

Cytotoxic and genotoxic investigation on barbatimão [*Stryphnodendron adstringens* (Mart.) Coville, 1910] extract

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Stryphnodendron adstringens (Mart.) Coville, 1910 is a small tree, distributed widely throughout the Cerrado region of Brazil and named “barbatimão” by the Tupi-Guarani tribes, which presents astringent properties. Its ethnopharmacological uses comprise, among others, anti-inflammatory and wound healing action, and it is used in the treatment of diarrhea and gynecological problems. The phytotherapeutic use of ‘barbatimão’ is largely related to its tannin content, which is abundant in its bark. The main goal of the present study was to evaluate the cytotoxic, mutagenic, and genotoxic potential of the lyophilized solution of the stem bark of *S. adstringens*, using the Ames test, the SOS-Inductest and the SOS-Chromotest. *S. adstringens* presented cytotoxic activity in all tested systems, did not present mutagenic activity detectable by the Ames test and SOS-Chromotest, and showed some genotoxic effect on the SOS-Inductest. However, the metabolization of the extract by S9 fraction attenuated its genotoxic and cytotoxic activities.

Uniterms: *Stryphnodendron adstringens*/cytotoxicity. *Stryphnodendron adstringens*/genotoxicity. Barbatimão/phytochemical study.

Stryphnodendron adstringens (Mart.) Coville, 1910 é uma pequena árvore amplamente distribuída nas regiões de cerrado do Brasil, chamada de “barbatimão” pelas tribos Tupi-Guarani, que apresenta propriedade adstringente. Seu uso etnofarmacológico compreende, entre outros, efeitos antiinflamatório e cicatrizante, sendo empregada no tratamento de diarreias e problemas ginecológicos. Grande parte das aplicações do fitoterápico de barbatimão está relacionada aos taninos, abundantes em sua casca. O objetivo do presente trabalho foi avaliar os potenciais citotóxico, mutagênico e genotóxico da solução liofilizada da casca de *S. adstringens*, utilizando Teste de Ames, SOS-Induteste e SOS-Cromoteste. *S. adstringens* apresentou atividade citotóxica em todos os sistemas testados, não apresentou atividade mutagênica detectável pelo teste de Ames e SOS-Cromoteste e mostrou certo efeito genotóxico no SOS-Induteste. Porém, a metabolização do extrato pela fração S9 atenuou suas atividades genotóxica e citotóxica.

Unitermos: *Stryphnodendron adstringens*/citotoxicidade. *Stryphnodendron adstringens*/genotoxicidade. *Stryphnodendron adstringens*/mutagênese. Barbatimão/estudo fitoquímico.

INTRODUCTION

Since the origin of mankind and to this day, medicinal herbs have been used to treat diseases worldwide. Although countless plant species have long been used in folk medicine (González-Ávila *et al.*, 2003), only recently have the pharmacology and toxicity of these plants begun

to receive attention from scientists. Many studies have been carried out to verify the claimed pharmacological and/or therapeutic properties of these plants, isolate their active constituents, and investigate their possible toxicities (Rebecca *et al.*, 2002).

Stryphnodendron adstringens (Mart.) Coville, 1910 (Mimosideae) is a small tree abundantly distributed throughout the central Cerrado of Brazil, where it is commonly known as “barbatimão”, a name of indigenous Tupi-Guarani origin. It has been largely used in folk medicine as an anti-inflammatory and gastric anti-ulcerogenic agent, to

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heal wounds, and treat various diseases, such as diarrhea, leucorrhea, and gynecological problems (Audi *et al.*, 1999; Bersani-Amado *et al.*, 1996; Lima, Martins, Souza Jr., 1998; Neves *et al.*, 1992a,b; Panizza *et al.*, 1988; Santos; Torres; Leonart, 1988). Due to its importance as a phytotherapeutic agent, this plant was included in the Brazilian Pharmacopoeia and so far its pharmacological activities have been attributed mainly to the tannins present in the bark (Brandão *et al.*, 2006; Santos *et al.*, 2002).

