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Evaluation of extract from leaves of *Calophyllum brasiliense* Cambess. (Clusiaceae) using micronucleus assay in mouse bone-marrow cells

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This study evaluated the chomosomal damage potential of the extract from leaves of *Calophyllum brasiliense* Cambess. (Clusiaceae) in mouse bone-marrow cells. The extract was analyzed by high-performance liquid chromatography (HPLC) to assess the concentration of coumarin (-) mammea A/BB, a compound with antileishmanial activity. The chromosomal damage potential of the extract was evaluated by the micronucleus test in erythrocytes of mouse bone marrow. The animals were treated with cyclophosphamide (50 mg) as positive control, DMSO (1%) as negative control, or the crude extract from *C. brasiliense* (100 or 200 mg/kg). The HPLC analyses showed that the extract contained 25.97 ± 0.91 µg of (-) mammea A/BB per mg of extract. The extract of *C. brasiliense* did not show chromosomal damage potential at the concentrations used for the preparation of creams with antileishmanial activity. This study may contribute to the registration of a topical phytomedicine containing the extract of *C. brasiliense* to treat cutaneous leishmaniasis.

Key words: *Calophyllum brasiliense*, genotoxicity assay, (-) mammea A/BB, high-performance liquid chromatography (HPLC), antileishmanial activity, medicinal plants.

INTRODUCTION

Calophyllum brasiliense Cambess. (Clusiaceae), popularly known as "guanandi", is a large tree that grows mainly in the Brazilian Atlantic rainforest, and is distributed in South America from Brazil to Mexico. This tree has been used in folk medicine for the treatment of several diseases such as rheumatism, varicose veins, hemorrhoids and chronic ulcers (Corrêa et al., 1978).

Previous studies showed that *C. brasiliense* is a rich

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source of bioactive compounds, including coumarins (Ito et al., 2003; Reyes-Chilpa et al., 2004), xanthones (Sartori et al., 1999; Ito et al., 2002), triterpenoids (Reyes-Chilpa et al., 2004), and biflavanoids (Da Silva et al., 2001).

The compounds isolated from *C. brasiliense* showed cytotoxic activity against some tumor cell lines (Kimura et al., 2005; Ito et al., 2006), mainly the coumarin mammea A/BB (Reyes-Chilpa et al., 2004; Ruiz-Marcial et al., 2007). Previous studies have also demonstrated that extracts, fractions, and especially the coumarin (-) mammea A/BB isolated from *C. brasiliense* leaves show significant molluscicidal activity against *Biomphalaria glabrata* (Gasparotto et al., 2005) and potent *in vitro* and *in vivo* leishmanicidal activity against *Leishmania amazonensis* and *Leishmania braziliensis* (Brenzan et al., 2007; 2008a; Honda et al., 2010; Tiuman et al., 2012). Honda et al. (2010) demonstrated that footpad lesions of mice infected with *L. amazonensis* decreased in size when the mice were treated topically with 10% dichloromethane extract or intraperitoneally with 100 mg/kg and 200 mg/kg of the hexane fraction of *C. brasiliense*.

This study contributed to the development of a topical phytomedicinal formulation containing compounds isolated from *C. brasiliense,* to treat cutaneous leishmaniasis (patent BR200904042-A2). Intraperitoneal administration of the extract at doses of 100 and 200 mg/kg also revealed healing lesions. The parasite load in the popliteal lymph nodes was significantly reduced in treated animals, showing that the plant components actually control infection (Honda et al., 2011). Other studies have also demonstrated the antileishmanial activity of the (-) mammea A/BB derivatives against *L. amazonensis* (Brenzan et al., 2012). Mammea A/BB isolated from *C. brasiliense* leaves showed trypanocidal activity against *Trypanosoma cruzi* (Reyes-Chilpa et al., 2008). Considering these effects, a method for quantitative analysis of the biologically active compound (-) mammea A/BB in the extract from *C. brasiliense* by means of high-performance liquid chromatography (HPLC) was validated by Brenzan et al. (2010).

Taken together, the results of the studies involving biological effects, mainly the antileishmanial activity of this plant, led us to evaluate the potential for chromosomal damage of the extract from *C. brasiliense* leaves, in erythrocytes from mouse bone marrow

MATERIALS AND METHODS

Plant

Aerial parts of *C. brasiliense* were collected at the Botanical Institute of São Paulo, Brazil, on 13/09/2012. The plant material was identified by Prof. Dr. Maria Claudia M. Young, and a voucher specimen (SP 363818) was deposited and authenticated at the herbarium of this institute. The leaves were dried at 45°C in a circulating-air oven and triturated in a knife mill (Usi Ram®), and the resulting powder was stored in a dry location in the dark.

