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Protective action against chemical-induced genotoxicity and free radical scavenging activities of *Stryphnodendron adstringens* (“barbatimão”) leaf extracts

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Abstract: The present study describes for the first time the antigenotoxic and antioxidant properties of *Stryphnodendron adstringens* (Mart.) Coville, Fabaceae (“barbatimão”) leaves. The aqueous (AEB), water fraction (WFB) and ethanolic (EEB) extracts of leaves were obtained and they presented high content of phenolic compounds, flavonoids and proanthocyanidins. WFB and EEB inhibited the genotoxicity induced by cyclophosphamide. WFB inhibited the DNA lesion formation and both WFB and EEB decreased significantly ($p < 0.05$) micronucleus formation. All extracts also showed high DPPH radical scavenging activity and reducing power. In conclusion all extracts presented antioxidant activity and WFB and EEB protected cells from genotoxicity induced by cyclophosphamide in rat bone marrow cells. Thus, our results support the beneficial effects of the barbatimão extracts as an anticarcinogenic agent.

Introduction

The use of herbal medicine (leaves, flowers, roots, stems and berries) is a traditional form of healthcare (Diwanay et al., 2004). In this context, an important focus of the search is to understand the true efficacy of plant materials and determine its mechanism of action in different experimental models (Romero-Jiménez et al., 2005). Although human body is equipped with self defense mechanisms such as detoxification process through various enzymes, serious exposure to dangerous chemicals can cause changes in the genetic material (mutations) leading to carcinogenic events (Edenharder et al., 1998; Belitsky & Yakubovskaya, 2008). Cyclophosphamide (CP), an antineoplastic agent, and its metabolites can bind DNA, causing damage that may result in chromosome breaks, micronucleus formation and cell death (Murata et al., 2004). Beyond that, CP alters the redox balance of tissues suggesting that physiological and biochemical disturbances may result from oxidative stress (Sugumar et al., 2007).

Oxidative stress is an important factor in the genesis of several pathologies, from cancer to cardiovascular and degenerative diseases. Human cells are constantly exposed to reactive oxygen species (ROS) generated by a number of biotic and abiotic factors that

can harm the cells. Antioxidants provide protection to living organisms from damage caused by uncontrolled production of ROS and the concomitant oxidation of nucleic acids, proteins, lipids and carbohydrates, which may subsequently determine mutagenesis and diseases related to DNA damage (Halliwell, 2007a; 2007b). Anticarcinogens/anti-mutagens, often used in traditional medicine, can prevent or revert some of the effects produced by carcinogens. However, knowledge about the mechanism of action of many anti-carcinogens, e.g., phytochemicals, is still poor (Paolini & Nestle, 2003).

Because of their wide spectrum of activities, constituents such as phenolic compounds and flavonoids have been the subject of many investigations. An important but often neglected group of polyphenolic compounds is that of the tannins, which are widely distributed in higher plants and they have proven to possess health benefits (Okuda, 2005).

Stryphnodendron adstringens (Mart.) Coville, Fabaceae, known as ‘barbatimão’, occurs in the central savannah region of Brazil and is a rich source of tannins (Santos et al., 2002) and proanthocyanidins (Mello et al., 1996a; 1996b; 1999; Ishida et al., 2006; Lopes et al., 2008). Stem bark is the main part of the plant used popularly as an anti-inflammatory, astringent, in the treatment of wounds and vaginal infections (Panizza et

al., 1988; Lopes et al., 2005). Several reports have proven the anti-ulcerogenic, anti-inflammatory, antinociceptive and antimicrobial (Lima et al., 1998; Audi et al., 1999; Ishida et al., 2006; Ishida et al., 2009; Melo et al., 2007) activities of bark extract of *S. adstringens*. However there are few reports on the toxicity of barbatimão leaf extracts (Almeida et al., 2010). Thus, the aim of the present study was to determine the antigenotoxic activity of *S. adstringens* leaf extracts on genotoxicity induced by CP in rat bone marrow cells and the *in vitro* antioxidant potential of the extracts.

