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Artigo

Determining the genotoxicity of an aqueous infusion of *Bauhinia monandra* leaves

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RESUMO: "Determinação da genotoxicidade do infuso aquoso das folhas da *Bauhinia monandra*". *Bauhinia monandra*, popularmente conhecida como "pata-de-vaca", é nativa da Ásia e amplamente utilizada em todo o mundo para o tratamento de várias doenças, em especial diabetes. Diante da grande utilização dessa planta no Brasil, e de forma a satisfazer a necessidade de regulamentação das plantas medicinais pelo Ministério Público de Saúde, objetivamos determinar a genotoxicidade, citotoxicidade e mutagenicidade do infuso aquoso das folhas da *B. monandra*. Os resultados foram correlacionados com os compostos químicos encontrados após realização de uma triagem fitoquímica. Os testes foram realizados em sistema *in vitro* como o DNA plasmidial, na presença e ausência de exonuclease III, e *in vivo* empregando sistema procarioto (transformação com bactérias competente DH10B) e eucarioto (teste *Allium cepa*). As concentrações do infuso foram 0,8 μg/μL, 4 μg/μL, 20 μg/μL e 100 μg/μL. Nessas concentrações o infuso não causou mutagenicidade ou citotoxicidade, porém as concentrações mais elevadas foram capazes de induzir quebra nas ligações fosfodiéster do DNA e formar sítios abásicos, efeito sugerido pela presença de hidroxilas fenólicas. Os resultados revelaram riscos e benefícios desse extrato vegetal para uso terapêutico e seus efeitos sobre integridade do material genético, especialmente quando empregados como hipoglicêmico.

Unitermos: *Bauhinia monandra*, Leguminosae, mutagenicidade, citotoxicidade, genotoxicidade, *Escherichia coli*, teste de micronúcleos.

ABSTRACT: *Bauhinia monandra***,** commonly known as "cow's-foot", is native from Asia and widely used all over the world to treat a variety of illnesses, in particular diabetes. The high usage of the plant in Brazil and to fulfill the need of medicinal plant regulation by the Ministry of Public Health, we aimed at determining the genotoxicity, cytotoxicity and mutagenicity of an aqueous infusion from *B. monandra* leaves. The results were correlated to the chemical compounds found after phytochemical selection. Tests were performed in an *in vitro* system with plasmid DNA, in the presence and absence of exonuclease III, and *in vivo* system employing prokaryotic (transformation into competent DH10B bacteria) and eukaryotic (*Allium cepa*) assays. The infusion concentrations were 0.8 μg/μL, 4 μg/μL, 20 μg/μL and 100 μg/μL. The infusion concentrations did not cause mutagenicity or cytotoxicity, however the highest concentrations were able to induce breaks in DNA phosphodiester bonds and form abasic sites, an effect suggested by the presence of phenolic hydroxyls. The results revealed risks and benefits of plant extracts for therapeutic use and their effect on genetic integrity, especially when commonly employed as a hypoglycemic.

Keywords: *Bauhinia monandra*, Leguminosae, mutagenicity, cytotoxicity, genotoxicity, *Escherichia coli*, micronucleus assay.

INTRODUCTION

Plant-derived products are an important therapeutic resource in the treatment of many illnesses, especially in developing countries. Data from the World Health Organization (WHO, 2000) show that 70-80% of the world's population use alternative medicine (Chan, 2003). The socioeconomic conditions of the Brazilian population along with the vast medicinal flora in the country have led a growing number of people to make therapeutic use of plant extracts.

The genus *Bauhinia*, known as "cow's-foot" or "ox nail", belong to the family Leguminosae (Caesalpiniaceae), and is among the countless plant

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species of medicinal interest. *Bauhinia* consists of approximately 300 species (Miyake et al., 1986) mainly distributed in tropical areas of the planet. They are widely used in Brazil and in other countries to treat various diseases, especially infections, pain processes and diabetes. Particularly prominent are aqueous extracts of the leaves, stalks and roots of *B. cheilantha*, *B. forficata*, *B. monandra*, *B. glabra*, *B. rufescens*, *B. splendens* and *B. ungulata* (Achenbach et al., 1988; Ritter et al., 2002; Pereira et al., 2004; Macedo and Ferreira, 2004; Morais et al., 2005; Silva et al., 2006; Agra et al., 2007; Agra et al., 2008).

