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Evaluation of the protective cytogenetic activity of *Parahancornia fasciculata* (Apocynaceae)

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Abstract

Parahancornia fasciculata is a large fruit tree, native to the Amazon region, where it is popularly known as "Amapazeiro". It is widely used in folk medicine to assist in the treatment of several pathologies: malaria, lung problems, among other diseases. Thus, considering the usual use of this plant, the present study evaluated the protective cytogenetic action of *Parahancornia fasciculata* on polychromatic erythrocytes of Swiss mice. The animals received different concentrations of *Parahancornia fasciculata* (250, 500, 1000 and 2000 mg/kg b.w.) including positive (Doxorubicin, DXR, 16 mg/kg b.w.), negative (water), and vehicle (Dimethylsulfoxide, DMSO) control groups. Dosages were administered to the animals via gavage, as well as the negative control and vehicle; the positive control was administered intraperitoneally. The animals remained in the daily treatment with the respective doses for 15 days for genotoxic evaluation. Caudal peripheral blood samples were collected at 24h, 48h, 7 and 15 days. For the antigenotoxic evaluation, on the 14th day the mice were treated with intraperitoneal injections of DXR. Peripheral caudal blood samples were obtained at 24h and 48h. Subsequently, 2,000 polychromatic erythrocytes were counted per animal in each group, to assess the frequency of micronuclei. The results showed that the methanolic extract of *Parahancornia fasciculata* was not genotoxic, as it did not present statistically significant differences when compared to the negative control. Animals treated with the different concentrations of *Parahancornia fasciculata* extract associated with DXR, obtained a significant reduction in Micronucleated Polychromatic Erythrocytes when compared to the positive control. Therefore, the methanolic extract of *Parahancornia fasciculata* demonstrated a protective action against DNA damage induced by DXR and did not demonstrate a genotoxic effect.

Keywords: Lifestyle. Body composition. Student Health. Food Service.

INTRODUCTION

Brazil is responsible for 15 to 20% of the total biodiversity in the world, with about 60,000 plants existing in the Brazilian flora, of which only 4,800 species have been studied regarding their bioactive compounds. In this context, there is a clear imperative to develop research aimed at evaluating the therapeutic potential of medicinal plants[1].

Among the little studied plants is *Parahancornia fasciculata*, it is a large, fruitful tree, native to the Amazon region, it has an erect and elevated trunk, and the crown is

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formed by many opposite and independent branches[2]. The leaves are lanceolate, opposite, with 12 to 15 major secondary veins[3]. The fruits are globular, about 8 cm in diameter, the seed has a dark brown and light-yellow color and may contain a brown border with a yellow center. Morphologically, it is smooth, ovoid-flat, and has a central and oval-shaped hilum[4].

The species is popularly known as "Amapazeiro", "Mapá" or "Amapá", its bark produces a medicinal exudate in the form of latex that is popularly called "Leite do Amapá" and is used in folk medicine to cure Malaria, pulmonary problems, gastritis, weakness and scarring[5]. Although biological activities have been observed empirically, they can be scientifically explained according to the chemical composition of *Parahancornia fasciculata*[6].

In the dichloromethane extract of the bark and latex, the pentacyclic triterpenes lupeol, β-amyrin and α-amyrin and their acetylated derivatives were isolated and identified6. Using the same methodology, steroids β-sitosterol, stigmasterol, and β-sitosterone were isolated from the roots. In another study, using methanolic extract, a large mixture of carbohydrates, methylmyosinosol, and phenylethanoid derivatives was identified with cornoside as the main constituent[6].

Studies on the evaluation of the protective cytogenetic action of the species Parahancornia fasciculata are not reported in the scientific literature, which made it necessary to carry out this research due to the completion of steps to register a probable herbal medicine based on this plant with the National Health Surveillance Agency (ANVISA)[7].

Since cytogenetic tests are a condition for the safety and viability of registration and commercialization of any medicine, this research proposed to evaluate the genotoxic and antigenotoxic activities of the methanolic extract of the leaves of *Parahancornia fasciculata* in polychromatic erythrocytes of Swiss mice.