According to Santos and coworkers (2002), the condensed tannins from *Stryphnodendron* genus present molecules composed of prodelphinidin units, formed by gallo catechin and/or epigallocatechin, which contain ortho-trihydroxyl groups in the β -ring. Free radical scavenging activity is enhanced by the presence of three β -ring hydroxyl groups in the condensed tannin structure (De Bruyne *et al.*, 1999a,b; Hagerman *et al.*, 1998). Ortho-trihydroxyl groups in β -ring potentiate the antiviral activity of condensed tannins, while galloylation of condensed tannins in a specific position (3-position), as found in this genus, presents anticancer activity (Dufresne; Farnworth, 2001). On the other hand, some authors have reported that the consumption of herbal tea rich in tannins has been proven to develop esophagus cancer and present chirogenic, abortive, hepatocarcinogenic and hepatotoxic effects (Angell, Kassirer, 1998; Zhu *et al.*, 1997).

Based on these facts, the aim of the present study was to evaluate the cytotoxic and genotoxic effects of the lyophilized solution of the stem bark of *Stryphnodendron adstringens* (Mart.) Coville, 1910 using the Ames Test, the SOS-Inductest, and the SOS-Chromotest.

MATERIAL AND METHODS

Plant material and preparation of Barbatimão extract (BE)

The ethanolic extract of "barbatimão", obtained from bark decoction, was purchased from Klein Laboratories Ltda. (It 001, Porto Alegre, RS, Brazil). The aqueous extract was obtained from the ethanolic extract, according to the technique of vaporization under low pressure using a rotary vacuum evaporator, at 40 °C. After complete evaporation of ethanol, we added an equal volume of distilled water and lyophilized this solution, obtaining the lyophilized "barbatimão" extract (BE).

Strains

Escherichia coli SOS-Inductest tester strains, WP2s(λ) (*uvrA*)(Amp^S) and RJF013 (*uvrD3*)(Amp^R),

and *Escherichia coli* SOS-Chromotest tester strain PQ37 (*rfa*)(*uvrA*)(Amp^R)(*sulA*: *lacZ*) were kindly provided by the Laboratório de Radiobiologia Molecular from the Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro (Rio de Janeiro, RJ, Brazil). *Salmonella typhimurium* strains TA97a (*hisD6610*) (*rfa*)(*uvrB*)(Amp^R), TA98 (*hisD3052*)(*rfa*)(*uvrB*)(Amp^R), TA100 (*hisG46*)(*rfa*)(*uvrB*)(Amp^R), and TA102 (*hisG428*)(*rfa*)(*uvrB*)(Amp^RTet^R), as described by Maron and Ames (1983), were kindly supplied by Dr. B. N. Ames, from the University of California (Berkeley, CA, USA).

Microsomal fraction

The postmicrosomal fraction S9 prepared from livers of Sprague-Dawley rats pre-treated with polychlorinated biphenyl mixture (Aroclor 1254) was purchased from Molecular Toxicology Inc. (MoltoxTM, Annapolis, MD, USA). The S9 metabolic activation mixture was prepared according to Quillardet and Hofnung (1985) for the SOS-Chromotest and according to Maron and Ames (1983) for the SOS-Inductest and *Salmonella*/microsome assay.

Ames Test

Following the *Salmonella typhimurium* histidine point mutation assay, proposed by Maron and Ames (1983), a 0.1 mL bacterial suspension (1-2 x 10⁹ cells/mL) of each *Salmonella typhimurium* strain (TA97a, TA98, TA100, and TA102) with 0.0, 0.25, 0.5, 1.0, 5.0, or 10 mg/plate BE was incubated, with or without S9 mix, at 37 °C for 25 min. Subsequently, 2.0 mL of top agar (0.6% agar Difco, 0.5% NaCl, 50 μ M L-histidine, 50 μ M biotin, 45 °C) was added to test tubes and poured onto Petri dishes with minimal agar (1.5% agar, 2% glucose, and Vogel-Bonner E medium). All assays included negative (distilled water) and positive [15 μ g 4-nitroquinoline 1-oxide (4NQO) per plate for TA97a and TA98, 1.5 μ g of sodium azide for TA100 and 2.5 μ g mitomycin C for TA102, data not shown] controls. After incubation at 37 °C for 48 h, colonies (His⁺ revertants) were counted and the results expressed as mutagenic index (MI = number of His⁺ induced in the sample/number of spontaneous His⁺ in the negative control). Bacterial survival was determined for TA98.