Plant extraction

The powdered leaves (825.0 g) were extracted by exhaustive maceration in ethanol/water (9:1) at room temperature, until depleted of all compounds. The extract was filtered and concentrated under vacuum at 40°C to obtain an aqueous extract and a dark-green residue. The residue from the extract, stored in glass bottles, was dissolved with dichloromethane, and the solvent was then completely evaporated at room temperature; this extract was termed the crude extract (31.5 g). The aqueous extract was lyophilized (148.0 g). Both were stored at -10°C, in the dark, until use (Brenzan et al., 2007).

HPLC analyses

The HPLC analyses of the crude extract from *C. brasiliense* leaves were carried out according to Brenzan et al. (2010). A Shimadzu LC-10 liquid chromatography equipped with a quaternary pump (LC-10 AD), automatic injection valve (Rheodyne) with a 20 ml loop, degasser (DEU-14), thermostatted column compartment from (CTO-10Avp), and a detector UV/vis (SPD-10A), controlled by the software CLASS LC-10 were used. The sample extracts were prepared in methanol at 3 mg/ml and filtered through a Millex® 0.45 µm membrane filter (Millipore, Brazil); then, 20 µl was injected into the HPLC system. A Metasil ODS column, 5 mm, 150 × 4.6 mm maintained at 30°C was used in the chromatographic analysis. The separation was performed in a gradient system, using as mobile phase a mixture of acetonitrile-water 5:95 v/v at 55:45 (0 to 10 min), 55:45 v/v at 80:20 (10 to 20 min), 80:20 v/v at 100% acetonitrile (20 to 30 min), and 100% acetonitrile (30 to 40 min), with a flow rate of 0.6 ml/min. The detection of the compounds was carried out at 254 nm and the run time was 40 min. Three determinations were carried out for each sample.

Animals and treatments

The experimental protocol was approved by the Animal Ethics Committee of Maringá State University (protocol number 037/2011).

The *in vivo* assay for chromosomal damage was carried out in female albino Swiss mice, approximately 7 to 8 weeks old and with a mean weight of 39.8 g. The mice were obtained from the Animal Facility of the State University of Maringá. The animals were kept in polyethylene cages with controlled temperature (25°C) in a 12/12 h light-dark cycle. Food and water were provided *ad libitum*. The feed was a standard commercial rodent chow (Nuvilab®).

The mice were housed in groups of six per cage and randomly divided into four groups, which received via orogastric gavage: (1) 1% DMSO (negative control), (2) 50 mg/kg of Cyclophosphamide (positive control), (3) 100 mg/kg of crude extract from *C. brasiliense* and (4) 200 mg/kg of crude extract from *C. brasiliense*. After 24 h, the animals were anesthetized through intraperitoneal injection of ketamine (60 mg/kg) and xylazine (12 mg/kg). The bone marrow was removed from the femur, and the animals were euthanized.

Micronucleus assay

The genotoxic effects of the *C. brasiliense* extract were evaluated in bone-marrow cells of mice by the micronucleus test, according to Schmid (1975). Immediately after the animals were anesthetized, the femurs were removed. The bone marrow was removed, emulsified, and transferred to centrifuge tubes containing 2 ml of fetal bovine serum (FBS). The bone-marrow suspension was centrifuged at 1000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in one drop of FBS, and one drop of this suspension was applied to a slide to prepare the