Materials and Methods

Plant material

Leaves of *Stryphnodendron adstringens* (Mart.) Coville, Fabaceae, were collected in Alfenas (Minas Gerais, Brazil) in Julho/2007 and deposited at the Herbarium of the Universidade Federal de Alfenas, Brazil (voucher specimen N. 196).

Preparation of leaf extract

The leaves were dried at 30 °C and then pulverized. The aqueous extract (AEB) was obtained by decoction of 10 g of leaves in 100 mL of deionized water, at 90 °C, for 10 min and filtered. The water fraction (WFB) remained after powder leaf extraction (100 g) with 70% (v/v) acetone aqueous solution (1 L) and partitioning with ethyl acetate. The ethanol extract (EEB) was obtained by maceration of 100 g of powder in 1 L of 70% (v/v) ethanol aqueous solution and the solvent was evaporated in a rotavapor under reduced pressure. All extracts were lyophilized and stored at -20 °C, until use. For use the extracts were dissolved in deionized water.

Determination of phenolic, flavonoid and proanthocyanidins content

The total phenolic compounds were determined using Folin-Ciocalteu's method (Rai et al., 2006), total flavonoids using aluminum chelating method (Ebrahimzadeh et al., 2008) and proanthocyanidins content was obtained after acid depolymerization to the corresponding anthocyanidins (Rösch et al., 2003).

Animals and treatment

Male Wistar rats (120±20 g) were housed with access to food and water *ad libitum* and light-dark cycles of 12 h were used. The animals were divided into eight groups (each group consisted of six rats). Control group received water; CP group received cyclophosphamide (40 mg/kg, per gavage), 24 h before euthanasia; extract

groups received 500 mg/kg body wt, per gavage, for 7 days and extract+CP groups received the extract for 7 days and CP 24 h before euthanasia. After euthanasia, with overdose of anesthesia, the femur was quickly removed and the bone marrow cells collected.

DNA isolation and electrophoresis

The DNA of the bone marrow cells from the left femur was isolated using the SDS/Proteinase K/RNase A extraction method (DeSierve et al., 2002). Samples were electrophoresed in 0.8% (w/v) agarose gel and DNA was visualized by ethidium bromide staining. The presence of apoptosis was indicated by the appearance of a ladder of oligonucleosomal DNA fragments that are approximately 180-200 bp multiples and the necrosis by the appearance of a smear on the agarose gel.

Micronucleus assay

This assay was performed according to Nakagawa et al. (2008), with modifications. Bone marrow cells of the right femur of each animal was flushed out as a fine suspension with 2.5 mL fetal bovine serum (FBS) and centrifuged at 1,000 x g for 5 min. The pellet was resuspended in 0.5 mL saline. The suspension was smeared onto the precleaned air-dried slides following fixing in 100% methanol for 5 min and stained with Giemsa. One thousand total erythrocytes (polychromatic and normochromatic) were scored per animal. The frequency of micronucleated polychromatic erythrocytes (MNPCE) and the total PCE and NCE were determined.

Antioxidant assays

Total antioxidant capacity

The total antioxidant capacity was determined (Prieto et al., 1999), and the results were expressed as equivalents of ascorbic acid.

DPPH assay

The hydrogen atoms or electron donation ability of the extracts was measured from the bleaching of purple colored methanol solution of DPPH according to Yen & Wu (1999). L-ascorbic acid and BHT were used as the reference standards.

Reducing power

The reducing power of the extracts was determined according to the method of Oyaizu (1986), using L-ascorbic acid and BHT as the reference standards.

Statistical analysis

Data were analyzed using ANOVA with post hoc analysis by Tukey test, when $p < 0.05$. Results are expressed as mean \pm standard error of the mean (SE).

