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Research Article

Genotoxic effects of *Tabebuia impetiginosa* (Mart. Ex DC.) Standl. (Lamiales, Bignoniaceae) extract in Wistar rats

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Abstract

Tabebuia sp. is native to tropical rain forests throughout Central and South America. Although the biological and pharmacological effects of bark extracts have been intensely studied, little is known on the extract obtained from the flower. Herein, the genotoxic potential of a flower extract from *T. impetiginosa* ("ipê roxo") on the blood and liver cells of Wistar rats was evaluated. Experimental procedures involved only male animals. Graduated concentrations of the extract, viz., 100, 300 and 500 mg kg^{-1} of body weight, were gavage-administered and 24 h latter cells were collected and processed for analysis. With the exception of the 100 mg kg^{-1} dose, a significant increase in DNA damage was noted, when compared with a negative control group. Although the genotoxic potential of this extract was higher in liver cells, the response in both tissues was related to dose-dependency. Even though DNA damage can be corrected before conversion into mutations, further study is recommended to arrive at a better understanding of incurred biological effects.

Key words: Tabebuia impetiginosa, comet assay, Wistar rats, natural compounds.

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Introduction

Over the centuries, plants have been the main source of crude drugs used in the cure or alleviation of human sicknesses (Skalani and Kutty, 2008). In America, herbal therapies, together with various other traditional remedies, are generally classified under the heading of "alternative medicine". The combination of this medical form, mainly involving the aforementioned traditional and folk remedies used worldwide, with conventional western medicine is termed "integrative medicine" (Itokawa *et al.*, 2008).

Tabebuia spp. (Bignoniaceae) is a native of Tropical Rain Forests throughout Central and South America (Son *et al.*, 2006). Extracts from the bark of this plant are known as "taheebo", "lapacho", "pau d'arco" or "ipê". Their most important components include naftoquinones, quinines, furanonaftoquinones, bezoic acid, ciclopentenes dialdehydes and flavonoids (Sharma *et al.*, 1988; Koyama *et al.*, 2000). These extracts are traditionally used in the treatment of ulcers, syphilis, gastrointestinal problems, candidiasis, cancer, diabetes, prostatitis, constipation and allergies (Park *et al.*, 2006). More recently, Melo *et al.* (2011) revised several of those medicinal plants employed in ethnobotanic research in Brazil that presented certain antitumor effects. Among the 84 studied, *T. impetiginosa* was the second most cited in ethnopharmocological literature, in the treatment or prevention of cancer and tumors (Melo *et al.*, 2011).

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Evidence on the biological effects of plant extracts is constantly on the increase. Nevertheless, their composition, apparently involving only beneficial properties, may include chemical components with mutagenic, teratogenic or carcinogenic activities (Santos *et al.*, 2008). If a genotoxic compound is present, it is capable of interacting with DNA molecules, thereby leading to genetic damage in regions of fundamental importance for cycle-control and apoptosis, with the subsequent initiation of neoplastic processes (Santos *et al.*, 2008). Whereby, the extreme importance of including a genotoxic approach in the toxicological evaluation of therapeutical compounds.

Numerous endpoints are commonly used in toxicological genetics. The alkaline version of Comet assaying, or rather Single Cell-Gel Electrophoresis (SCGE), represents a rapid, visual and quantitative technique for measuring DNA damage in eukaryote cells, such as single and double-strand breaks, as well as incomplete repair and alkali labile sites, and DNA-protein and DNA-DNA cross-links, in virtually any eukaryotic cell population obtainable as a single-cell suspension (Burlinson *et al.*, 2007). Although this method is not included among the current genotoxicological tests recommended by the Organization for Economic Co-operation and Development (OECD), interna-

tional expert groups have published recommendations describing standards for comet assaying based on the current OECD guidelines for other *in vivo* genotoxicity tests, such as MN assay (Tice *et al.*, 2000; Hartmann *et al.*, 2003).

Accordingly, in the present study, the genotoxic activities of a *Tabebuia impetiginosa in vivo* extract were assessed, using blood and liver cells from Wistar rats. The extent of DNA damage was detected through Comet assay.