MATERIAL AND METHODS

Chemical agent inducing chromosomal damage in DNA *in vivo*

The DXR ampoule containing 50 mg at 98% purity was purchased from Sare Drogarias Ltda, São Paulo, SP, Brazil. This compound dissolved in distilled water was used as an inducer of micronuclei in peripheral blood cells of mice (positive control). The dose of DXR (16 mg/kg body weight, b.w.) was selected based on its effectiveness in inducing chromosomal damage[8].

Evaluation of the Frequency of Polychromatic Erythrocytes

The peripheral blood micronucleus assay was performed according to the protocol described by MacGregor *et al.*[9,10]. A total of 2000 polychromatic erythrocytes (PCEs) were analyzed per animal to determine the frequency of micronucleated polychromatic erythrocytes (MNPCEs). A total of 400 erythrocytes per animal were scored to calculate the Nuclear Division index [NDI, PCE/PCE+NCE (normochromatic erythrocytes) to determine treatment

cytotoxicity[11]. The slides were analyzed blindly using an optical microscope with a 100x immersion objective.

For the protective evaluation, the percentage of reduction in the frequency of MNPCEs was calculated using the formula:

$$\% \text{ redução} = \frac{\text{Frequência de EPCsMN em A} - \text{frequência de EPCsMN em B}}{\text{Frequência de EPCsMN em A} - \text{frequência de EPCsMN em C}} \text{ x100}$$

Where A corresponds to the group treated with DXR (positive control), B corresponds to the group treated with *Parahancornia fasciculate* and DXR, and C corresponds to the group treated with water (negative control).

Obtaining the methanolic extract from the bark of *Parahancornia fasciculata* (Apocynaceae)

The *Parahancornia fasciculata* bark was collected in the municipality of Macapá-AP, at KM 09, with GPS location: Lat. 00° 04' 06.2" S; Long. 51° 08' 01.5" W. From the specimen collected an exsiccatae was prepared, which was composed with branches of the species mentioned. These branches, after cleaning, were dried by compression, and then were sent and deposited as No. 019140 at the Herbário Amapaense of the Institute of Scientific and Technological Research of Amapá.

The *Parahancornia fasciculata* bark collected were dried in a circulating air oven at 45ºC until complete dehydration.

After drying, the shells were crushed in a knife mill, and the crushed material was macerated with methyl alcohol (liquid extractor), in a proportion of 1:5 (1 kg of plant material/5 L of methanol). The mixture was homogenized for 10 days, in order to break the concentration zones inside the flask, providing better contact between the solvent and the plant material.

After maceration, the extracted solution was vacuum filtered. Then, the liquid was placed in a rotary evaporator (Q344b2, Quimis) at 78 rpm and 60ºC in order to completely remove the solvent through evaporation, resulting in the methanolic extract of the *Parahancornia fasciculate* bark.

Animals used in the experiment

For the experiment, Swiss male mice weighing approximately 25 grams were purchased. The animals came from the Multidisciplinary Center for Biological Investigations in the Area of Laboratory Animal Science - CEMIB of the University of Campinas - UNICAMP. The mice remained in collective cages in a quarantined experimental room, under controlled conditions of temperature (23±2°C), humidity (50±10%), 12 hours of light-dark cycle, with *ad libitum* access to food and water. The project was submitted to the Research Ethics Committee of the Federal University of Amapá (CEUA/UNIFAP) in accordance with the Arouca Law 11.794/2008, which regulates the use of animals in research, approved and filed under number 14/2017 .

Experimental design

Swiss mice were divided into different experimental groups of six animals each. The doses of *Parahancornia fasciculata* were selected based on the guidelines of the National Health Surveillance Agency

(ANVISA) 8. In order to investigate its potential genotoxicity, *Parahancornia fasciculata* was administered by gavage in a volume of 0.5 ml per 25 g of weight in doses of 250, 500, 1000 and 2000 mg/kg b.w. per day for each animal for 15 days. Peripheral blood samples were collected at 24 and 48 hours and 7 and 15 days after the start of treatment. For protective evaluation, animals treated with different concentrations of *Parahancornia fasciculata* received intraperitoneal (0.3 ml per 25 g b.w.) DXR (16mg/kg b.w.) on the 14th day. Peripheral blood samples were collected 24 and 48 hours after treatment with DXR. The different doses of *Parahancornia fasciculata* were prepared from a stock solution of 400 g. The vehicle group was treated by gavage (0.5 ml per 25 g of b.w.) with the same amount of DMSO used to prepare 2000 mg of *Parahancornia fasciculata* per kg/b.w.

