic heterosides, tannins and alkaloids, by methods described by Wagner & Bland (1996).

Ames mutagenicity test

The test used to detect mutagenicity was the Ames test, with a 30-min preincubation with strains TA98 and TA100 of *Salmonella typhimurium*, with and without metabolic activation (Maron & Ames, 1983). These strains were kindly provided by Dr. Eliana Varanda, of São Paulo State University at Araraquara, Brazil. Five different doses (0.51, 1.03, 2.06, 3.09 and 4.12 mg/plate) of SBC and LBC, previously suspended in dimethyl sulfoxide (DMSO), were assessed. The concentrations employed were based on a cytotoxicity test against the bacterium *S. typhimurium* (data not shown). To each 100 µL of the test mixture was added 500 µL of 0.2M phosphate buffer, pH 7.4 (without metabolic activation) or 500 µL of S9 mix (with metabolic activation) and 100 µL of bacterial culture (4×10^8 cells/mL) and this mixture was incubated at 37 °C for 20-30 min. The lyophilized rat liver S9 fraction induced by Aroclor 1254 was purchased from Moltox (Molecular Toxicology, Annapolis, USA). The S9 mix (50 mL) was prepared as follows: 19.75 mL of distilled water, 25 mL of 0.2 M phosphate buffer pH 7.4, 2 mL of 0.1 M NADP, 0.25 mL of 1 M D-glucose-6-phosphate, 1 mL of 1.65 M KCl + 0.4 M $MgCl_2.6H_2O$ salt solution and 2 mL of the lyophilized S9 fraction that had been reconstituted with distilled water. After this period, 2 mL of top agar was added and the mixture plated on glucose minimal medium. The plates were incubated at 37 °C for 48 hours and *His+* revertants were counted. The tests were conducted in triplicate. The standard mutagens used as positive controls in the test without metabolic activation were 4-nitro-O-phenylenediamine for TA98 and sodium azide for TA100 and, with metabolic activation, 2-aminoanthracene for both strains. DMSO was used as a negative control. Analysis of Variance (ANOVA, F-test) was used to assess the differences between the average numbers of revertants at the various doses tested and the negative control, followed by a linear regression for the dose response. The mutagenic index (M.I.), which is the average number of revertants on the test plate divided by the average number of revertants per negative control plate, was calculated for each dose analyzed. The result was considered positive when the M.I. was higher than or equal to 2 for at least two of the tested doses. Extracts which, at some concentrations, induced a significant difference in revertant frequency from the negative control and M.I. higher than 1.5 but lower than 2 were considered to be weak mutagens (Santos et al., 2010).

Plasmid treatment

In order to assess the genotoxic potential of the plant extracts, the plasmid treatment test was performed, following the method described by De Mattos et al. (2000). SBC and LBC, in doses of 0.51, 1.03, 2.06, 3.09 and 4.12 mg (same as those in the mutagenicity tests) were incubated with 42 µg of pUC18 plasmid DNA, in 20 µL of buffer for 2.5 h at 37 °C. Later, the volume of each sample was brought up to 100 µL with Tris-EDTA (TE) and the DNA was precipitated with 100% ethanol and 3M sodium acetate (pH 5.3). The precipitated plasmid DNA was resuspended in 15 µL of ultra-pure water. This aliquot of 15µL DNA was loaded on to 0.8% agarose gel; after electrophoresis, the gel was colored with ethidium bromide (0.1 µg/mL) to allow visualization of DNA bands. As a positive control, the plasmid DNA was incubated with 0.5 mg of stannous chloride ($SnCl_2$), while DNA treated with distilled water and DNA treated with DMSO were used as negative controls. The experiment was repeated at least three times, with the same results. The result of the plasmid DNA test was assessed by noting the presence or absence of the relaxed-circular conformation (form III), helical conformation (form II) and strained superhelical conformation (form I). A comparison between the test DNA bands and the positive and negative control DNA bands was used to diagnose any possible genotoxic action of the extracts.

RESULTS

Chemical authentication of plant extracts constitutes a key stage in quality control procedures for such products, as well as the basis for producing, developing and using herbal medicines safely. Thin-layer chromatography (TLC) is currently the most widely-used method for the authentication of plant drugs, allowing the identification and comparison of several samples at the same time (Tistaert et al., 2011). In this study, TLC was used to perform a qualitative comparison of extracts from the bark and leaves of the same species. The results of the phytochemical screening performed on the ethanolic extracts of the bark and leaves of *B. cuspidata* are presented in Table 1.

From the results obtained, a difference can be seen between the chemical composition of groups of

secondary metabolites in the stem bark and leaf extracts of *B. cuspidata*. In addition to the presence of triterpenes/steroids, flavonoids and tannins in both extracts, the bark also contained coumarins and alkaloids and it is therefore not advisable to use this part of the plant in therapeutic preparations.