Material and Methods

Chemical compounds and extracts

Normal melting point agarose, low melting point agarose, Ethylenediamine tetraacetic acid (EDTA), Tris base, ethidium bromide and dimethyl sulfoxide (DMSO) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin (DXR) was purchased from Pharmacia (Brazil, Ltda), for dissolution in distilled water, immediately preceding use as positive control.

The *Tabebuia impetiginosa* extract, kindly donated by the Chemical Research Group of the University of Franca, Franca, São Paulo State, Brazil, was dissolved in DMSO (10% in distilled water) just prior to use. The inflorescences used in the preparation of the extract were collected from "ipê roxo" (*T. impetiginosa* Standl., Bignomiaceae) trees, in Franca. Drying in an air-circulating oven at 60 °C gave origin to 1.1 kg of dried material in powder form. After four weeks, 60.1 g of hydroalcoholic extract was obtained by macerating. This was refrigerator stored until use. On the occasion, the extract was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10% of the solution. The desired concentrations (100, 300 and 500 mg kg^{-1} per b.w. of the animal) were based on the dosage used in pertinent analgesic activity in rats, according to a project developed in the University (unpublished data).

Animals and treatments

Experiments with Wistar rats were carried out with the approval of the University of Franca Ethics Committee (process 007/07A). Thirty male Wistar rats, each weighing 100 g, were acclimatized for 3 days prior to the experiments. Maintenance was under controlled conditions of temperature (24 ± 1 °C) and humidity (55 ± 5%), in a 12:12 h light/dark cycle, with water and a commercial meal (Purina®) *ad libitum*.

The experimental design comprised 6 groups treated for 24 h prior to euthanasia. Group 1 (negative control) mice received only drinking-water (0.5 mL by gavage), Group 2 (positive control), a single injection of doxorubicin (90 mg kg^{-1} b.w., i.p.), Groups 3-5, treatment by gavage (0.5 mL) with solutions of *T. impetiginosa* extract (100, 300 and 500 mg kg^{-1} b.w., respectively), and Group 6 (solvent control), treatment by gavage (0.5 mL) with DMSO 10% (v/v) in distilled water. Euthanasia was by an overdose of thiopentone sodium (45 mg kg^{-1} b.w.).

Sample collection

Blood samples were collected from the tail into heparinized microtubes. An aliquot of 10 μL each was used for comet assaying. Immediately after collection, the animals underwent euthanasia. Liver cells, obtained by excising a single fragment (approximately 1 g) from the right lobe, were washed in NaCl (0.9%) and minced in 1 mL of cold Hanks solution (pH 7.4, DMSO 10% and EDTA 20 mM). Aliquots of 30 μL of cell suspension were used in comet assaying. The samples, which were kept on ice as previously recommended (Collins *et al.*, 2008), underwent trypan blue exclusion staining for cell viability.

Comet assay

Evaluation of the extent of DNA damage in both blood and liver cells was by way of the alkaline version of comet assaying (Singh *et al.*, 1988). Briefly, the cell suspension was first homogenized with 100 μL of low melting point agarose (0.5%), then spread onto slides previously coated with normal melting point agarose (1.5%), and finally covered with a coverslip. After 5 min at 4 °C, the coverslip was removed, and the slides immersed in a cold lysis solution (2.4 M NaCl; 100 mM EDTA; 10 mM Tris, 10% DMSO and 1% Triton-X, pH 10) for 24 h. Subsequently, they were placed into an electrophoresis chamber and covered with an electrophoresis buffer (300 mM NaOH per 1 mM EDTA, pH > 13) for an additional 20 min in order to facilitate DNA unwinding. Electrophoresis proceeded for 20 min (1 V/cm and 300 mA) and the slides were then submerged for 15 min in neutralization buffer (0.4 M Tris-HCl, pH 7.5), dried at room temperature and fixed in 100% ethanol for 5 min. Slides were stained with 20 μg/mL ethidium bromide immediately before analysis and prepared in duplicate. 100 cells were screened per sample (50 from each slide) by means of a fluorescent microscope (Zeiss, Germany) equipped with an excitation filter of 515-560 nm, a barrier filter of 590 nm and a 40X objective. Nuclei were visually classified according to fragment migration, as follows: class 0 (no damage), class 1 (little damage, with tail-length less than the diameter of the nucleus), class 2 (medium damage, with tail-length one to two times the diameter of the nucleus), class 3 (significant damage with tail-length between two and a half to three times the diameter of the nucleus), and class 4 (very significant damage with tail-length more than three times the diameter of the nucleus). This scoring system was based on extensive recommendations in the literature (Tice *et al.*, 2000; Collins *et al.*, 2008; Liao *et al.*, 2009).