B. monandra is distinguishable from other species by its hypoglycemic activity, proven when administered at 10% dry leaf extract (100 µg/µL) (Minto and Pereira, 2000), the concentration used by popular medicine. Studies performed on malnourished mice demonstrate the plant's capacity to restore normal insulin secretion; also shows considerable antioxidant activity, and is capable of acting on free radicals in cells (Argôlo et al., 2004). Although some compounds of the species have been isolated and identified, there are few studies correlating the biological and adverse effects of its continuous use.

Assays performed on other medicinal species correlate the presence of certain chemical plant substances to cytotoxic and genotoxic damage. Some are capable of causing bacterial mutations and can potentially slow the development of cancers in humans (Agner et al., 1999). Several plants have already had their genotoxic studied, such as *Schinus terebinthifolius* extract (Carvalho et al., 2003), the methanol extracts from whole plants of *Helichrysum simillimum*, *Helichrysum herbaceum* and *Helichrysum rugulosum*, which indicated mutagenicity (Reid et al., 2006). The detection and evaluation of damage caused by toxic plant components is important in reducing risk when used therapeutically, particularly during prolonged treatment (Cardoso et al., 2006).

The aim of this work was to perform a phytochemical survey of aqueous extract obtained from dried *B. monandra* leaves and determine its cytotoxic, genotoxic, and mutagenic potential through *in vitro* and *in vivo* assays with prokaryote and eukaryote systems, to minimize possible human health risks.

MATERIAL AND METHODS

Sample

Leaves of *B. monandra* Kurz were collected from ornamental trees at Banco de Germoplasma de Plantas Medicinais na Estação Experimental de Itapirema (Goiana City, State of Pernambuco, Northeast of Brazil). The botanical classification of registered species was recorded as nº 57462, IPA, at the "Dárdano de Andrade Lima" (Empresa Pernambucana de Pesquisa Agropecuária, Recife, Brazil). The leaves were roughly ground and dried at a constant weight. The infusion with a concentration of 200 µg/µL was prepared according to the description in the Farmacopéia dos Estados Unidos do Brasil 2nd Edition (1959). It was filtered through filter paper, sterilized using a 0.45 µm acetate filter (Millipore, São Paulo), separated into aliquots in 2 mL microtubes and stored at -20 °C.

Plasmid and bacterial strains

The pBCKS plasmid was derived from the pBluescript® II phagemid and the ampicillin-resistant gene was replaced with the chloramphenicol-resistant gene.

Escherichia coli DH10B used in the bacterial transformation test was of the DH10B Genotype: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G /bMON14272/pMON7124.

Phytochemical selection

The phytochemical selection of dried *B. monandra* leaves was performed by precipitation and coloration reactions as proposed by Professor S. J. A. Matos and colleagues at the Faculdade de Farmácia e Bioquímica, Universidade Federal do Ceará - UFC/Brazil (Matos, 2000). The qualitative analysis of secondary metabolites were performed with *B. monandra* leaves dried and stabilized in an incubator between 40 °C to 45 ºC for 24 h. The chemical groups were: cyanogenetic heteroside, lactone, including coumarins, quinone, catechin, steroid and/or triterpenoid/carotenoid, resin, alkaloid, organic acids and bases, phenol, saponin, tannin, gum and mucilage, tannins or other substances containing phenol hydroxyls, proanthocyanidin, pyrogallic tannin, flavone heteroside, mucilage. Dehydrated material was powdered; following aqueous and alcoholic extracts were prepared and used in the assays described below.

Alcohol extraction

B. monandra ethanol leaf extract was obtained by addition of alcohol 94.6 °GL (200 mL) to dust from dried leaves (20 g), for about 10 minutes. The mixture was then filtered through a funnel to produce an ethanol extract.