Statistical analysis

The data went through tests of normality and homogeneity of variances, to verify the parametric nature of the data. After these statistical analyses, they underwent analysis of variance for entirely randomized experiments (ANOVA), with calculations of the F statistic and its respective p-value. In cases where $p \leq 0.05$, the treatment averages were compared using the Tukey method, with the calculation of the minimum significant difference $p = 0.05$, using the Graph Pad Prism® program.

RESULTS

There was no statistically significant difference in micronucleus induction between groups treated with different doses of *Parahancornia fasciculata* and the negative control (P> 0.05; Table 1). These findings indicated the absence of a genotoxic effect of the different concentrations of P*arahancornia fasciculata* at different sampling times used in the present study.

With a single intraperitoneal dose of DXR associated with gavage administration of each concentration of *Parahancornia fasciculata* extract, a reduction between 43.76% to 50.68% could be observed for the 24-hour treatment, and in 48 hours it was observed from 51.50% to 63.62% (Table 2), when compared to the group of animals treated only with DXR. It is worth mentioning that there was no increase in the reduction of genotoxicity in the 24h and 48h periods in relation to the increase in extract dosages, thus indicating a lack of a dose-response relationship. In the protective action tests, where doxorubicin was used, it was observed that the frequency of Micronucleated Polychromatic Erythrocytes (MNPCEs) was lower in animals treated with DMSO + DXR than in those treated only with DXR; however, these differences were not statistically significant.

For the cytotoxic evaluation the results of nuclear division index (NDI), in the treatment of 24h, 48h, 7 and 15 days (table 3) and 24 and 48h (table 4) associated with DXR, no differences were observed between the treated groups with the concentrations of the methanolic extract of *Parahancornia fasciculata* and their respective controls, demonstrating the absence of cytoxicity.

Table 1 – Frequency of Micronucleated Polychromatic Erythrocytes (MNPCEs) in peripheral blood of male Swiss mice, treated with different concentrations of *Parahancornia fasciculata* and their respective controls after 24, 48 h, and 7 and 15 days after treatment.

Treatment (mg.kg^{-1}p.c.)	MNPCEs/1000 PCEs[a]			
	24h	48h	7 days	15 days
Control	1.08 ± 0.79	1.17 ± 0.71	1.17 ± 0.71	1.08 ± 0.79
DMSO	0.92 ± 0.90	0.91± 0.90	1.25 ± 0.45	1.25 ± 0.45
P. fasciculata 250 mg	1.67 ± 0.77	1.33 ± 0.49	1.33 ± 0.49	1.50 ± 0.52
P. fasciculata 500 mg	2.25 ± 0.75	1.83 ± 0.71	1.33 ± 0.49	1.33 ± 0.49
P. fasciculata 1000 mg	2.00 ± 0.85	1.67 ± 0.49	1.6 7± 0.49	1.67 ± 0.65
P. fasciculata 2000 mg	2.75 ± 1.06	1.67 ± 0.49	1.67 ± 0.49	1.33 ± 0.65
DXR				24.8 ±0.86*

Two thousand PCEs were analyzed per animal, for a total of 12,000 cells per group;
[a]Values of mean ± standard deviation;
*Statistically significant difference when compared to the control (P <0.05);
DXR, Doxorubicin;
DMSO, Dimethylfulfoxide.

Table 2 – Frequency of Micronucleated Polychromatic Erythrocytes (MNPCEs) in peripheral blood of male Swiss mice treated with different concentrations of *Parahancornia fasciculata* and their respective controls after 24, 48 hours of treatments.

