

Chemoprotective effect of leaf extracts of *Cecropia distachya* Huber (Urticaceae) in mice submitted to oxidative stress induced by cyclophosphamide

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ABSTRACT

The use of medicinal plants has been occurring for many years as a result of information that is passed on through successive generations. Interest in the discovery of new and safe antioxidant drugs has increased. The interest in the discovery of new and safe antioxidant drugs has increased, since plants are rich in secondary metabolites. With this purpose, the objective of this work was to evaluate the chemoprotective effects of the embaúba *Cecropia distachya* Huber using two methanolic fractions (F1 and F2), on oxidative stress induced by cyclophosphamide (75 mg kg^{-1}) in mice, as well as some phytochemical analyzes such as LC-MS/MS. The flavonoids quercetin-3 β -D-glucoside, rutin and luteolin were identified in F1, but only quercetin-3 β -D-glucoside and rutin in F2. In the experimental model, the fractions increased the hepatic and cardiac catalase (CAT), reduced glutathione (GSH) of the kidney and the heart. Besides, both fractions increased the levels of red blood cells (RBC), hematocrit (HCT) and hemoglobin (Hb) and decreased white blood cells (WBC). The effects attributed to F1 only were a reduced liver lipoperoxidation (TBARS) and aspartate aminotransferase (AST) activity, increased platelet count (PLT) and decreased glucose (GLU) concentration. As for F2, only it decreased the frequency of micronucleus in bone marrow cells. In some cases, the fractions were also hepatotoxic. These results demonstrated that both fractions stimulate the antioxidant defenses, being hypoglycemic (F1) and antimutagenic (F2), and, the harmful effects attributed to the fractions may be the association of compounds that were not elucidated in this work.

Keywords: Histology, oxidative lesions, hypoglycemic, immunosuppression.

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Man has always sought resources in nature to improve living conditions, especially in relation to the use of plants to combat disease, a habit that has always accompanied the history of mankind (Firmo et al. 2011). Medicines produced from plants are called herbal medicines, in which the active principle is added to other plant substances (Falzon & Balabanova 2017). They are made up of medicinal teas or extracts. The cost to prepare these products is much lower than phytopharmaceuticals, and for this reason the World Health Organization encourages their development (Brandão et al. 2010).

In this perspective, the use of medicinal plants has aroused the interest of research for several biological activities, among them being their antioxidant potential. The antioxidant action of plants is attributed to the presence of secondary metabolites, such as phenolic compounds and flavonoids; this property is associated with the presence of hydroxyl groups in these compounds (Wang et al. 2018). Antioxidants can act on the free radicals preventing diseases, because oxidative stress leads to the oxidation of biomolecules, the consequence being the loss of their biological functions. This process favors the development of some diseases such as atherosclerosis, diabetes, obesity, neurodegenerative diseases, cardiovascular diseases and even cancer (Green et al. 2004).

Cyclophosphamide is a drug used to treat diseases of the immune system exerting suppressive effect and antineoplastic activity (Ghosh et al. 2015). Cyclophosphamide is oxidized by P450 enzymes in the liver to become pharmacologically active, where it is converted to highly toxic metabolites, acrolein and phosphoramid mustard (Golan et al. 2014) which induce oxidative stress and mutagenesis.

The Embaúba species, plants of the genus *Cecropia*, are used in traditional medicine to treat asthma, bronchitis, diabetes, hypertension, cough suppression and diarrhea (Quintela et al. 2013). In pharmacological studies, the leaves of species of the genus *Cecropia* were investigated. For example, the ethanolic extract of the species *C. peltata* presented healing potential in rats (Nayak et al. 2006); the aqueous extract of the *C. pachystachya* species showed anti-inflammatory and nephroprotective activity in Wistar rats (Maquiaveli et al. 2014) and the aqueous extract of *C. glaziovii* showed anti-inflammatory and antioxidant action also in Wistar rats (Müller et al. 2016).