SOS-Inductest Prophage λ induction

The SOS-Inductest was performed according to Moreau, Bailone and Devoret (1976). An exponential

growth phase culture (2×10^8 cells/mL) of *Escherichia coli* WP2s(λ), grown in LB medium [1% bacto tryptone (Difco), 0.5% bacto yeast extract (Difco) and 1% NaCl] was centrifuged (5000 rpm, 20 min), resuspended, and incubated at 37°C for 25 min with 0.0, 0.25, 0.5, 1.0, 5.0, or 10 mg/tube BE (with or without S9 mix). The mixture was then diluted in M9 buffer (0.6% Na_2HPO_4 , 0.3% KH_2PO_4 , 1% NH_4Cl , and 0.05% NaCl) and 0.1 mL of this lysogenic culture [WP2s(λ)] was added to 0.3 mL indicator strain (RJF013), after which 2.0 mL top agar at 45 °C (0.6% agar Difco, 0.5% NaCl) was added to the mixture and poured onto Petri dishes with LB. All assays were performed in duplicate and included negative (distilled water) and positive [Cyclophosphamide (CP) and ultraviolet radiation, $\lambda = 254\text{nm}$, data not shown] controls. The results were expressed as induction factor (IF = plaques of treated culture/plaques of untreated culture). Bacterial survival was determined for WP2s(λ).

SOS-Chromotest

The SOS-Chromotest was carried out as described by Quillardet and Hofnung (1985) with subsequent modifications (von der Hude *et al.*, 1988). According to the standard procedure, BE was incubated with a 10-fold diluted exponential phase culture (10^8 cells/mL) of the tester strain (PQ37) and the culture diluted in fresh medium (LB) with or without S9 mix. After incubating this mixture for 2 h with 0.0, 0.25, 0.5, 1.0, 5.0, or 10 mg/tube BE, it was divided into two samples, one used for the β -galactosidase (βg) assay and the other for the alkaline phosphatase (ap) assay. Appropriate buffers were added to disrupt the cell membranes and specific substrates for each enzyme (4 mg/mL *o*-nitro-phenyl-galactopyranoside and 4 mg/mL *p*-nitro-phenyl-phosphate for βg and ap activities, respectively). After adding the buffers to stop the enzymatic reaction (NaCO_3 for βg and HCl and Tris-HCl for ap), the absorbance of each assay was read against a colorimeter blank. βg and ap activities were calculated according to the simplified method recommended by Quillardet and Hofnung (1993): enzyme units (U) = $(A_{420} \times 1000)/t$ (A_{420} = optical density at 420 nm; t = substrate conversion time in minutes). Negative (distilled water) and positive (mitomycin - MMC, data not shown) controls were also included in these experiments.

Exponential phase cultures of TA98 or WP2s(λ) were centrifuged, resuspended and incubated at 37 °C for 25 min with 0.0, 0.25, 0.5, 1.0, 5.0, or 10 mg/tube BE. Each mixture was then diluted in M9 buffer and 0.1 mL of each treatment poured onto Petri dishes with Nutrient Agar or LB, for TA98 and *E. coli*, respectively. The results were

expressed as a Survival Fraction (SF), obtained as the rate between colonies of the treated culture and colonies of the untreated culture.

RESULTS

Ames test

The mutagenic activity of BE was evaluated using the tester strains TA97a, TA98, TA100, and TA102 with or without S9 mix. All the results were generated by four independent experiments carried out in duplicate. A positive result is generally considered when (i) MI in one or more treatment groups is higher than in the control group by a given multiple, such as twice the negative control MI (positive results = MI in treatment group ≥ 2 spontaneous MI), and (ii) a dose-response relationship exists for at least several doses (Maron, Ames, 1983). Mutagenic Index (MI) as a function of concentrations of 0.25 to 10 mg/tube BE (with or without S9 fraction) is shown in Figures 1, 2, 3 and 4. All tested strains had similar profiles and the MI did not reach twice the respective numbers of spontaneous revertants.