Aqueous extraction

Boiling water was added to 20 g of the drug for extraction (rapid decoction) and allowed to stand for approximately 10 min. The mixture was filtered in a cotton wool-lined funnel and the solution was concentrated to 10% (200 mL). The color and smell were observed.

Analysis of the occurrence of breaks in plasmid DNA

The pBCKS plasmid is a derivation from pBluescript® II phagemid, had approximately 3400 base pairs and the following characteristics: a b-galactosidase ORF (Open Reading Frame), T7 promoter transcription initiation site, a multiple cloning site, a T3 promoter transcription initiation site, f1 (-) origin of single-stranded DNA replication (for secretion as single-stranded DNA packaged phages), chloramphenicol resistance ORF, a Lac promoter and a bacterial origin of replication (Flieger et al., 2004).

The vector preparation was performed according to the alkaline method described by Sambrook et al. (1989). After plasmid treatment with the infusion, we were able to determine the presence or not of breaks in the DNA phosphodiester bonds through agarose gel electrophoresis. The plasmid (1 µg) was incubated at 37 °C for 1 hour with up to 100 µg/µL of the *B. monandra* infusion in a final volume of 20 µL. The treated plasmid was electrophoresed at 80 V in 0.7% agarose gel, containing ethidium bromide (0.5 µg/mL) in 1XTBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 7). The assay included a negative control (distilled water plasmid) and a positive control (plasmid treated with Bam *HI*). The bands were visualized with an UV transilluminator and then photographed with a Kodak EDAS290 system.

Abasic site assay with exonuclease III

The exonuclease III enzyme has two distinct functions: as an exonuclease hydrolyzing the DNA phosphodiester bond, and as an endonuclease hydrolyzing the DNA phosphodiester bonds at AP sites. The enzyme and its buffer are combined with distilled water to form the MIX, from which 10 µL is separated and incubated at 37 °C for 1 hour with 10 µL of each sample analyzed in the DNA plasmid test. After incubation, 10 µL is removed from the samples, 2 µL of bromophenol blue is added and the mixture is placed in 0.7% agarose gel and electrophoresed and photographed as described above.

Bacterial transformation

For the survival assay, the pBCKS$^-$, treated as described above, were transferred, by electroporation (25 µF 200 Ω 1800 V), into *Escherichia coli* DH10B. Cells were plated in LB medium and grown overnight at 37 ºC in the presence of X-gal (1.6 µg/ml) and chlorophenicol (25 µg/mL) for a blue/white and resistance selection, respectively. The survival rate was calculated from the ratio between the number of colonies with the treated plasmid and the number of colonies with the untreated plasmid control. Positive control was the Plasmid DNA digested by Bam *HI*. All these experiments were performed in triplicate and the significance of the results was tested by analysis of variance and the Tukey test (Sokal and Rohlf, 1995).

***Allium cepa* assay**

In order to observe whether *B. monandra* infusion was able to generate micronucleus, the *Allium cepa* test was performed according to Ma et al. (1995). Onions were germinated for 48 hours in distilled water and then treated with *B. monandra* infusion at concentrations of 0.0 µg/µL, 0.8 µg/µL, 4 µg/µL, 20 µg/µL and 100 µg/µL for 24 hours (five onions per sample), followed by a 24 h recovery time in distilled water. The roots were then collected and treated with a 3:1 solution of ethyl alcohol and frozen acetic acid and underwent hydrolysis in 1 N HCl at 60 ºC for 10 minutes. Coloration was achieved using Schiffs reactive. The roots were washed in sulfur water and placed on slides with a few drops of 45% acetic acid. The cowl was removed and the F1 area gently soaked before placing the coverslip. For each onion, 3000 cells were analyzed for micronuclei, totaling 15000 per sample. One slide was prepared per bulb and all slides were coded and examined blind using bright-field microscopy at a total magnification of 40x.

RESULTS

Phytochemical selection confirmed the presence of a number of therapeutically significant compounds in the alcoholic and aqueous solutions, including: lactones, steroids, triterpenoids, resins, alkaloids, acids, organic bases and saponins, gums, mucilage, phenol hydroxyls, proanthocyanidins, tannins (hydroxyphenols) and pyrogallic tannins.

