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Assessment of the cytotoxic, genotoxic, and antigenotoxic activities of Celtis iguanaea (Jacq.) in mice

Assessment of the cytotoxic, genotoxic, and antigenotoxic activities of *Celtis iguanaea* (Jacq.) in mice

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ABSTRACT

Ethnobotanical surveys of Cerrado native plants show that leaves of *Celtis iguanaea* (Jacq.) Sargent (Cannabaceae), popularly known in Brazil as "esporão de galo", are used in folk medicine for body pain, asthma, cramps, poor digestion, urinary infection, kidney dysfunctions, as well as a stimulant and diuretic. This work aimed at evaluating possible *C. iguanaea* aqueous leaf extract (CALE) cytotoxicity, genotoxicity, and antigenotoxicity using the mouse bone marrow micronucleous test. To assess CALE genotoxicity, Swiss mice were orally treated with three different extract concentrations (100, 300, and 500 mgkg^{-1}). To evaluate its antigenotoxicity, the same doses were used simultaneously with a single i.p. dose of mitomycin C (MMC, 4mg.kg^{-1}). The frequencies of micronucleated polychromatic erythrocytes (MNPCE) were evaluated 24 h and 48 h after administration except for the negative control (24 h). Genotoxicity was evaluated using the frequency of micronucleated polychromatic erythrocytes (MNPCE), whereas cytotoxicity was assessed by the polychromatic and normochromatic erythrocytes ratio (PCE/NCE). The results showed that CALE did not exhibit a significant reduction in the PCE/NCE ratio, neither a considerable increase in the frequency of MNPCE. Nonetheless, CALE reduced bone marrow toxicity (increased PCE/NCE ratio) and decreased the micronuclei frequency induced by MMC. We can conclude that CALE presented no cytotoxic and genotoxic effects, but showed antigenotoxic and anticytotoxic actions under the experimental conditions applied in this study.

Key words: Cytotoxicity, genotoxicity, mice, medicinal plant.

INTRODUCTION

The use of plant products for the treatment, cure, and prevention of general disorders is one of the earliest forms of medical practice, and probably almost as old as the human species (Halberstein 2005). It is estimated that natural products and herbal preparations are responsible for 25% of the medical prescriptions in developed countries and about 80% in developing countries. The number of bioactive plant compounds exceeds 100,000, but

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the actual number must be much higher because only 20 - 30% of plants have been investigated so far (Wink 2009). Also, a significant number of synthetic drugs was obtained from natural precursors (Raskin et al. 2002, Rates 2001).

The Brazilian Cerrado is the richest savanna formation in the world, responsible for about 5% of global biodiversity, and considered one of the world's hotspots. (Myers et al. 2000). It has an endemic level of 44% for vascular plants and 70% for herbaceous plants, representing a valuable spectrum of organic molecules with medical and therapeutic potential (Machado et al. 2008).

Safety and toxicity studies of plants used in therapy are vital, due to its considerable range of applications and its widespread use in folk medicine, which, according to Maciel et al. 2002, often represent the only therapy of many communities and ethnical groups.

In the Brazilian Cerrado traditional medicine, the leaf infusion of *Celtis iguanaea* (Jacq.) Sargent, popularly known as "esporão-de-galo" (Souza and Lorenzi 2005), is used in the treatment of body pains, asthma, colic, poor digestion, urinary infection, kidney dysfunctions, as well as a stimulant and diuretic (Carneiro 2009, Silva and Proença 2008, Piliackas et al. 2001). According to ethnobotanical surveys of the Cerrado native plants, this species showed a considerable consensus of popular usage (Silva and Proença 2008), which may indicate potential medical properties and strengthen the need of proper pharmacological and toxicological studies (Vendruscolo and Mentz 2006). However, despite the wide use of this plant, a rather scarce literature is dedicated to the species and little information regarding the biological effects of its constituents can be found, especially when it comes to their potential toxicological properties.