The genus *Cecropia* presents several works described in the literature, however, the species *Cecropia distachya* Huber is the first study involving antioxidant activity. The aim of this study was to investigate the chemoprotective effects of methanolic fractions of *C. distachya* leaves against oxidative events induced by cyclophosphamide in mice, as well as phytochemical analysis of fractions.

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METHODOLOGY

CHEMICAL PRODUCTS

Cyclophosphamide (CP) - Baxter, amentoflavone, apigenin, canferol, luteolin, quercetin, quercetin-3 β -D-glucoside, rutin, taxifoline, aluminum chloride (AlCl₃), 1,1-diphenyl-2-picryl hydrazyl (DPPH[•]), Triton X-100, hydrogen peroxide (H₂O₂), reduced glutathione (GSH), 2-thiobarbituric acid (TBA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), silica (60Å, 40-63 μ m), Bradford's reagent, trichloroacetic acid (TCA), potassium phosphate monobasic potassium phosphate, sodium phosphate monobasic, sodium phosphate dibasic, ethylenediamine tetra acetic acid (EDTA), trisaminomethane (Tris), bovine serum albumin, all purchased from Sigma-Aldrich (St. Louis, USA). The solvents used for the tests were all from Merck. Glucose, cholesterol, aspartate aminotransferase (AST) and alkaline phosphatase (ALP) dosage were used kits purchased from Labtest[®], Diagnóstico S.A, Minas Gerais, Brazil.

COLLECTION AND BOTANICAL IDENTIFICATION

The leaves were collected in the Federal University of Mato Grosso - Campus of Sinop, MT-Brazil, under coordinates S 11°51'49.5864" and W 55°28'59.1996". Popularly known as Embaúba, the plant species was identified as *Cecropia distachya* Huber, by Márcia Vilela Santos. The exsiccate was deposited in the collection of the Acervo Biológico da Amazônia Meridional (ABAM), Campus de Sinop under registration number 4472.

PREPARATION OF THE EXTRACT AND FRACTIONATION

To obtain the crude extract, 7.768 g *C. distachya* leaves were collected, dried in the oven with forced air circulation at 50 °C for five days. After drying, they were ground until a fine powder was obtained and extracted by maceration with absolute ethanol for 7 days to obtain 2.390 g of crude extract, the solvent removed at reduced pressure in a rotary evaporator at 40 °C. Subsequently, the removal of chlorophyll by partition with methanol and water in a ratio of 1 : 1, obtained 219 g of extract without chlorophyll.

The fractionation was performed in column chromatography using silica gel. For the elution, solvents, chloroform, ethyl acetate and methanol were used. The fractions were collected and rotated.

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After fractionation, two methanolic fractions which presented more flavonoids were chosen in this work for in vivo studies and identified as fraction 1 (F1) and fraction 2 (F2).

DETERMINATION OF FLAVONOIDS AND DPPH[•] (2,2-DIPHENYL-1-PICRYLHYDRAZYL) TEST

The determination of the amount of total flavonoids was performed using quercetin as the standard curve (Sá et al. 2012). The result was expressed in milligrams of quercetin equivalent per gram of extract (mg EQ g⁻¹).

The antioxidant potential of the vegetable extract was evaluated (Sousa et al. 2007). First, 50 mL of a stock solution of DPPH[•] in methanol at a concentration of 40 µg/mL were prepared, kept refrigerated and protected from light. For the calibration curve, dilutions of the stock solution (35, 30, 25, 20, 15, 10, 5 and 1 µg/mL) were made and read in triplicate at 515 nm.

The DPPH[•] radical is purple, when it is reduced by the action of an antioxidant, it becomes yellow. From this reaction we could determine the remaining amount of DPPH[•] sequestered by the antioxidant by means of readings in the spectrophotometer.

PHYTOCHEMICAL SCREENING

The presence of other secondary metabolites in the extract was evaluated through qualitative tests; the colorimetric tests were used to verify the presence of alkaloids, coumarins, steroids, saponins, polysaccharides, purines and tannins (Teixeira et al. 2013).