The extracts were first tested for toxicity against *S. typhimurium*, to determine the range of appropriate doses for the mutagenic test. For the *in vitro* mutagenic assessment of the species *B. cuspidata*, five different SBC and LBC concentrations were tested on two strains of *S. typhimurium*, TA98 and TA100, both without and with metabolic activation (Table 2).

From the mutagenic index (M.I.), SBC and LBC showed no mutagenicity for the strains in the absence of metabolizing factor. However, SBC at 3.09 mg/plate resulted in the M.I. 1.8 for strain TA100 without S9 activation, which constitutes a sign of mutagenicity.

On the other hand, after undergoing metabolization with S9 mix, SBC showed mutagenic index values close to 2 for strain TA98, when tested in doses of 0.51 mg (M.I.

1.74) and 1.03 mg (M.I. 1.98), and a statistical difference (by the F-test) at the highest dose, 4.12 mg. This result suggests traces of mutagenicity in SBC. A significant increase in the frequency of reversion in strain TA100 (M.I. 2.07) was also seen for SBC after metabolization by S9, when tested at the dose 0.51 mg. These results indicate some mutagenic action for SBC, according to the Ames test.

Genotoxic assessment by plasmid treatment makes use of the topological changes seen in DNA in contact with genotoxic agents, such as stannous chloride ($SnCl_2$) (Dantas et al., 1999). Thus, it can be seen in the band patterns in Figure 1 that neither SBC nor LBC showed any genotoxic action, since treatments performed with increasing extract concentrations (lanes 4 to 8 for SBC and lanes 9 to 13 for LBC) did not change the DNA conformation, having a similar profile to the negative controls (lanes 1 and 2). By contrast, a known genotoxic agent, stannous chloride, used as a positive control, induced changes in forms I, II and III of plasmid DNA, which no longer had well-defined conformations (lane 3).

Table 1. Results of phytochemical screening by TLC of SBC and LBC.

Class of secondary metabolites	SBC	LBC
Coumarins	+	-
Anthraderivatives	-	-
Triterpenes and steroids	+	+
Saponins	-	-
Flavonoids	+	+
Cardiotonic heterosides	-	-
Tannins	+	+
Alkaloids	+	-

+ : positive; - : negative.

Table 2. Mutagenic activity expressed as the average number of revertants, standard deviation and mutagenic index (in brackets) in strains TA98 and TA100 of *S. typhimurium*, without (-S9) and with (+S9) metabolic activation, exposed to various concentrations of SBC and LBC.

Sample (mg/plate)	TA98				TA100			
	SBC		LBC		SBC		LBC	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0.00a	28.0 ± 3.4	15.3 ± 2.6	28.0 ± 3.4	15.3 ± 2.6	125.3 ± 6.0	77.0 ± 9.2	125.3 ± 6.0	77.0 ± 9.2
0.51	28.0 ± 3.4 (1.10)	26.7 ± 8.3 (1.74)	38.6 ± 9.5 (1.40)	23.7 ± 8.0 (1.54)	162.0 ± 17.3 (1.30)	159.3 ± 31.6 (2.07)	153.3 ± 9.8 (1.20)	122.3 ± 31.9 (1.59)
1.03	44.0 ± 1.7 (1.60)	30.3 ± 3.7 (1.98)	44.0 ± 7.8 (1.60)	24.3 ± 1.7 (1.59)	202.0 ± 7.9 (1.60)	75.0 ± 22.5 (0.97)	162.0 ± 6.0 (1.30)	116.0 ± 27.6 (1.51)
2.06	38.6 ± 9.0 (1.20)	17.7 ± 4.9 (1.15)	31.0 ± 5.2 (1.10)	22.7 ± 2.9 (1.48)	180.3 ± 4.0 (1.40)	44.3 ± 33.0 (0.57)	196.0 ± 1.7 (1.50)	105.3 ± 28.5 (1.37)
3.09	44.0 ± 7.8 (1.00)	7.7 ± 5.8 (0.50)	39.0 ± 5.5 (1.40)	14.7 ± 3.1 (0.96)	229.6 ± 12.7 (1.80)	124.3 ± 10.5 (1.61)	193.6 ± 15.8 (1.50)	60.3 ± 27.1 (0.78)
4.12	31.0 ± 5.2 (1.20)	11.0 ± 11.6 (0.72)*	36.0 ± 8.5 (1.30)	19.7 ± 6.2 (1.28)	181.3 ± 17.3 (1.40)	88.3 ± 15.1 (1.15)	183.0 ± 3.6 (1.40)	-
Control +	161.3 ± 52.1c*	2018.7 ± 181.3b*	161.3 ± 52.1c*	2018.7 ± 181.3b*	1296.0 ± 62.4d*	1440.0 ± 169.7b*	1296.0 ± 62.4d*	1440.0 ± 169.7b*