Several studies on phytotherapic substances have already reported that many medicinal plant compounds present undesirable properties, such as mutagenicity, carcinogenicity and toxicity, limiting their use as therapeutic agents (Déciga-Campos et al. 2007, Marques et al. 2003). On the other hand, a number of studies have also shown that phytotherapic compounds may possess antigenotoxic/anticarcinogenic effects (Aruoma 2003, Gupta et al. 2001, Waters et al. 1996). Therefore, the investigation of traditionally used medicinal plants is valuable both as a source of potential chemotherapeutic drugs, and as a measure of safety for the continuous use by the population (Verschaeve et al. 2004).

Short-term assays have been used for more than 30 years to identify chemical, physical, and biological genotoxic agents, as well as to assess their carcinogenic potential. Although the genetic toxicity is not a direct measure of carcinogenicity, it is often used as an indicator for cancer, since genotoxicity tests measure an initial or intermediary event in tumorigenesis (Fearon and Volgelstein 1990).

Among the methods for *in vivo* genotoxicity investigation, the micronucleus test has been widely accepted by regulatory agencies and governmental institutions (Mateuca et al. 2006, Choy 2001). This assay was initially developed in mouse bone marrow erythrocytes (Schmid 1975). Since then, it has been used to assess the genotoxic potential of physical and chemical agents (Ding et al. 2003, Chung et al. 2002), biomonitor human populations occupationally exposed to mutagens (Bolognesi et al. 2004, Majer et al. 2001), in the search for carcinogenesis inhibiting compounds (Roy et al. 2003, Izzotti et al. 2001), and in ecotoxicological studies (Llorente et al. 2002, Gauthier et al. 1999).

The micronucleus test detects genetic alterations arising from chromosomal damage and/or damage to the mitotic apparatus caused by clastogenic or aneugenic agents, respectively. As micronuclei (MN) are indicative of irreversible DNA loss, their frequency may be used as a mutation index (Azevedo et al. 2003). It is already known that there is a positive correlation between increased frequency of MN and the appearance of tumors in rodents and humans (Clare et al. 2006, Azevedo et al. 2003).

Thus, considering the widespread use of this plant by the Brazilian population, the present work aimed at evaluating the cytotoxic, genotoxic, and antigenotoxic activities of *Celtis iguanaea* aqueous leaf extract (CALE) using the *in vivo* mouse bone marrow micronucleus test.

MATERIALS AND METHODS

PLANT MATERIAL: *Celtis iguanaea* Extract

Celtis iguanaea (Jacq.) Sargent ("esporão de galo") leaves were collected in a riparian forest located in the municipality of Campestre (16°45'44" S; 49°41'40" W; altitude = 651 m), in the state of Goiás, Midwestern Region of Brazil. The botanical material was identified and a voucher specimen deposited in the Herbarium of the Universidade Federal de Goiás, Goiânia, GO, under the number 40110/UFG.

The plant material was prepared according to Paula, 2009: The leaves were dried in an oven at 40°C with forced ventilation and then ground into a powder. CALE was obtained by infusion of the powder at 3% at 80°C for 30 min, with agitation every 10 min. After vacuum filtration, the filtrate was concentrated under reduced pressure at 45°C. The yield of the extract was determined by the dry weight method (20%), and the final concentration was 60 mg.mL^{-1}. At this concentration, CALE showed pH of 7.22, was odorless, had a greenish aspect and presented mild viscosity, probably due to the presence of mucilage. CALE solutions were always prepared with distilled water (extract concentrations of 100, 300, and 500 mg.kg^{-1}) immediately before their use in the experiments.