Treatment (mg.kg^{-1}b.w)	MNPCEs/1000 PCEs[a,b]			
			100% REDUCTION	
	24h	48h	24h	48h
Control	1.1 ±0.80	1.8± 0.72	-	-
DMSO + DXR	22.8 ± 0.70[c]	24.2 ±1.03[c]	-	-
DXR	23.2 ± 1.19[c]	24.5 ± 0.90[c]	-	-
P. fasciculata 250mg + DXR	13.5 ± 1.24[c,d]	12.5 ± 1.24[c,d]	43.76%	51.50%
P. fasciculata 500mg + DXR	13.1 ± 0.90[c,d]	12.1 ± 1.16[c,d]	45.70%	53.22%
P. fasciculata 1000mg + DXR	13.0 ± 1.04[c,d]	11.7 ± 1.83[c,d]	46.15%	54.94%
P. fasciculata 2000mg + DXR	12.4 ± 1.04[c,d]	9.67 ± 0.98[c,d]	50.68%	6352%

[a]Values of mean ± standard deviation;
Two thousand PCEs were analyzed per animal, for a total of 12,000 cells per group;
*Statistically significant difference when compared to the control (p-value);
Statistically significant difference when compared to the DXR group.

Table 3 – Frequency of Cell Division Index - NDI in peripheral blood of male Swiss mice treated with different concentrations of *Parahancornia fasciculata* and their respective controls after 24, 48 h, 7 and 15 days of treatments.

CONCENTRATION mg/kg b.w.	24 hours[a,b]	48 hours[a,b]	7 days[a,b]	15 days[a,b]
Control	0.11 ± 0.05	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.02
DMSO	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.02
DXR				0,09 ± 0.02
P. fasciculata 250 mg	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.02
P. fasciculata 500 mg	0.09 ± 0.01	0.08 ± 0.02	0.08 ± 0.02	0.08 ± 0.01
P. fasciculata 1000 mg	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.10 ± 0.01
P. fasciculata 2000 mg	0.10 ± 0.01	0.08 ± 0.02	0.08 ± 0.02	0.09 ± 0.01

Positive Control (DXR);
Vehicle (DMSO) Dimethylsulfoxide.
[a]400 blood cells were analyzed per animal, for a total of 2400 cells per treatment (PCE/PCE + NCE);
[b]Mean values ± standard deviation.

Table 4 – Frequency of Cell Division Index - NDI in peripheral blood of male Swiss mice treated with different concentrations of *Parahancornia fasciculata* associated with DXR and their respective controls after 24 AND 48 h of treatments.

CONCENTRATION mg/kg b.w.	24 horas[a,b]	48 horas[a,b]
Controle Negativo	0.11 ± 0.05	0.09 ± 0.01
DXR	0,08 ± 0.02	0,08 ± 0.02
DMSO + DXR	0.09 ± 0.02	0.09 ± 0.01
P. fasciculata 250 mg + DXR	0.07 ± 0.01	0.08 ± 0.01
P. fasciculata 500 mg + DXR	0.07 ± 0.02	0.07 ± 0.01
P. fasciculata 1000 mg + DXR	0.11 ± 0.01	0.10 ± 0.02
P. fasciculata 2000 mg + DXR	0.09 ± 0.02	0.10 ± 0.01

Positive Control (DXR);
Vehicle (DMSO) Dimethylsulfoxide;
[a]400 erythrocytes were analyzed per animal, for a total of 2400 cells per treatment (PCE/PCE + NCE);
[b]Mean values ± standard deviation.

DISCUSSION

Pre-clinical tests are of fundamental importance in screening studies of medicinal plants[12]. Among toxicological tests are cytogenetics such as the micronucleus test. The micronucleus induction methodology in peripheral blood erythrocytes has been used to assess genotoxic and antigenotoxic activities as it is effective for identifying agents capable of inducing or preventing chromosomal damage[13].

The micronucleus test has the characteristic of evaluating the effects of certain substances, which are observed in PCEs, which in turn have a short life span, thus, indicating recently induced damage[14].

The presence of a micronucleus results in damage to chromosomes, is capable of breaking chromosomes or affecting the formation of the metaphase plate, and also compromises the mitotic spindles responsible for the unequal distribution of chromosomes during the cell division process[15].