Statistical analysis

Experimental data are expressed as means ± SD of 5 animals. Differences among groups treated with different concentrations of *T. impetiginosa* in comparison to nega-

tive and positive control were evaluated by Student-t testing with GraphPad Prism 4.1.

Results

The results obtained in blood cells of Wistar rats after 24 h of treatment with *T. impetiginosa* extract and the respective controls (negative and positive), appear in Table 1. Statistical analysis of significant differences between groups treated with the extract (100, 300 and 500 mg kg^{-1} b.w.) and the negative control ($p < 0.05$), indicated a dose-dependent response.

DNA damage in nucleoids of liver cells was significantly higher in the groups treated with the extract than in the negative control ($p < 0.05$) (Table 2). Moreover, the response to treatment with *T. impetiginosa* was dose-dependent. DNA damage was higher in the highest concentration tested (500 mg kg^{-1} b.w.) than in the positive control ($p < 0.05$). According to the DNA damage index, damage in the DMSO group, though higher than in the negative control, was not significantly so (Table 2). The distribution of comet nucleoids did not differ in any of the groups (negative control and DMSO) (Figure 1).

Distribution of the various levels of DNA damage observed in comet nucleoids is presented in Figure 1. The frequency of comet nucleoids at levels 2 and 3 was higher in the liver than in the blood. DNA damage in the blood at level 1 accounted for 50% of damaged nucleoids. Taken as a whole, in the liver, frequencies at levels 2, 3 and 4 were higher than at level 1.

Discussion

The active principles present in extracts from a variety of plant sources from tropical and sub-tropical regions of the world, have been studied for their possible application in human-health treatment (Aruoma, 2003). For many years, stem-bark of the South American tree *Tabebuia impetiginosa* has been extensively used in both North and South America as an anticancer, antifungal, antibacterial and anti-inflammatory drug (Abbott *et al.*, 1967; Zani *et al.*, 1991).

Genotoxicity assaying is a means of evaluating the capacity of different compounds to induce genetic damage, such as single- and double-strand breaks, chromosomal aberrations and genetic mutations. This can be done *in vitro* or

Table 1 - Distribution of comet cells (mean ± SD) and DNA damage index in blood cells of Wistar rats treated with *T. impetiginosa* extract.

Treatments	Comet cells					DNA damage index[a]
	Level 0	Level 1	Level 2	Level 3	Level 4	
Neg. control	78.0 ± 8.7	18.0 ± 7.4	3.0 ± 0.8	1.0 ± 0.8	0	27.0
DMSO	88.3 ± 7.6	5.8 ± 1.7	3.8 ± 4.3	2.3 ± 2.6	0	20.3
DXR	39.8 ± 8.1	22.8 ± 5.8	17.8 ± 7.7	11.8 ± 5.3	8.0 ± 2.9	125.8[b]
TI 100	44.2 ± 30.7	28.2 ± 14.5	21.2 ± 30.1	3.8 ± 4.6	2.6 ± 4.0	92.4
TI 300	22.4 ± 13.0	43.2 ± 9.3	26.0 ± 9.7	5.4 ± 6.0	3.0 ± 4.1	123.4[b]
TI 500	33.0 ± 12.5	31.8 ± 14.3	14.4 ± 5.4	10.6 ± 5.4	10.2 ± 3.5	133.2[b]

[a]DNA damage Index = $(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)$, where n = number of cells at the corresponding level of DNA damage (0-4).
[b]significantly different when compared to negative control ($p < 0.05$).
DXR = 90 mg kg^{-1} b.w.; DMSO = 10%; TI: *Tabebuia impetiginosa* extract (mg kg^{-1} b.w.).