ANIMALS

This study was approved by the Human and Animal Research Ethics Commitee of the Universidade Federal de Goiás (CEPMHA/HC/UFG n° 014/09). Healthy young male adults (8–12 weeks) outbred mice (*Mus musculus*, Swiss Webster), weighing 30–40 g, obtained from the Central Animal Facility of Universidade Federal de Goiás (Goiás, Brazil) were randomly allocated to treated groups. All animals were brought to the laboratory 7 days before the experiments and housed in polyethylene cages (40 cm x 30 cm x 16 cm), in groups of five animals, lined with wood shavings, in air-conditioned rooms at 25 ± 2°C and 50 ± 10% relative humidity, with a 12-h light/dark natural cycle. Food (appropriate commercial rodent diet Labina, Ecibra Ltda.) and water were given *ad libitum*.

EXPERIMENTAL PROCEDURE

To evaluate the genotoxicity of the extract, five animal groups were orally treated with three different doses (100, 300, and 500 mg.kg^{-1} body weight) of CALE. A positive control group (4 mg.kg^{-1} i.p. mitomycin C, $C_{15}H_{18}N4O5$, MMC, Bristol-Myers Squibb) and another negative (sterile distilled water) control group were included. In order to assess antigenotoxicity, the same CALE doses were administered simultaneously with a single i.p. dose of MMC (4 mg.kg^{-1}). All treatments were evaluated 24 h and 48 h after administration except for the negative control (24 h). The animals were euthanized by cervical dislocation, femurs were dissected, opened, and the bone marrow was gently flushed out using fetal calf serum (Soralli). After homogenization of the bone marrow in serum, it was centrifuged at 1,000 rpm for 5 min. The bone marrow cells were smeared on glass slides, coded for blind analysis, air-dried, and fixed with absolute methanol (CH_4O, LabSynth) for 5 min. The smears were stained with Giemsa (Doles), dibasic sodium phosphate ($Na_2HPO_412H_2O$, Sigma-Aldrich Chemical Co.), and monobasic sodium phosphate ($NaH_2PO_4H_2O$, Sigma-Aldrich Chemical Co.) to detect micronucleated polychromatic erythrocytes (MNPCE). For each mouse, three slides were prepared and a minimum of 2,000 polychromatic erythrocytes (PCE) were counted to determine the frequency of MNPCE. To evaluate CIE cytotoxicity,

1,000 normocromatic erythrocytes (NCE) were counted, as well as the frequency of polycromatic erythrocytes (PCE) within the same microscope fields, and the PCE/NCE ratio was then calculated to measure bone marrow toxicity. The slides were analyzed by microscopy (Olympus BH-2 10x100). The micronucleus test and MNPCE scoring were carried out according to Schmid (1975).

STATISTICAL ANALYSIS

To evaluate the genotoxic activity of CALE, the frequency of MNPCE in the treated groups was compared to the results of the negative control group (genotoxicity assessment) or to the results from the positive control group (antigenotoxicity evaluation), using one-way analysis of variance (ANOVA), followed by the multiple comparison test (Tukey). P values lower than 0.05 ($p < 0.05$) were considered indicative of statistical significance. In order to assess CALE cytotoxicity, the polychromatic/normochromatic erythrocytes ratio (PCE/NCE)

of all treated groups was compared to the result of the negative control group (cytotoxicity assessment) or to the result of the positive control group (anticytotoxicity evaluation), using qui-square test (χ^2). A value of $p < 0.05$ was taken as the criterion of statistical significance.

RESULTS

Table I summarizes the frequencies of MNPCE and PCE/NCE ratio in mouse bone marrow cells treated with CALE.

The results obtained showed no significant increase in MNPCE frequency either 24 h (4.6, 5.0, 5.4) or 48 h (4.8, 5.4, 4.8) after the administration of CALE at any tested dose (100, 300, 500 mg.kg^{-1}) when compared to the negative control ($p > 0.05$). There was significant increase of MNPCE frequency in the positive control group compared to the negative control group ($p < 0.05$). This result was already expected, since MMC is described as a highly genotoxic and mutagenic agent (Kang et al. 2006).