The results also show that the extract of Parahancornia fasciculata causes a significant reduction in the frequency of MNPCEs induced by DXR, although the mechanisms underlying the protective (antigenotoxic) effects of Parahancornia fasciculata are not completely understood, and the properties of the compounds of this plant are probably responsible for effects observed in the present study. The genotoxic activity of the chemotherapeutic agent DXR has been attributed to its ability to produce free radicals[16], which cause different types of cell damage, including DNA cleavage. The chemical structure of DXR favors the generation of free radicals and the compound can bind to iron and form complexes with DNA, inducing a break in the double strand[17]. Some studies have shown that oxidative damage is probably related to the formation of free radicals, accompanied by reduced antioxidant capacity[18]. Here are some mechanisms considered: (1) intercalation in DNA, leading to the inhibition of macromolecule synthesis; (2) generation of free radicals, leading to DNA damage or lipid peroxidation; (3) DNA binding and alkylation; (4) cross-linking of DNA; (5) interference with DNA unwinding or DNA strand separation and helicase activity; (6) direct effects of the membrane; (7) initiation of DNA damage via inhibition of toposoimerase II; and (8) induction of apoptosis in response to inhibition of toposoimerase II[19,20].

It is understood that the formation of free radicals occurs when the release of an electron, which the anthracycline ring of DXR suffers, can react with tissue macromolecules[21]. Thus, DXR is used as a micronucleus inducer, frequently used in mutagenicity tests as a positive control, which is the compound used in the referred study[22].

The results show that the methanolic extract of *Parahancornia fasciculata* caused a significant reduction in the frequency of MNPCEs induced by DXR. Although many mechanisms underlying the antigenotoxic effects of *Parahancornia fasciculata* are not yet fully understood, the antioxidant properties of the compounds in this plant are probably responsible for the effects observed in the present study.

The concentrations administered of the extract of *Parahancornia fasciculata* did not show proportional increases in the reduction of genotoxic activity in the period of 24h and 48h, thus inferring that there is no relationship with the dose-response, leading to an inconsistent bioavailability of the compound in the cell. On the other hand, the assessment of dose effects is complicated by the fact that many chemoprotective compounds act simultaneously at different levels of protection[23]. On this basis, it is suggested that the observed lack of response to the doses used can be attributed to the activation of different mechanisms depending on the dose levels of the extract of *Parahancornia fasciculata*.

It is important to consider that the proportion of drug absorption decreases with an increase in body surface, and the relationship between body surface and weight is not linear. Therefore, conversion must be done using allometric calculations to determine the expected dose for humans[24].

The NDI results obtained in this study demonstrated that there was no reduction in the percentage of PCEs in relation to the total number of erythrocytes in all treatment groups, when compared to the negative control animals, showing the absence of cytotoxic potential of the different treatments under the experimental conditions used.

Compounds with a high cytotoxicity index are able to completely inhibit dividing cells. The cytotoxic effect in the assay is necessarily evaluated on the cells' ability to divide[25], so that their cytotoxicity is directly proportional to the sample concentration and exposure time[26].

According to a study by Silva *et al.*[27], a bioassay was carried out to verify the preliminary toxicity of the extract of the species *Parahancornia fasciculata* with Artemia salina. In their study, it was demonstrated that through the acute preclinical toxicological assay in concentrations of up to 2000 mg/kg no toxic effect was observed. Therefore, the present study corroborates that described by Silva *et al.* (2016) because it was performed with lower concentrations of the extract (250, 500, 1000 and 2000 mg/kg b.w.) of the species *Parahancornia fasciculata* for the investigation of the complementary toxicological tests.

CONCLUSION

Indeed, the present study demonstrated that the extracts of *Parahancornia fasciculata*, did not have genotoxic effects, but demonstrated efficacy in reducing DXR-induced chromosome damage in the micronucleus assay in peripheral blood of Swiss mice. Although the exact mechanism underlying the protective effect (antigenotoxicity) of *Parahancornia fasciculata* is not completely understood, its antioxidant activity may explain its effect on the genotoxicity of DXR. Therefore, the ability of *Parahancornia fasciculata* extract to reduce the frequency of DXR-induced MNPCEs is an indication of its promising and potential chemoprotective properties. Further studies should be carried out to better characterize the mechanism of action of *Parahancornia fasciculata* as well as to quantify its different constituents at plasma levels for a rational implementation of chemopreventive strategies.

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