A cell- free assay was performed with plasmid pBCKS, with the objective of intervening if the compounds present in the *B. monandra* extract were able to generate breaks or abasic sites on DNA. The pBCKS was treated with different concentrations of the infusion. Results of the plasmid DNA test showed breaks in the phosphodiester DNA bonds at concentrations of 20 µg/µL and 100 µg/µL, converting some of its supercoiled DNA (form I) to nicked circular DNA (form II) (Figure 1). Even generating breaks on the phosphodiester bound it does not exclude the possibility that other types of damages could be occurring. To access whether abasic sites were generated by *B. monandra* infusion, after the treatment of pBC with this infusion, they were incubated at 37 ºC with an AP. The results showed changes in plasmid DNA conformation at concentrations of 4 µg/µL 20 µg/µL and 100 µg/µL, as seen in Figure 2.

Another test employed was to see whether *B. monandra* infusion could generate other lesions that could block replication or induce mutations, treated plasmid DNA with this infusion were transformed into

DH10B strain. Neither cytotoxicity nor mutagenic potential (presence of white colonies) were observed (Table 1).

The analyses of micronuclei in *A. cepa* showed no increase in the frequency of small acentric chromosome fragments or whole chromosomes in any of the concentrations tested, suggesting there were no chromosome breaks or disturbances in mitotic spindle (Table 2).

DISCUSSION

Determination of the safety of natural products is very important since toxicity represents an important obstacle in drug development. In the ethnopharmacological context, the lack of toxicity and genotoxicity are important for the whole population.

Diabetes affects about 10 million Brazilians and is the fourth leading cause of deaths worldwide (WHO, 2000). The *B. monandra* leaf infusion is highly used as a diabetes treatment and in this study it was characterized according to its cytotoxic, genotoxic and mutagenic potential.

There are many plant extracts that reduce the blood-glucose level and the large number of chemical groups shows that various activity mechanisms are involved in lowering the blood-glucose level. Some of these substances may have therapeutic value, while others can cause hypoglycemia as a side-effect owing to their toxicity, particularly hepatotoxicity (Pérez Gutiérrez, 2002; Lamba et al., 2000).

In vitro analysis with plasmid DNA has been widely used to determine breaks in DNA molecule caused by interaction with natural products (Kovary et al., 2001; Carvalho et al., 2003; Petta et al., 2004; Varela-Barca et al., 2007; Santos et al., 2008). Gel electrophoresis of the plasmid DNA treated with *B. monandra* infusion and with exonuclease III, showed interaction of leaf compounds with the DNA, suggesting a genotoxicity of infusion only at higher concentrations. However, it is not excluded the possibility of this infusion in generate another kind of lesions besides breaks and abasic sites, not observed in electrophoresis. So, it was better investigated transforming the treated plasmid DNA into DH10B cells, deficient in recombinational repair. Neither citotoxicity nor mutagenicity was observed suggesting that *B. monandra* infusion is not able to induce mutagenic lesion in a cell free system.

Studies assess the toxic action of plant extracts through various tests (Silva et al., 2006; Vasconcelos et al., 2007; Dias et al., 2007, Batistuzzo de Medeiros et al., 2003), within which the test is *Allium cepa*, which is a plant species commonly used to evaluate genotoxicity of chemical and environmental products (Smaka-Kincl et al., 1996; Moreira et al., 2002; Jos et al., 2005; Bagatini et al., 2007; Fachinetto et al., 2007) through the presence of micronuclei (MN), what derived from whole chromosomes or acentric fragments not incorporated into the nucleus during cell division. The chromosome fragments can result from clastogenic effects caused by chemical substances or aneugenic effects where chromosome is lost from mitotic spindles (Yi and Meng, 2003). Cellular activity can also correct errors in cell division, preventing damage to cell genome in the form of micronuclei. According to the ontogenetic structure of the onion, most MN occurs in F1 cells except in rare cases where there is a delay in mitosis. Studies show greater efficiency when these acentric fragments are analyzed in the F1 region in relation to the meristematic region, facilitating the score (Yi et al., 2007). This is an efficient system in detecting the clastogenicity of physical agents and chemical products, confirming the non-significance of *B. monandra* infusion analysis in relation to micronuclei found in different infusion concentrations and the lack of mutagenicity in the present approach.