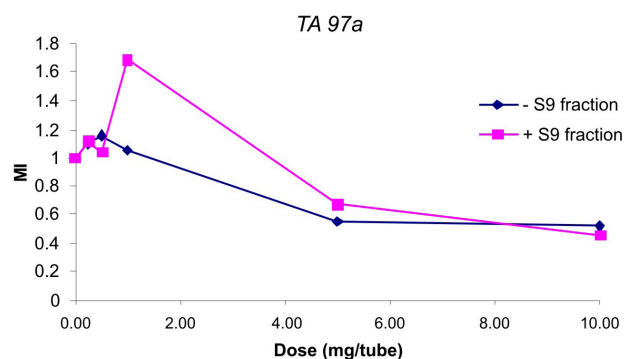


FIGURE 1 - Mutagenic Index (MI) as a function of dose (mg/tube) of “barbatimão” extract (BE), with or without S9 fraction on TA97a. The maximum coefficient of variation (CV) of experiments was 10%. Plots represent average of four independent experiments.

The highest MI value was 1.74 (TA102, 0.5 mg/plate in the presence of metabolic activation), suggesting that neither BE nor its metabolites exhibited a mutagenic profile at any of the tested doses. Nevertheless, bacterial survival was considerably affected, as can be observed in Figure 5.

SOS-Inductest

To determine whether BE itself or its metabolic derivatives would act as inducing agents, the lysogens

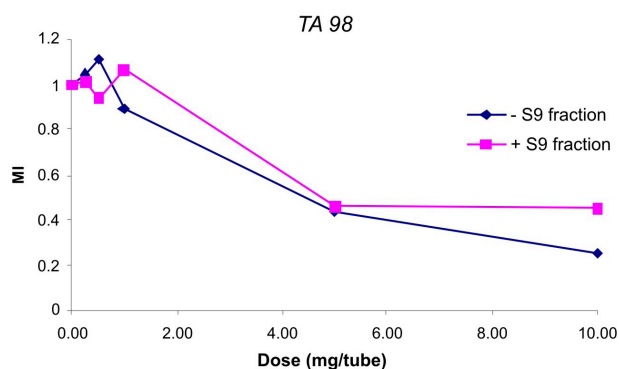


FIGURE 2 - Mutagenic Index (MI) as a function of dose (mg/tube) of “barbatimão” extract (BE), with or without S9 fraction on TA98. The maximum coefficient of variation (CV) of experiments was 10%. Plots represent average of four independent experiments.

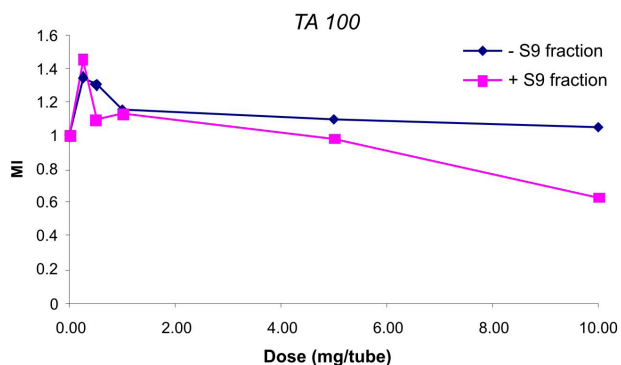


FIGURE 3 - Mutagenic Index (MI) as a function of dose (mg/tube) of “barbatimão” extract (BE), with or without S9 fraction on TA100. The maximum coefficient of variation (CV) of experiments was 10%. Plots represent average of four independent experiments.