Results and Discussion

In our study, the chemopreventive and antioxidant properties of barbatimão leaf extracts were evaluated. We used cyclophosphamide (CP), a genotoxic agent widely used both, in clinical treatment and experimentally (Murata et al., 2004; Nagar, 2010). CP belongs to the group of N-lost compounds. It requires CYP450-dependent activation, yielding the active compound 4-hydroxy-cyclophosphamide. This molecule undergoes spontaneous decomposition to the biologically active alkylating species acrolein and phosphoramidate mustard, which preferentially alkylates the N7 position of guanine. Since CP is a bifunctional compound, DNA alkylation gives rise to interstrand cross-links that are thought to be responsible for the induction of cell death (Murata et al., 2004). From the DNA fragmentation analysis, it can be seen that CP induced apoptosis, confirmed by the DNA ladder pattern (Figure 1). The AEB and WFB were not genotoxic, as they did not induce DNA lesion. The WFB was antigenotoxic, as it protected cells from the apoptotic effect of CP and it could be postulated that proanthocyanidins contributed to its activity, once, amongst the studied extracts, WFB presented the highest content of these compounds.

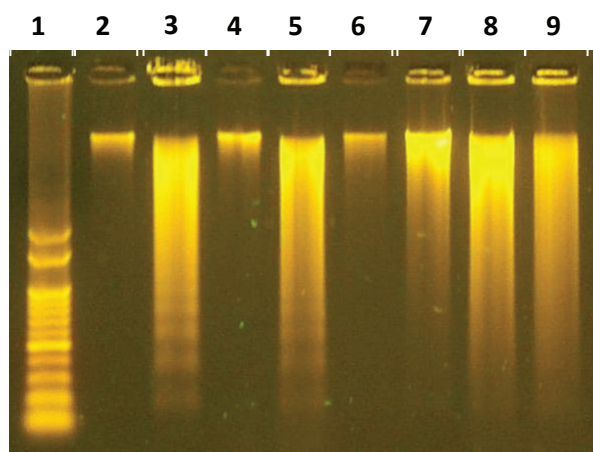


Figure 1. Agarose gel electrophoresis of bone marrow cells genomic DNA of control and treated rats. 1, 123 bp ladder marker; 2, Control; 3, Cyclophosphamide (CP); 4, Aqueous extract (AEB); 5, AEB plus CP; 6, Water fraction (WFB); 7, WFB plus CP; 8, ethanolic extract (EEB); 9, EEB plus CP. DNA ladder formation indicates apoptosis as seen in lanes 3 and 5 and DNA smear as seen in lanes 8 and 9 indicates necrosis. The results are representative of five independent experiments.

The quantitative assessment of micronucleus containing cells serves as a good indicator for the induction of structural and numerical chromosomal aberrations. Breakage of chromatids or chromosomes can result in micronuclei formation if an acentric fragment is produced; therefore assays detecting either chromosomal aberration or micronuclei are acceptable for detecting clastogenicity (Natarajan, 2002) and the micronucleus test is recommended by the Brazilian Health Surveillance Agency for phytotherapeutic preclinical toxicity studies (Anvisa, 2004). The results of micronucleus assay are shown in Table 1. The data of this test corroborates the findings of the DNA fragmentation test. A significant ($p < 0.05$) increase in the percentage of micronuclei was observed after treatment with CP as compared to the control and treated groups. Administration of CP in combination with extracts showed significant decrease ($p < 0.001$) in the percentage of micronuclei as compared to CP treated rats. In the groups treated with extracts the PCE/NCE ratio increased, indicating, possibly, a cell proliferation stimulation activity of the extracts. These results further confirm that the extracts were antigenotoxic. Our results are in agreement with Costa et al. (2010) who observed no genotoxic effect of the proanthocyanidin polymeric-rich fraction of stem bark of *S. adstringens*. This work reported no significant differences in the frequency of MNPCE induced by the fraction treatment when compared to the negative control. The fraction was also antimutagenic and this activity could be attributed to the tannin content and to its antioxidant activity, due to its ability to form complexes with other macromolecules. In the present work we observed an antigenotoxic activity of all extracts and that all of them exhibited antioxidant activity.

Table 1. Frequencies of micronucleated polychromatic erythrocytes (MNPCE), polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) in mouse bone marrow cells of control and treated groups.