PHYTOCHEMICAL IDENTIFICATION OF *C. DISTACHYA* LEAVES BY LC-MS/MS

For the identification of flavonoids present in *C. distachya* leaves, the analytical standards for comparison, amentoflavone, apigenin, canferol, luteolin, quercetin, quercetin-3β-d-glucoside, rutin and taxifolinade luteolin were used. The fractions were subjected to liquid chromatography coupled to mass spectrometry (LC-MS/MS) using a 1290 Infinity UHPLC system (Agilent Technologies) coupled to a 6460 Triple Quad LC/MS (Agilent Technologies) in which a system pumps with 20 µL of sample injected via the self-injection system. Separation of the compounds occurred on a C-18 column (Zorbax Eclipse AAA of 4.6x150 mm diameter, 3.5 µm particle size). The sample elution method used a flow rate of 0.5 mL/min and an elution gradient composed of Solvent A (water: formic acid, 99.9: 0.1%

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(v/v) and Solvent B (acetonitrile: formic acid, 99.9: 0.1% (v/v)) having the following characteristics: 0-30 min: 95-5% B, 30-32 min: 0-100% B, 32-33 min: 95-5% B. The samples were detected by mass spectrometry using electrospray ionization. The method of elution of the samples in gradient, source temperature 300°C and desolvation temperature 250°C (Duan et al. 2011).

ANIMALS AND EXPERIMENTAL DESIGN

This research was approved by the Comitê de Ética no Uso de Animais (CEUA) - UFMT under protocol number 23108.722993/2017-28. Male *Swiss* mice, mean weight of 30g – 40 g were used. During the acclimation period (2 weeks) and the whole experimental period, the animals were kept in polyethylene and stainless steel crates and remained under controlled conditions of temperature (22 ± 2 °C), relative humidity ($55 \pm 10\%$), light cycle (12 hours light/dark), commercial diet and filtered water ad libitum. The animals received oral treatments (water with vehicle or extract) for 15 days and an intraperitoneal injection of CP or saline on the 15th day. The substance used was CP at the concentration of 75 mg Kg⁻¹ (Oboh et al. 2013).

For the in vivo tests 6 groups (48 animals) were used, according to the figure 1:

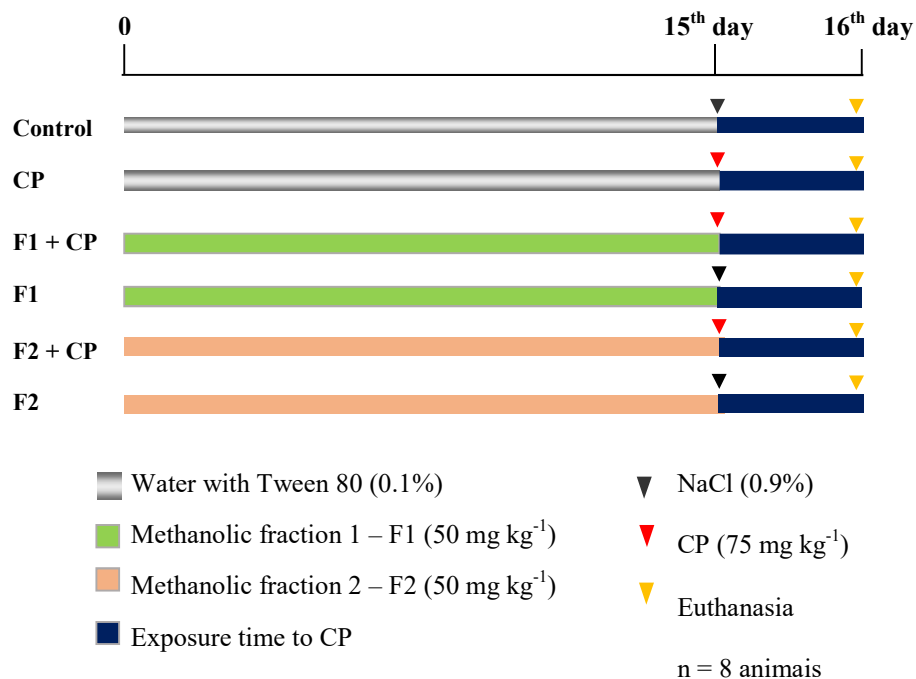


Figure 01. Experimental design for the evaluation of antioxidant and antimutagenic effects of F1 and F2. Source: Author