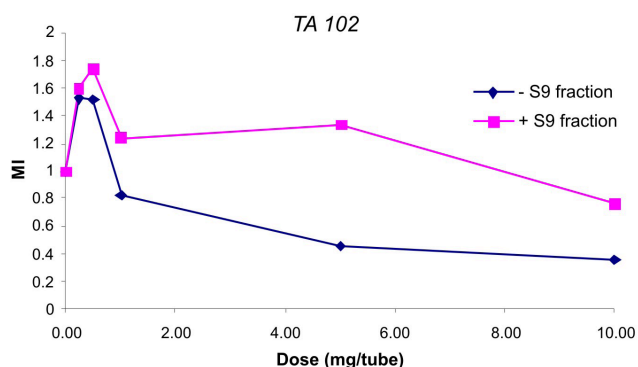


FIGURE 4 - Mutagenic Index (MI) as a function of dose (mg/tube) of “barbatimão” extract (BE), with or without S9 fraction on TA102. The maximum coefficient of variation (CV) of experiments was 10%. Plots represent the average of four independent experiments.

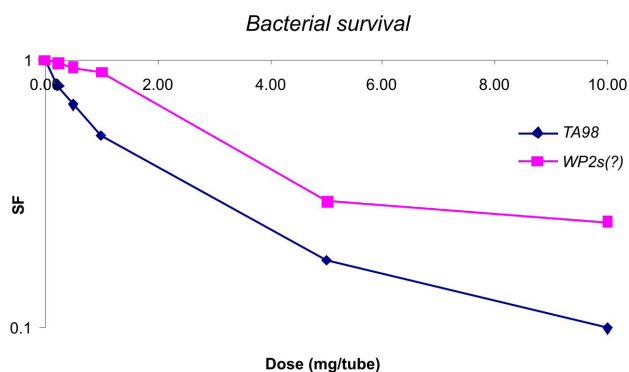


FIGURE 5 - Survival Fraction (SF) as a function of dose (mg/tube) of “barbatimão” extract (BE) on *S. typhimurium* TA98 and *E. coli* WP2s(λ). The maximum coefficient of variation (CV) of experiments was 10%. Plots represent the mean of four independent experiments.

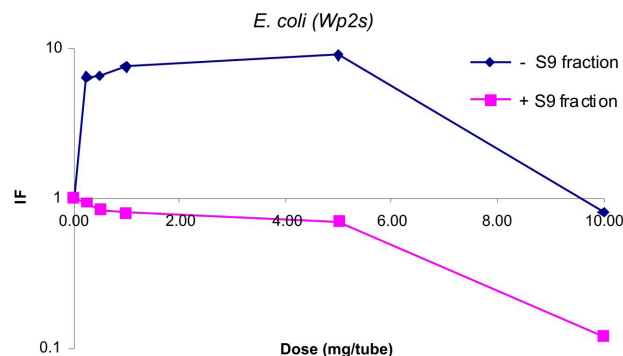


FIGURE 6 - Induction Factor (IF) as a function of dose (mg/tube) of “barbatimão” extract (BE), with or without S9 fraction, on *E. coli* WP2s(λ). The maximum coefficient of variation (CV) of experiments was 10%. Plots represent average of four independent experiments.

were incubated in different doses of BE. The IF induced by BE, in the absence or presence of S9 fraction, is shown in Figure 6.

We observed an increase in the number of plaques as a function of BE dose and the maximum induction was observed at the dose of 5 mg/tube, which resulted in a 9-fold IF value compared to the negative control. The metabolized effect, however, did not increase the induction of the prophage λ . The IF at the dose of 10 mg/tube in the absence of S9 remained at the levels of the negative control. Bacterial survival was reduced by the treatment with BE, as observed in Figure 5.

SOS-Chromotest

When applying the SOS-Chromotest, genotoxic effects are assumed to exist whenever the induction

surpasses a defined level, and a positive result is generally considered when (i) the enzymatic ratio increases at least 0.5 compared to the negative control, and (ii) a dose-response relationship exists for at least several doses. The decrease in the constitutive ap levels reflects the cytotoxicity of a compound while the augmentation of the inducible SOS-dependent β g activity shows its mutagenic potential (Von Der Hude *et al.*, 1988). In the present study, BE did not induce SOS mutagenesis when the SOS-Chromotest was applied, neither in the absence nor in the presence of metabolic activation (Figures 7 A and B).