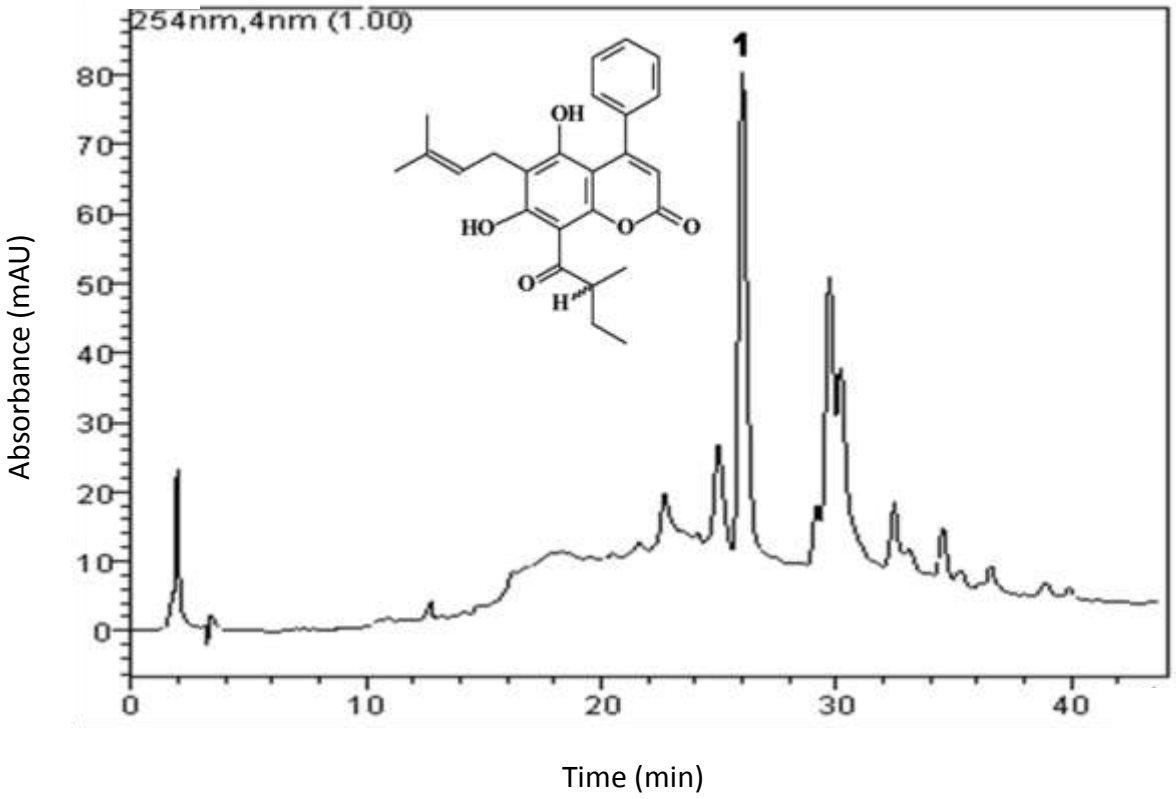

Figure 1. Chromatogram of crude extract from *Calophyllum brasiliense* leaves. (1) (-) mammea A/BB (Rt: 26.2 min). Chromatographic conditions: Metasil ODS column; mobile phase: acetonitrile:water 5:95 v/v at 55:45 (0–10 min), 55:45 v/v at 80:20 (10–20 min), 80:20 v/v at 100% acetonitrile (20–30 min) and 100% acetonitrile (30–40 min); flow rate of 0.6 ml/min; temperature: 30°C; detection: 254 nm.

smears. After 24 h of drying at room temperature, the smears were stained (May-Grünwald Giemsa) and the slides were again dried at room temperature.

The smears were examined under an optical microscope (Olympus, Japan) at 1000 × magnification. The criteria for counting micronuclei were based on the diameter, shape and coloration of micronuclei, according to Krishna and Hayashi (2000). To assess the induction of micronuclei formation (MN), the number of micronucleated polychromatic erythrocytes (MNPCE) in 1000 polychromatic erythrocytes (EPC) per animal and per slide was determined (Schmid, 1975).

Statistical analysis

The results were expressed as mean ± standard deviation (SD), to compare the frequencies of MNPCE between the treated and control groups. The micronucleus assay data were statistically analyzed by ANOVA followed by Tukey's test. P≤ 0.05 was considered statistically significant.

RESULTS

HPLC analysis of the *C. brasiliense* extract

In the chromatogram of the extract from *C. brasiliense* leaves, with a retention time of 26.2 min, the coumarin (-) mammea A/BB was identified as the majority compound (Figure 1). This extract showed a concentration of 25.97 ± 0.91 µg of (-) mammea A/BB per mg of crude extract. The content of this compound in the *C. brasiliense* extract was analyzed according to the methodology proposed by Brenzan et al. (2010), and the results were in agreement with this previous study.

Micronucleus test

The frequencies of micronucleated polychromatic erythrocytes (% MNPCE ± standard deviation (SD)) of the negative control and the groups that received 100 or 200 mg/kg of crude extract of *C. brasiliense* were 2.34 ± 1.6, 1.84 ± 1.2, and 3.0 ± 3.0%, respectively. The frequencies of MNPCE for mice treated with 100 or 200 mg/kg of the crude extract were similar to the negative control (Tukey's ANOVA Test, p ≤ 0.05). The frequency of MNPCE for the positive control group (16.83 ± 8.4%) (Figure 2) differed significantly (p ≤ 0.05) from the negative control group and from the groups treated with 100 or 200 mg/kg of the crude extract of *C. brasiliense*