TABLE I
MNPCE frequencies and PCE/NCE ratio in mouse bone marrow cells treated
with *Celtis iguanaea* aqueous extract (CALE) at different doses and times.

Treatment (mg.kg^{-1})	Time (h)	MN/2000 PCE						PCE/NCE ratio [4]
		Individual data					Mean ± SD[4]	
Negative control [1]	24	5	4	6	4	4	4.60 ± 0.89a	1.13 ± 0.10d
Positive control [2]	24	36	29	34	31	33	32.60 ± 2.70b	0.51 ± 0.04e
	48	14	12	11	11	15	12.60 ± 1.81c	0.39 ± 0.03f
CALE [3]								
100 mg.kg^{-1}	24	4	7	3	4	5	4.60 ± 1.51a	1.22 ± 0.08d
300 mg.kg^{-1}	24	5	5	6	4	5	5.00 ± 0.70a	1.08 ± 0.04d
500 mg.kg^{-1}	24	7	4	4	6	6	5.40 ± 1.34a	1.18 ± 0.07d
100 mg.kg^{-1}	48	3	6	4	5	6	4.80 ± 1.30a	1.13 ± 0.05d
300 mg.kg^{-1}	48	6	7	4	7	3	5.40 ± 1.81a	1.16 ± 0.07d
500 mg.kg^{-1}	48	5	3	4	5	7	4.80 ± 1.48a	1.27 ± 0.15d

[1] Sterile distilled water.

[2] Mitomycin C (4 mg.kg^{-1}).

[3] CALE doses are compared to their respective positive controls at the same exposure times.

[4] Same symbols in the same column – $p > 0.05$; different symbols in the same column – $p < 0.05$.

Regarding cytotoxicity, no significant decrease in the PCE/NCE ratio was observed when comparing mice treated with CALE with the negative control group for all tested doses and different times of evaluation ($p > 0.05$). As expected, the PCE/NCE value of MMC was much lower compared to the negative control or CALE doses, confirming its well-known cytotoxic activity (Estrem and Vanleeuwen 2000, Kraut and Drnovsek-Olup 1996).

Table II summarizes the frequency of MNPCE and PCE/NCE ratio in mice bone marrow cells treated simultaneously with different doses of CALE and 4.0 mg.kg^{-1} of MMC.

TABLE II

**MNPCE frequencies and PCE/NCE ratio after simultaneous treatment
with *Celtis iguanaea* extract (CALE) and mitomycin C (MMC).**

Treatment (mg.kg^{-1})	Time (h)	MN/2000 PCE						PCE/NCE ratio [4]
		Individual data					Mean ± SD[4]	
Negative control [1]	24	5	4	6	4	4	4.60 ± 0.89a	1.13 ± 0.10d
Positive control [2]	24	36	29	34	31	33	32.60 ± 2.70b	0.51 ± 0.04b
	48	14	12	11	11	15	12.60 ± 1.81c	0.39 ± 0.03c
CALE [3]								
100 mg.kg^{-1} + MMC	24	24	26	27	21	22	24.0 ± 2.54d	0.64 ± 0.07a
300 mg.kg^{-1} + MMC	24	21	21	25	24	20	22.2 ± 2.16d	0.71 ± 0.09a
500 mg.kg^{-1} + MMC	24	23	20	19	20	18	20.0 ± 1.87d	0.69 ± 0.06a
100 mg.kg^{-1} + MMC	48	10	8	9	11	10	9.60 ± 1.14e	0.58 ± 0.07f
300 mg.kg^{-1} + MMC	48	9	8	8	9	8	8.40 ± 0.54e	0.55 ± 0.06f
500 mg.kg^{-1} + MMC	48	8	7	10	7	9	8.20 ± 1.30e	0.62 ± 0.04f

[1] Sterile distilled water.

[2] Mitomycin C (4 mg.kg^{-1}).

[3] CALE doses are compared to their respective positive controls at the same exposure times.