In the absence of the S9 mix, even at the initial doses, BE inhibited the ap enzyme while SOS-dependent β g activity decreased at almost the same level. Nevertheless, when S9 mix was added to the complex, the cytotoxic effect of BE was attenuated and remained almost at the levels of ap U (enzymatic units) of the negative control. On the other hand, the enzyme U levels in the β g assay in the presence of S9 fraction recovered slightly, and were clearly seen at the dose of 0.5 mg/tube).

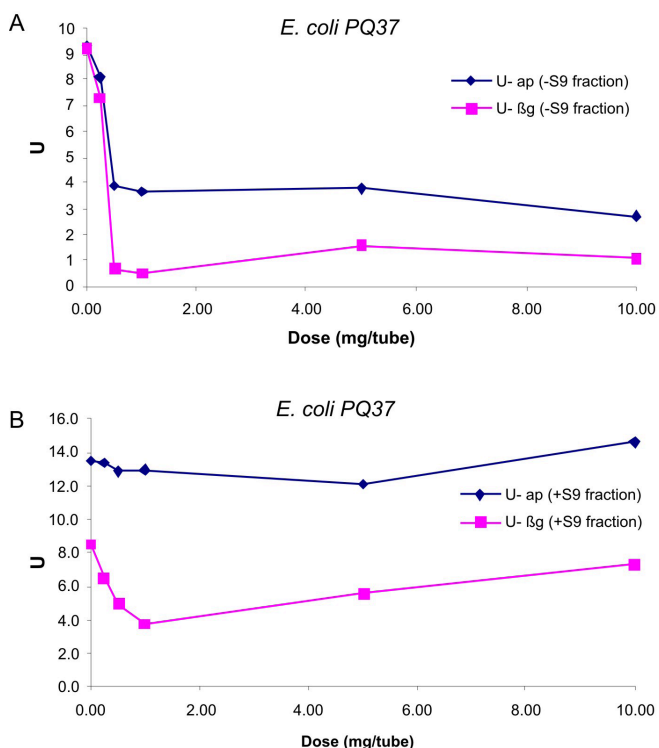


FIGURE 7 - Enzymatic Units (U) of enzymes β -galactosidase (β g) and alkaline phosphatase (ap) as a function of dose (mg/tube) of “barbatimão” extract (BE), in the absence (A) or presence (B) of S9 fraction on *E. coli* PQ37. The maximum coefficient of variation (CV) of experiments was 10%. Plots represent average of four independent experiments.

DISCUSSION

Although there are differences in metabolism, DNA repair, and other physiological processes affecting chemical mutagenesis according to species, the universality of DNA and the genetic code provides a rationale basis for using various non-human test systems aimed at predicting the intrinsic mutagenicity of the chemicals tested. It is known that a great variety of genetic events may lead to mechanisms that are likely to induce carcinogenicity and cytotoxicity. However, nowadays there is no specific test that can be used alone to detect the full spectrum of different endpoints that reveal all manifestations of genotoxic actions (Dearfield *et al.*, 2002).

The Ames test was employed in this study, a test which is widely accepted to detect substances that can produce genetic damage leading to gene mutations. The *Salmonella* strains we employed are able to detect both frameshift mutations (TA97a and TA98) and base-pair substitutions (TA100 and TA102) (Mortelmans; Zeiger, 2000). Nevertheless, BE did not induce direct or indirect mutagenicity (Figures 1, 2, 3, and 4). Cytotoxicity was also observed on TA98 (Figure 5).