The cytotoxic and genotoxic effects of plant extracts have been studied through years, involving the indiscriminate use of these products, which may be due to their chemical compounds. Secondary metabolites in plants are of great interest at present, whether for their pharmacological, chemical, medicinal or toxic aspects.

The knowledge of the components of a plant from phytochemical selection helps the understanding of its action and its adverse reactions (Andrade et al., 2005; Peitz et al., 2003; Mariz et al, 2006; Carlos et al., 2005). Phytochemical studies of *Bauhinia* genus confirm the presence of various isolated and identified compounds, including lactones, flavonoids, terpenoids, glycolipids, steroid glycosides, steroids, tannins and quinines (Cechinel-Filho et al., 1996, Silva et al., 2000; Mendes et al., 2006). Bhartiya and Gupta (1981) identified, in *B. purpurea* seeds, the compound: 3.4-dihydroxychalcone-4-*O*-β-L-arabinopyranosyl- *O* - β - D-galactopyranoside. Previous chemical studies with methanol extract of *B. purpurea* isolated and identified 6-butil-3-hydroxyflavanone (Kuo et al., 1998). Steroid glycosides of *B. candicans* were isolated and identified as: sitosterol 3-*O*-α-D-riburonofuranoside (Irribarren and Pomilio, 1985). In a phytochemical study of *B. manca,* Achenbach et al. (1988) isolated 3-*O*-galloylepicatechin, one of its primary components. They also identified gallic acid, cinnamic acid, β- sitosterol and β- D-glucosyl-sitosterol in significant concentrations. Rutin and quercetin were also identified from *B. splendens* leaves (Cechinel-Filho et al., 1996). In *B. forficata* it was possible to isolate and identify: 3,7-di-O- α-(rhamnopyranosylkaempferol, kaempferitrin, and 3,7-di-O- rhamnopyranosylquercetin (Menezes et al., 2007) .

Among *B. monandra* components are: flavonoids, steroids and lectins (Argôlo et al., 2004). Menezes et al. (2007) isolated and identified the compound: 3,7-di-*O*-β-rhamnopyranosylquercetin. According to the author, hypoglycemic activity can

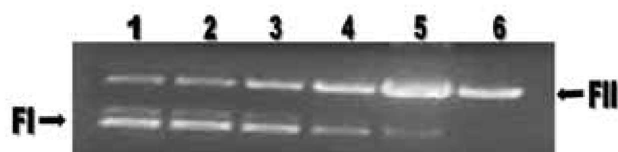

Figure 1. Electrophoretic run in 0.7% agarose gel of dry *B. monandra* leaf infusion at concentrations: 2 (0.8 µg/µL), 3 (4 µg/µL), 4 (20 µg/µL) and 5 (100 µg/µL), negative (1) and positive (5) controls. The conformation of plasmid DNA was indicated by form F1 (supercoiled), form FII (nicked circular) and form FIII (linear).

Figure 2. Electrophoresis in 0.7% agarose gel after treatment of plasmid DNA with exonuclease III enzyme and with infusion of dry *B. monandra* leaves at concentrations: 2 (0.8 µg/µL), 3 (4 µg/µL), 4 (20 µg/µL), 5 (100 µg/µL), negative (1) and positive (2) controls.

Table 1. Result of DH10B bacterial transformation at study concentrations of the infusion of *B. monandra* and leaf infusion negative control (plasmid DNA treated with distilled water).

Infusion concentration (µg/µL)	Bacterial colonies
NC	7987 ± 413
0.8	9451 ± 558
4	9152 ± 595
20	7021 ± 306
100	5848 ± 1.012**

NC (Negative control with distilled water), Test performed in triplicate. Mean ± S.D. ** Significant values ($p < 0.01$) by analysis of variance (ANOVA) and Dunnett test.