Key: a: DMSO (100 µL/placa) (negative control). Control + (positive control): b: 2-aminoanthracene (5 µg/plate); c: NPD (4-nitro-O-phenylenediamine) (2.5 µg/plate); d: Sodium azide (1.25 µg/plate); -: non-tested dose. *Significant compared to negative control by ANOVA (p<0.05).
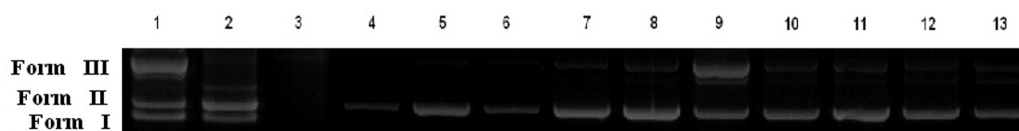
Figure 1. Genotoxic action of SBC and LBC. Lanes: 1: negative control (42 μg DNA + 100 μL H2O); 2: negative control (42 μg DNA + 100 μL DMSO); 3: positive control (42 μg DNA + 0.5 mg SnCl2); 4: (42 μg DNA + 0.51 mg of SBC); 5: (42 μg DNA + 1.03 mg of SBC); 6: (42 μg DNA + 2.06 mg of SBC); 7: (42 μg DNA + 3.09 mg of SBC); 8: (42 μg DNA + 4.12 mg of SBC); 9: (42 μg DNA + 0.51 mg of LBC); 10: (42 μg DNA + 1.03 mg of LBC); 11: (42 μg DNA + 2.06 mg of LBC); 12: (42 μg DNA + 3.09 mg of LBC); 13: (42 μg DNA + 4.12 mg of LBC). The figure depicts an electrophoretic gel representative of the three experiments.

DISCUSSION

Although similarities in the chemical composition of the leaf and bark of *B. cuspidata* were observed for some groups of secondary metabolites, it is clear that the phytochemical analysis also revealed qualitative differences.

As for mutagenicity, it was noted that both the traces of mutagenicity and the significant differences in the rate of reversion in one of the strains employed in the study were found only for SBC which, unlike LBC, showed the presence of coumarins and alkaloids. These compounds could be the reason for the difference in mutagenic action between the extracts from the leaves and the bark. There are published reports of mutagenicity for groups of secondary metabolites found in SBC, such as alkaloids and coumarins (Goeger et al., 1999; Ansah et al., 2005). Additional studies should be performed to investigate the possible toxic compounds. It was also found that SBC has traces of mutagenicity both for strain TA98, which indicates DNA base frameshifts, and strain TA100, which detects DNA base-pair substitutions, showing that SBC induces mutation in different ways and corroborating the mutagenic data obtained (Maron & Ames, 1983). No genotoxic action was observed for the tested extracts within the concentration ranges employed. Genotoxicity is caused by substances that act directly on DNA (Felzenszwalb et al., 1998; Reiniger et al., 1999), leading to strand breaks or reducing the intramolecular interactions of the DNA. The negative result for genotoxicity indicates that, at the tested concentrations, neither SBC nor LBC act as genotoxic agents of plasmid DNA. However, given the chemical composition of the extracts, this does not mean that at higher concentrations they will not prove genotoxic, since among the compounds detected, such as flavonoids and alkaloids, some can act as pro-oxidants and genotoxic substances (Moreira et al., 2002).

The potential mutagenic effect shown by the extract obtained from stem bark of *B. cuspidata* suggests that medicinal preparations of this species should be used with greater caution, since the bark is the part of the plant usually employed in Brazilian herbal medicine.

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RESUMO

Avaliação do potencial genotóxico de extratos etanólicos de cascas e folhas de Bathysa cuspidata (A.St.-Hil.) Hook

Bathysa cuspidata (A.St.-Hil.) Hook. é uma árvore nativa do bioma Mata Atlântica largamente utilizada na medicina popular brasileira. Apesar do amplo uso, ainda não existe relato sobre a toxicologia dos compostos obtidos dessa espécie vegetal. O presente estudo avaliou efeitos mutagênicos e genotóxicos de extratos etanólicos de *B. cuspidata* obtidos das cascas do caule (SBC) e das folhas (LBC). A mutagenicidade dos extratos foi avaliada utilizando o teste de Ames nas linhagens TA98 e TA100 de *Salmonella typhimurium*, na ausência e presença de ativação metabólica. Os efeitos dos extratos diretamente sobre o DNA foram avaliados por clivagem plasmidial. Análises fitoquímicas foram realizadas para comparar a composição de metabólitos secundários dos extratos. Não foi encontrada atividade mutagênica para LBC quando avaliados utilizando as cepas TA98 e TA100, sem ou com ativação metabólica. No entanto, SBC apresentou atividade mutagênica. Clivagem plasmidial não indicou ação genotóxica tanto para SBC quanto para LBC. Diferenças na composição dos metabólitos secundários presentes nos cascas e folhas, detectadas pelas análises fitoquímicas, devem ser determinantes para a variação do efeito mutagênico entre SBC e LBC. Os resultados sugerem cautela no emprego de cascas de *B. cuspidata*.

Palavras-chave: *Bathysa cuspidata.* Genotoxicidade. Mutagenicidade. Teste de Ames.

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