Groups	PCE	NCE	PCE/NCE	MNPCE/ 1000 cells
Control	380.50 \pm 20.78 ^a	611.50 \pm 19,62 ^a	0.63 \pm 0.05 ^a	4.33 \pm 0.80 ^a
CP	240.16 \pm 26.91 ^b	697.33 \pm 31,42 ^a	0.35 \pm 0.06 ^b	31.83 \pm 6.00 ^b
WFB	664.00 \pm 44.32 ^c	323.16 \pm 42,53 ^b	2.23 \pm 0.29 ^c	9.16 \pm 2.02 ^a
WFB+CP	620.16 \pm 55.01 ^c	360.83 \pm 53,26 ^b	2.60 \pm 0.12 ^c	12.33 \pm 3.38 ^a
EEB	779.33 \pm 18.46 ^c	203.66 \pm 16.96 ^b	4.02 \pm 0.48 ^d	7.16 \pm 1.77 ^a
EEB+CP	635.50 \pm 65.49 ^c	341.50 \pm 61.25 ^b	2.51 \pm 0.82 ^c	12.16 \pm 2.08 ^a
AEB	803.00 \pm 43.54 ^c	191.83 \pm 43,67 ^b	5.19 \pm 0.93 ^d	2.16 \pm 0.54 ^a
AEB+CP	738.50 \pm 12.34 ^c	254.66 \pm 11.87 ^b	2.96 \pm 0.22 ^c	4.00 \pm 0.85 ^a

AEB, aqueous extract; WFB, water fraction and EEB, ethanolic extract of barbatimão leaves; CP, cyclophosphamide. N=6. A total of 6,000 cells were counted per group (1,000 cells per animal). The results are mean \pm SE. Different letters in the same column indicate significant difference ($p < 0.05$) by the Tukey test.

The determination of main components of the extracts (Table 2) showed that EEB presented the higher phenolics and flavonoids content while the WFB presented the higher proanthocyanidins content. Phenolic compounds are regarded to be the most important antioxidative constituent of plants; hence correlations between the concentrations of plant phenolics and the total antioxidant capacities have been well reported (Gulcin et al., 2002; Oktay et al., 2003). The content of phenolic compounds in the extracts was higher than that reported to other previously examined sources of antioxidants (Hu et al., 2000; Mathew & Abraham, 2004; Basile et al., 2005). It is important to point out that the WFB presented the highest amount of proanthocyanidins and it was expected since we used a recommend solvent system to better extract this class of compound (Foo & Porter, 1980). We further confirm the proanthocyanidin content of the WFB by heating it with 5% HCl in tert-butanol which yielded cyanidin as the principal pigment.

Table 2. Barbatimão leaf extracts total phenolic compounds, flavonoids and proanthocyanidins content.

Extracts	Content		
	Phenolics (%)	Flavonoids (%)	Proanthocyanidins (μM)
AEB	32,00 \pm 2,05 ^a	5,02 \pm 0,29 ^{ab}	23.32 \pm 1.75 ^a
WFB	23,93 \pm 1,29 ^b	3,63 \pm 0,22 ^a	35.40 \pm 2.10 ^b
EEB	34,94 \pm 2,95 ^a	6,44 \pm 0,19 ^b	24.75 \pm 1.28 ^a

AEB, aqueous extract; WFB, water fraction and EEB, ethanolic extract of barbatimão leaves. The results are mean \pm SE of three independent experiments (N=5). Different letters in the same column indicate significant difference ($p < 0.05$) by the Tukey test.

It was observed that the amount of flavonoids in the analyzed barbatimão leaf extracts showed a high correlation with the total amount of phenolics and antioxidant activity. Extracts with high content of phenols and flavonoids are known to exhibit high antioxidant activity (Boudet, 2007).

All of the extracts tested presented antioxidant activity, and it could be attributed to the extract constituents. The total antioxidant capacity of the extracts was: 147.85 \pm 12.68, 124.36 \pm 7.18 and 148.64 \pm 8.41 mg ascorbic acid/g extract, for AEB, WFB and EEB respectively. The extracts also showed strong DPPH scavenging activity (Figure 2A). The free radical DPPH scavenging activity of the extracts was concentration-dependent and the maximum activity was achieved with 62.00 \pm 0.91 $\mu\text{g}/\text{mL}$. The extracts showed a similar scavenging activity to ascorbic acid and higher than BHT. DPPH assay evaluates the ability of antioxidants to scavenge free radicals. Hydrogen-donating ability is an index of the primary antioxidant activity. In our study WFB, that had the highest content of proanthocyanidins, showed a similar radical scavenging activity to the extracts (AEB and EEB)