Table 2 - Distribution of comet cells (mean ± SD) and DNA damage index in liver cells of Wistar rats treated with *T. impetiginosa* extract.

Treatments	Comet cells					DNA damage index[a]
	Level 0	Level 1	Level 2	Level 3	Level 4	
Neg. control	66.3 ± 5.3	22.3 ± 6.8	7.8 ± 3.0	2.8 ± 1.0	0	46.3
DMSO	24.0 ± 11.5	51.0 ± 11.7	15.0 ± 0.8	9.0 ± 5.0	1.0 ± 1.2	112.0
DXR	33.5 ± 9.0	19.5 ± 5.4	11.8 ± 5.4	26.3 ± 6.8	9.0 ± 2.8	158[b]
TI 100	27.8 ± 18.2	31.2 ± 6.8	16.4 ± 10.7	14.8 ± 8.1	9.8 ± 9.0	147.6[b]
TI 300	14.8 ± 13.2	32 ± 10.4	28.4 ± 14.0	22 ± 7.1	2.8 ± 2.7	166.0[bc]
TI 500	9.8 ± 9.6	22.2 ± 17.3	13.2 ± 2.2	37.2 ± 24.2	17.6 ± 7.9	230.6[bd]

[a]DNA damage Index = $(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)$, where n = number of cells at the corresponding level of DNA damage (0-4).
[b]significantly different when compared to negative control ($p < 0.05$).
[c]$0.001 < p < 0.01$.
[d]$p < 0.001$.
DXR = 90 mg kg^{-1} b.w.; DMSO = 10%; TI: *Tabebuia impetiginosa* extract (mg kg^{-1} b.w.).