Table 2. Result of the *Allium cepa* test, assessing the presence of micronuclei in the F1 region, after treatment with the infusion of *B. monandra* at determinate concentrations and after treatment with a negative control (distilled water).

Concentration (µg/µL)	MN in F1 cells
NC	1.0 ± 1,7
0.8	0.6 ± 0.9
4	1.0 ± 0.7
20	1.2 ± 1.1
100	1.4 ± 1.5

NC (Negative control with distilled water). 15.000 cells analyzed per treatment. Mean ± S.D. The results showed no significant differences ($p < 0.05$) by analysis of variance (ANOVA) and Dunnett test.

be related to the presence of glucosyl flavonoids that have different qualitative and quantitative profiles in extracts. These had a very pronounced effect in the *in vitro* method used to establish activity, showing them as promising hypoglycemic agents. This study presents similar results in relation to the presence of flavonoids and steroids and identified other components that offer insights into infusion activity, such as: tannins, lactones, triterpenes, resins, alkaloids, organic acids and bases and saponins.

Flavonoids are a phytochemical group with extensive biological activity due to their antioxidant properties and ability to modulate various enzymes and cell receptors; however depending on the number and position of hydroxyl groups in the A and B ring they can act as a mutagenic or a pro-oxidant compound (Skibola and Smith, 2000; Silva et al., 2000; Hodek et al., 2002).

The flavonoid present in the extract (decoction) of stem bark from the pepper tree (*Schinus terebinthifolius* Raddi) has mutagenic potential as demonstrated by Carvalho et al., (2003) in several bacterial tests, like SOS chromotest, Salmonella reversion assay, and a forward mutagenesis test using CC104 strains of *E. coli*. Flavonoid- enriched fractions and the amentoflavone purified from the decoction of *Schinus terebinthifolius* Raddi, have shown to be able to damage DNA via oxidative stress producing lesions on DNA that are potential targets of FPG and MutY glycosylase from the base excision repair pathway, confirming the mutagenic potential of this flavonoid (Varela-Barca et al., 2007).

Studies on the free radical-scavenging properties of flavonoids have identified many natural phenol compounds as the main phytochemical antioxidants (Rice-Evans et al., 1997).

According to Argôlo et al. (2004) the antioxidant activity of *B. monandra* leaves is suggested by the presence of flavonoids and steroids. This may impact on the prevention of chronic cardiovascular and neoplastic diseases, neurodegenerative diseases such as Parkinson's and Alzheimer's, as well as immunological dysfunctions, premature aging and cancer. *B. monandra* also contains tannins, which were found to have antimutagenic properties according to many published studies (Deguchi et al., 2001; Chen and Chung, 2000) and saponins, reported in some studies as non-mutagenic or antimutagenic (Scarpato et. al., 1998; Berhow et al., 2000).

Species of the same genus such as *B. galpinii*, *B. purpurea* (Codington et al., 1975) and *B. variegata* (Rajkapoor et al., 2003) have been described as antimutagenic.

The infusion of dried *B. monandra* leaves was found to be genotoxic with *in vitro* experiments, at usual concentrations (100 µg/µL). However, it did not cause cytotoxicity or mutagenicity in tests where concentrations were determined by the study. This demonstrates

the feasibility of its use and commercialization as a hypoglycemic for oral use with medical follow-up, in order to be employed correctly and reasonably as a phytotherapeutic.

We can therefore conclude that the *B. monandra* Kurtz infusion was able to cause breaks in the phosphodiester DNA bonds and form abasic sites but only at the highest concentrations tested, which does not represent a therapeutic dose. This action can be explained by the presence of phenol hydroxyls in positions 3' and 4' of ring B and free hydroxyls or methoxyl groups in position 7 of ring A. However, the infusion did not cause cytotoxicity or mutagenicity in any of the concentrations tested, which may be due to the presence of tannins, steroids, lecithins, saponins, antimutagenic flavonoids._

The non mutagenic effects observed in the two *in vivo* systems used, *E. coli* and *Allium cepa*, is very interesting since this infusion has shown a potent hypoglycemiant effect.

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