with higher phenols and flavonoids content than WFB. Proanthocyanidins certainly contributed to the free radical scavenging activity of the extracts, as demonstrated by others (Kimura et al., 2008; Spranger et al., 2008). The extracts presented Fe^{3+} reducing capacity similar to ascorbic acid and higher than BHT and it was also concentration-dependent. The maximum reducing power was achieved with extracts at 250 $\mu\text{g}/\text{mL}$ (Figure 2B).

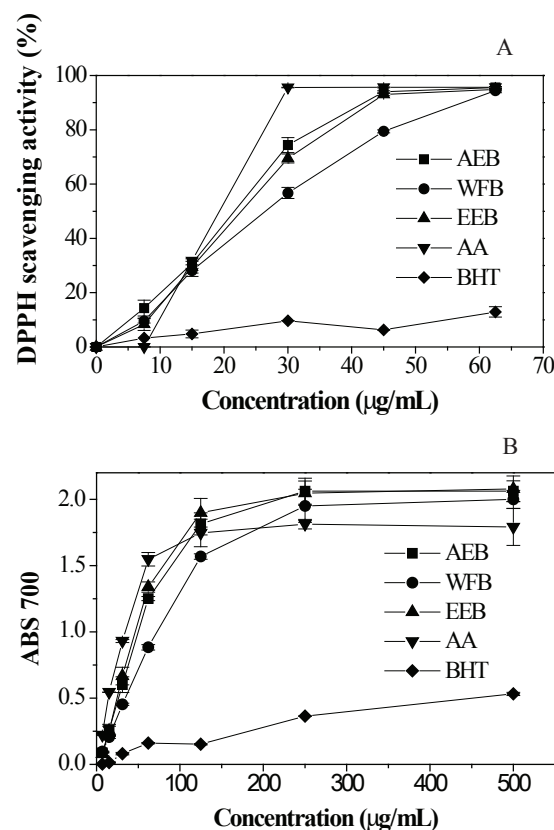


Figure 2. Antioxidant activity of barbatimão leaf extracts. A, DPPH scavenging activity; B, reducing power. AEB, aqueous extract; WFB, water fraction; EEB, ethanolic extract; AA, acid ascorbic (standard); BHT, butylated hydroxytoluene (standard). The results are mean \pm SE of five independent experiments performed in triplicate.

The interplay between free radicals and antioxidants is important in maintaining health and preventing the onset and progression of several pathologies (Halliwell, 2007a; Halliwell, 2007b).

The antioxidant activity of plant compounds has been attributed to various mechanisms, including prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Cos et al., 2003). The results of the present work indicate that the extracts act as antioxidants. The most important target for ROS in the carcinogenesis process is probably DNA. It is believed that antioxidant

properties of these phytochemical materials protect cells from ROS-mediated DNA damage that can result in mutation and subsequent carcinogenesis and they may donate hydrogen to free radicals, leading to non-toxic species and therefore to inhibition of the propagation phase of lipid oxidation (Hosseinimehr et al., 2008).

The barbatimão leaf extracts might be reducing the micronuclei formation by scavenging the DNA damaging free radicals generated following CP exposure. Another possible extracts mechanism of action might involve selective removal of cells with DNA damage by apoptosis, which seems to be case of EEB. Proanthocyanidins and flavonoids are known to form a complex with proteins and monooxygenases metabolizing xenobiotics, due to the fact that the formers can act as multidentate ligands and thus are able to bind simultaneously to more than one point of the protein surface. The more the oligomeric nature, the more its association produces an increased antimutagenic activity towards the inhibition of the enzyme mediated genotoxicity of indirect acting mutagens (Bruni et al., 2006).

In conclusion, the present study demonstrates that WFB and EEB protected cells from the CP chemical-induced genotoxicity and all extracts presented powerful antioxidant activity. Thus, our results support the beneficial effects of the barbatimão extracts as anticarcinogenic agent.

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