Agents that inhibit DNA replication in *E. coli* trigger the expression of a series of cellular functions which notably, lead to prophage λ induction. This property is explored in the SOS-Inductest and has been used to screen potential chemical carcinogens and antitumor antibiotics (Anderson *et al.*, 1980). We observed an increase in IF as a function of BE dose, except for the dose of 10 mg/tube (Figure 6). A reasonable explanation for this finding is the high cytotoxicity observed at this concentration of BE (Figure 5). However, the metabolized effect did not increase the induction of prophage λ . Therefore these results suggest that BE possesses direct mechanisms of genotoxicity and its metabolic activation by the post-microsomal S9 fraction is able to suppress the genotoxic effect, probably due to detoxification of some extract components.

The SOS-Chromotest reflects a possible aspect of the mutational process: error-prone DNA repair. It is also assumed that the majority of the cells respond when exposed to a mutagen (Rosenkranz, Mersch- Sundermann, Klopman, 1999). BE was not able to induce SOS mutagenesis in the SOS-Chromotest, with or without the oxidative metabolism (Figures 7A and 7B). In the absence of S9 mix, BE early inhibited ap and SOS-dependent β g activity also decreased. In spite of this, when S9 mix was added to the complex, the cytotoxic effect of BE was attenuated and remained almost at the same levels of ap U of the negative control. On the other hand, enzyme U levels in the β g assay in the presence of S9 fraction were not as low as they

were in its absence. This could be explained by the fact that the number of mutational events, and consequently the enzyme U levels in the β g assay, is affected by the number of viable cells. Our results suggest that BE possesses direct mechanisms of cytotoxicity and no genotoxic activity detectable by this test.

These data show that BE did not exhibit considerable mutagenicity. However, it presented some genotoxicity revealed by an increase in prophage λ induction data using the SOS-Inductest. A toxic effect was also observed for the strains employed in the three assays, especially on the SOS-Chromotest, since the constitutive enzyme ap was early inhibited. According to Houk and DeMarini (1988), differences in detection sensitivity could be at least partially explained by the fact that phage induction and the SOS response in general (i.e. prophage λ induction of the Inductest) are broader genetic endpoints than reverse mutation in bacteria (i.e. Ames test) because they occur due to a variety of mechanisms and involve a number of classes of genetic damage, including alkylations, intercalations, strand scissions, oxidative deaminations, and crosslinks. In contrast, there is a close correlation between the results yielded by the SOS-Chromotest and the Ames test. The capacity of the Ames test to identify carcinogens is higher than that of the SOS-Chromotest. However, because the number of false positive compounds is lower in the SOS-Chromotest, it is suggested that both tests must be used so that one can complement the other (Quillardet, Hofnung, 1993). Thus, the concurrent use of these tests improves the sensitivity of the screening procedure in the evaluation of plants commonly used in folk medicine.

We suggest that the decrease in enzyme U levels observed in the SOS-Chromotest and the genotoxic activity detected in the SOS-Inductest could be at least partially affected by the tannin fractions commonly found in *S. adstringens* bark. These tannin fractions possess a well known ability to bind to proteins (leading to the formation of large protein aggregates) and precipitate (Naczek *et al.*, 2001; Silber *et al.*, 1998), properties that could affect both the enzymatic activity and the DNA replication by the formation of crosslinks with required proteins. Literature data also support that tannic acid and its hydrolyzed products lack mutagenic activity in *Salmonella* tester strains (Chen, Chung, 2000). When the *S. adstringens* extract was tested in eukaryotic systems, using the micronucleus test in mice bone marrow (Mota, 1997) and the SMART test in *Drosophila melanogaster* (Sousa *et al.*, 2003), conflicting results were obtained, inasmuch as the genotoxic effect of BE was reported in mice, but not in fruit flies. In contrast to our results, the

findings could reflect some divergence in the *in vivo* metabolism of the extract constituents.

In conclusion, according to our results, *S. adstringens* presented cytotoxic activity in all tested systems, did not present mutagenic activity, but demonstrated some genotoxic effects. Moreover, the metabolization of the extract of this plant reduced its genotoxic and cytotoxic activities. Taken together, the data available on the genotoxicity of *S. adstringens* are not fully conclusive and thus further *in vitro* and *in vivo* studies are needed to clarify its mechanisms of action and to better determine its risk for human consumption.

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