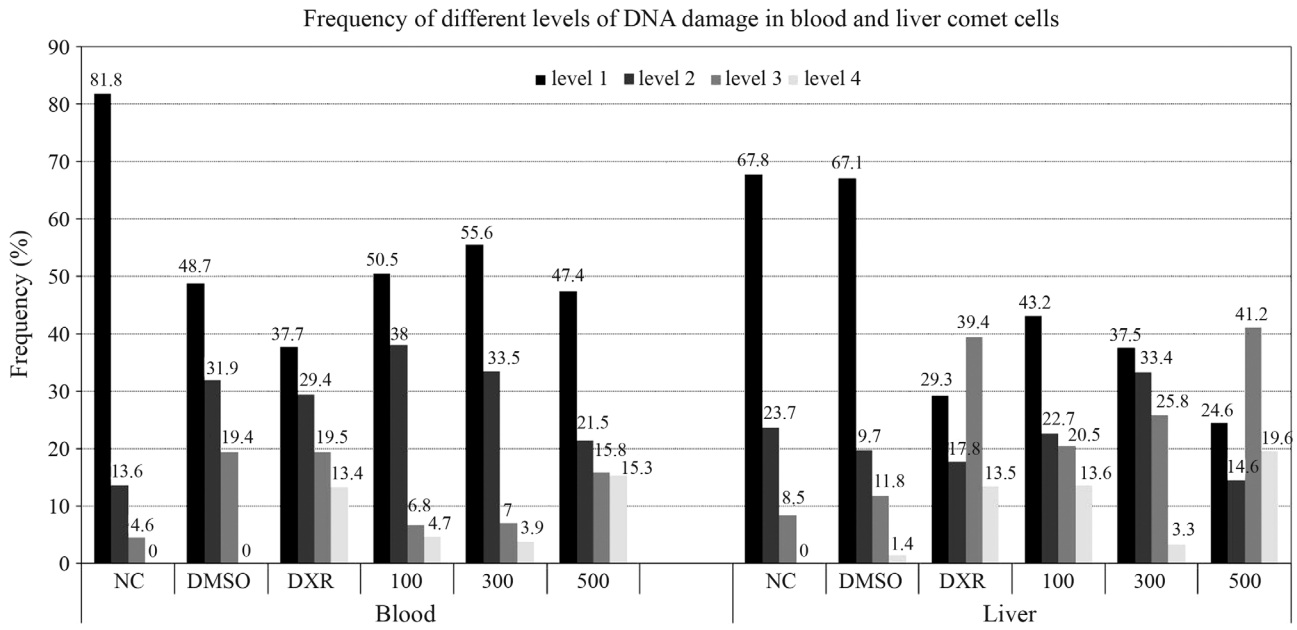

Figure 1 - Distribution of comet cells according to the level of DNA damage detected in blood and the liver. Nucleoids in *class 0*, *i.e.*, no DNA damage, were not shown. NC: negative control; DMSO: solvent control; DXR: doxorubicin at 90 mg kg^{-1} b.w.; *Tabebuia impetiginosa* extract at 100, 300 and 500 mg kg^{-1} b.w.

in vivo by way of comet assaying, a quick, simple and highly sensitive method for detecting single and double DNA strand breaks and alkali-labile sites under conditions of exposure to a chemical or physical compound with genotoxic potential (Gontijo and Tice, 2003; Kumaravel and Jha, 2006). During the battery of assays for genotoxic evaluation, it is important to include negative, positive and solvent control groups (Tice *et al.*, 2000). One of the most important advantages of the comet assay is its applicability to various tissues and organs simultaneously, through independence of cell division for revealing the extent of DNA damage (Tice *et al.*, 2000; Hartman *et al.*, 2003). In the present study, the genotoxic potential of a *T. impetiginosa* extract on liver and blood cells was evaluated. As the liver is the preeminent organ in metabolization, according to the different protocols for comet assaying *in vivo*, analysis of its cells is recommended (Tice *et al.*, 2000; Hartman *et al.*, 2003). Even so, in the present study, blood cells were also used, through representing a potential target for genotoxic compounds, independent of the parameter considered for analysis (Groover *et al.*, 2001; Rodeiro *et al.*, 2006).

The analysis of liver and blood cells at 24 h after treatment revealed highly extensive DNA damage when compared to the negative control, the effect being more pronounced in the former. Post analysis of the genotoxic effects of Stevia, a naturally sweeter compound extracted from *Stevia rebaudiana*, also revealed higher levels of DNA damage in the liver than in other tissues such as the brain, spleen and blood (Nunes *et al.*, 2007). The genotoxic and antigenotoxic effects of *T. impetiginosa* were evaluated with the Somatic Mutation and Recombination Test (SMART) (De Sousa *et al.*, 2009). These authors had previously noted that the plant, although not genotoxic in itself, was so through potentially inducing DXR genotoxicity. They also evaluated the antigenotoxic effects, without finding any significant results. The methanolic extract of the plant, especially the chloroform fraction, was capable of attenuating platelet aggregation and vascular smooth-muscle-cell proliferation, probably through the suppression of arachidonic acid liberation and ERK1/2 MAPK activation (Son *et al.*, 2006).

In the present case, collection was 24 h after treatment with the *T. impetiginosa* extract, as previously recommended for *in vivo* comet assaying (Tice *et al.* (2000). In a previous study, an increase in DNA damage had been detected by comet 3, 24 and 48 h after treatment in various organs, this including blood and the liver (Gary *et al.*, 2003). Even so, the maximum DNA damage was observed after intermediate exposure of 24 h in all the tested organs. Irrespective of the high level of DNA damage in the blood and liver cells encountered here, the mechanisms involved are unrecognizable. It is well known that *T. impetiginosa* is a naturally occurring source of naphthoquinones, previously extensively described by way of their genotoxic activities, through the generation of reactive oxygen species, inhibition of topoisomerase II and activation of topoisomerase I (Boothman *et al.*, 1989; Klaus *et al.,* 2010).

According to the findings presented here, *T. impetiginosa* extract presented high genotoxic activity in Wistar rats, whereby its use in alternative medicine requires caution. Further *in vivo* investigation is proposed to elucidate the mechanisms by which this compound induces genotoxic consequences.

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