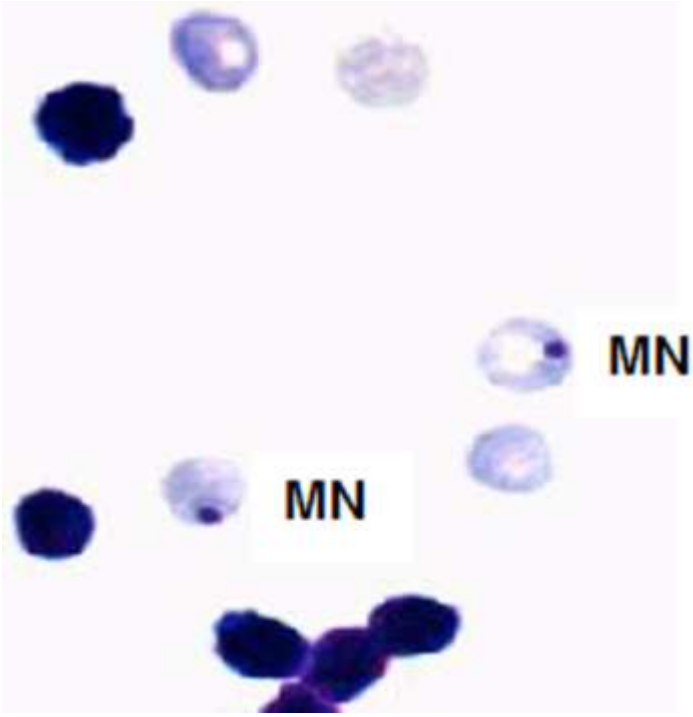

Figure 2. Micronuclei in bone-marrow cells of mice 24 h after the administration (orogastric gavage) of crude extract from *Calophyllum braziliense* leaves. MN: Micronuclei.

Table 1. Frequencies of micronuclei in the mouse femur bone-marrow cells treated with the crude extract from *Calophyllum brasiliense* leaves.

Treatment	n	nMN/1000 erythrocytes	Mean % (± SD)
C. brasiliense extract (100 mg/kg)	6	2,0,2,3,3,1	1.84 (±1.2)
C. brasiliense extract (200 mg/kg)	6	9,3,2,2,0,2	3.0 (±3.0)
Cyclophosphamide (50 mg/kg)	6	19,26,24,12,3,17	16.83* (±8.4)
DMSO 1%	6	4,2,0,1,3,4,	2.34 (±1.6)

*p≤0.05. (ANOVA-Tukey's) compared to the control (1% DMSO). n: Number of animals. nMN: number of micronuclei. SD: standard deviation.

(Table 1).

DISCUSSION

Coumarins are heterocyclic molecules that have been associated with beneficial effects in reducing the risk of cancer, diabetes, and cardiovascular and brain diseases. These effects are related to the elimination of free radicals, due to their antioxidant activities. Gonçalves et al. (2013) demonstrated that dichloromethane extracts and extracts obtained by supercritical fluid from *C. brasiliense* leaves showed a significant content of the coumarin (-) - mammea A/BB, with high antioxidant activity.

Micronuclei in interphase cells result from chromosome breakage or delayed chromosomal and DNA damage; in eukaryotic organisms or individual cells, this damage is often evaluated with the comet assay (García et al., 2004). The high content of (-) mammea A/BB in the dichloromethane extracts obtained from leaves of *C. brasiliense* may be effective in reducing oxidation of DNA by free radicals.

The crude extract of *C. brasiliense* leaves, with a concentration of (-) mammea A/BB at 25.97 ± 0.91 µg/mg of extract was used in two doses, that is, 100 and 200 mg/kg, to evaluate the induction of micronuclei in erythrocytes from bone marrow. This is the primary *in vivo* genotoxicity test recommended by regulatory agencies worldwide, as part of the safety assessment of chemicals

chemicals and natural products. This test, when performed correctly, detects both clastogenic and aneugenic effects (Krishna and Hayashi, 2000).

The micronuclei in young erythrocytes arise mainly from acentric fragments or chromosomes that are unable to migrate following the mitotic spindle during cell division of hematopoietic tissue (Salamone and Heddle, 1983; Ouanes et al., 2003). The increase in the frequency of MNPCE in animals treated with different compounds is an indication of induced chromosome damage (Krishna and Hayashi, 2000).

The frequency of MNPCE in groups treated with the crude extract from *C. brasiliense* was significantly lower than the frequencies observed in the positive control. Cyclophosphamide has been widely used as a positive control in the micronucleus test with rodents (Krishna and Hayashi, 2000), because of its ability to induce MNPCE. However, the decrease in frequency of MNPCE compared to the controls suggests that the crude extract from *C. brasiliense* leaves does not act as a genotoxin, and it may also contain compounds that have an antigenotoxic effect.

This study was the first to evaluate the potential for chromosomal damage of the *C. brasiliense* extract in mouse bone-marrow cells. The results indicate that the compounds present in this extract did not cause a significant increase in the number of cells with micronuclei at doses of 100 and 200 mg/kg. These doses are higher than that received by human patients using creams with antileishmanial activity (Honda et al., 2010, 2011).

Conclusion

The results of this study demonstrated that the extracts from *C. brasiliense* are safe, at least in terms of the potential for chromosomal damage.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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