[4] Same symbols in the same column – $p > 0.05$; different symbols in the same column – $p < 0.05$.

The results of the antigenotoxicity evaluation showed that CALE significantly decreased MNPCE frequency either 24 h (24.0, 22.2, 20.0) or 48 h (9.60, 8.40, 8.20) after the administration of any of the three tested doses (100, 300, 500 mg.kg^{-1} g co-treated with MMC) compared with the positive control ($p < 0.05$).

In relation to the anticytotoxic assessment of CALE, we observed an attenuation of the cytotoxic action provoked by MMC at all tested doses, either 24 h or 48 h after exposure when compared with the respective positive control ($p < 0.05$).

DISCUSSION

In the present study, we aimed to evaluate the cytotoxic, genotoxic, and antigenotoxic activities of CALE using the mouse bone marrow micronucleus test. This short-term assay is recommended by regulatory agencies all over the world as the first *in vivo* test to be conducted in chemical safety analyses (Khrishna and Hayashi 2000). It has proven to be a reliable method to investigate the genotoxic (clastogenic and/or aneugenic) effects of chemical and physical agents (Hayashi et al. 1990, Schmid 1976).

MN appear in erythrocytes due to damage induced in parental cells (Fenech 2000). These small masses of chromatin are originated from acentric fragments or lagging chromosomes that fail to incorporate into one of the daughter nuclei during telophase of the mitotic cells. MN frequency in polychromatic erythrocytes (PCE) of mouse bone marrow is a very sensitive index of damage (Suzuki et al. 2008), being induced by oxidative stress, exposure to clastogenic or aneugenic agents, genetic defects in cell cycle checkpoints, and/or DNA repair genes, and also by the deficiency of nutrients required as co-factors in DNA metabolism and chromosome segregation machinery (Bonassi et al. 2007). All of these events that cause MN formation are associated with the chromosomal instability commonly observed in cancer (Rajagopalan et al. 2004, Fenech 2002).

The results of the present work demonstrate that CALE did not provoke a significant increase in MNPCE frequency when compared with the negative control at all tested doses and times of exposure, indicating that this extract did not exhibit genotoxic effects in PCE of mouse bone marrow.

The micronucleus assay also detects cytotoxic effects by the PCE/NCE ratio. When normal proliferation of bone marrow cells is affected by a toxic agent, there is a decrease in the number of immature erythrocytes (PCE) in relation to the number of mature erythrocytes (NCE), reflecting bone marrow toxicity and cell depression (Shahrim et al. 2006).

Our results showed no significant reduction of the PCE/NCE ratio at any CALE doses and times of exposure compared with the negative control. Therefore, these results indicate that CALE did not present cytotoxic action.

The results of the antigenotoxicity evaluation (CALE + MMC) showed that CALE significantly reduced the frequency of MMC-induced MNPCE at all concentrations tested and times of exposure, attenuating the genotoxic activity of the alkylating agent.

In relation to the anticytotoxic activity of CIE, we observed an attenuation of MMC cytotoxic action at all CALE doses and times of exposure tested.

The phytochemical analysis of *Celtis iguanaea* leaves revealed the presence of coumarins, mucilage, and flavonoids (Paula 2009).

Coumarins present a wide variety of bioactivities, including anti-inflammatory, anticoagulant, antimicrobial, vasodilating, anthelmintic, sedative, hypnotic, analgesic, hypothermic, and antitumor-promoting activity (García-Argáez et al. 2000, Fujioka et al. 1999, O'Kennedy and Thornes 1997, Mizuno et al. 1994). The majority of tests for assessing mutagenic and genotoxic potential suggest that coumarins are not genotoxic agents, and exposure to coumarins from food, medicines, and/or cosmetic products poses no health risks to humans (Lake 1999). However, possible phototoxic effects of coumarin furanoderivatives should be kept in mind (Edwards et al. 1994).

Mucilage is used in medicine mainly as an emollient and a demulcent, but some mucilaginous plants have other applications, such as *Aloe vera* gel, which has been used since ancient times to treat burns and other wounds due to its ability to enhance the healing rate and to reduce the risk of infection (Choy and Chung 2003, Capasso et al. 1998).

Flavonoids have been recognized to possess anti-inflammatory, analgesic, antiallergic, hepatoprotective, anti-bacterial, antiviral, and anticarcinogenic activities (Havsteen 2002, Hodek et al. 2002). Although the mechanisms of flavonoid protection against DNA damage and potential protection against carcinogenesis are largely unknown, it is suggested that they may act as antioxidant, free radical scavengers, inhibitors of tumor cell growth, inducers of apoptosis, modulators of DNA repair, or carcinogen inactivators (Lee et al. 2003, Duthie and Dobson 1999).

Antioxidant agents are broadly known to significantly reduce cytotoxicity and genotoxicity

of compounds that generate free radicals (Borek 2005, Halliwell 2002, Marnett 2000). It is also known that the hazardous effect of MMC is related to its ability to alkylate DNA and produce reactive free radicals, causing different types of cellular damage, including DNA breaks (Kang et al. 2006). Consequently, the antigenotoxic and anticytotoxic activities of CALE detected in our experiments can be associated, at least partially, to the presence of flavonoids exerting protective effects by scavenging reactive oxygen, reducing alkylation and/or other antioxidant mechanisms. However, the complexity of plant extracts should not be overlooked, as the final response of a treatment using them is likely to be the result of synergistic, antagonistic, and other interactive effects among their biologically active components.

Concluding, our results in the present work indicate that CALE did not exhibit genotoxic or cytotoxic effects in mouse bone marrow micronucleus test. Nonetheless, this plant extract showed antigenotoxic and anticytotoxic effects under the experimental conditions tested.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

RESUMO

Levantamentos etnobotânicos de plantas nativas do cerrado evidenciam que as folhas de *Celtis iguanaea* (Jacq.) Sargent (Cannabaceae), popularmente conhecidas como "esporão-de-galo" são usadas na medicina popular para dores no corpo, asma, cólicas, má-digestão, infecções urinárias, disfunções renais, como estimulante ou como diurético. O presente trabalho teve como objetivo avaliar as possíveis atividades citotóxica, genotóxica e antigenotóxica do extrato aquoso das folhas de *C. Iguanaea* (CALE) através do teste do micronúcleo em medula óssea de camundongos. Para avaliar a genotoxicidade de CALE, os camundongos foram tratados por via oral com três diferentes concentrações de extrato (100, 300 e 500 mg.kg^{-1}). Para a investigação da atividade antigenotóxica, foram utilizadas as mesmas doses concomitantemente a uma única dose i.p. de Mitomicina C (MMC, 4mg. kg^{-1}). Com exceção do controle negativo (apenas 24h), todas as outras doses tiveram as frequências de eritrócitos policromáticos micronucleados (EPCMN) avaliadas em 24 e 48 horas após o tratamento. A Genotoxicidade foi avaliada por meio da frequência de eritrócitos policromáticos micronucleados (MNPCE), enquanto que a citotoxicidade foi mensurada pela razão entre eritrócitos policromáticos e normocromáticos (PCE / NCE). Os resultados mostraram que CALE não apresentou uma redução significativa na razão PCE/NCE, nem um aumento considerável na frequência de EPCMN. No entanto, CALE reduziu a toxicidade na medula óssea (aumento na razão PCE/NCE) e diminuiu a frequência de micronúcleos induzidos pela MMC. Nos resultados obtidos, CALE não apresentou efeitos citotóxicos e genotóxicos, contudo apresentou ações antigenotóxicas e anticitotóxicas nas condições experimentais aplicadas neste estudo.

Palavra-chave: Citotoxicidade, genotoxicidade, camundongos, planta medicinal.

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