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ANIMALS AND EXPERIMENTAL DESIGN

After 24 hours after the last dose of each treatment applied during the 15 days as explained in figure 1 and after 8 hours of fasting, the animals were anesthetized intraperitoneally with ketamine 50 mg kg⁻¹, xylazine 20 mg kg⁻¹ and acepromazine 20 mg kg⁻¹. This protocol is widely used to minimize the pain and discomfort of the animal during cardiac puncture and subsequent cervical dislocation. Ketamine has the function of pain relief, sedation and decreased mobility, while xylazine has the function of muscle relaxant and finally, acepromazine is a tranquilizer that further enhances other drugs. Blood was withdrawn via cardiac puncture with heparinized syringes and, after this step, the animals were sacrificed and liver, kidney and heart tissues were removed and frozen at -80°C.

The glutathione-S-transferase (GST) activity in hepatic tissue was quantified by the formation of S-2,4-dinitrophenyl glutathione using the spectrophotometer to monitor their activity and results were expressed in $\mu\text{mol GS-DNB min mg protein}^{-1}$ (Habig et al. 1974). Superoxide dismutase (SOD) activity in same tissue was measured according to Misra & Fridovich (1972). The result was expressed UI SOD mg protein⁻¹. For catalase (CAT) activity (liver, kidney and heart), it was observed by spectrophotometry the decomposition of H₂O₂, and the results expressed in $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$ according to Nelson & Kiesow (1972). For reduced glutathione (GSH) in three tissues, the formation of anilide thiolate was evaluated and compared to a standard GSH curve and the result was expressed in $\mu\text{mol GSH mg protein}^{-1}$ (Sedlack & Lindsay 1978). Thiobarbituric acid reactive substances (TBARS) were measured in liver, kidney and heart and the results were compared with an increasing concentration of MDA and the amount of lipid peroxidation was expressed in nmol MDA mg protein⁻¹ (Buege & Aust 1978). The protein content for all determinations was made by the Bradford method (1976), using bovine serum albumin as the standard for construction of the calibration curve. The samples were read at 595 nm.

Aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities, glucose (GLU) and cholesterol (CHO) were measured from plasma using commercial kits (Labtest[®]). Hematocrit (HCT), white cell count (WBC), red cell counts (RBC), hemoglobin (Hb) and platelets (PLT) were determined using the biochemical analyzer (XT-18000 Sysmex, Roche, Hitachi Ltd, Tokyo, Japan).

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MICRONUCLEUS TEST

For the micronucleus test 2,000 polychromatic erythrocytes (PCEs) were analyzed per animal blinded. The purpose of this test is to observe the micronucleated polychromatic erythrocytes (PCEMN) frequency, where increasing this frequency indicates DNA damage (MacGregor et al. 1987).

A formula was used to verify the percent harm reduction as the mean frequency decrease of micronucleated cells using the formula (Mahoharan & Banerjee 1985, Waters 1990):

$$(\%) \text{ reduction} = \frac{(\text{frequency of MNPCEs in A} - \text{frequency of MNPCEs in B}) \times 100}{(\text{frequency of MNPCEs in A} - \text{frequency of MNPCEs in C})}$$

Where A corresponds to the positive control group; B the group of analysis (group that was observed the reduction of micronuclei) and C the negative control group.

HYSTOLOGY OF THE LIVER

The mice livers were removed at the end of the experiment and fixed in 10% buffered formalin. Subsequently, they were cut transversely and dehydrated with ethanol and embedded in paraffin with a thickness of 4 μm stained with Hematoxylin and Eosin. Evaluation of this organ followed the observation of the sinusoids and the central vein as to the possible dilatation, infiltrations in the hepatic tissue by inflammatory cells or vacualization.

STATISTICAL ANALYSIS

The biochemical results obtained (mean \pm standard deviation) were analyzed by one way analysis of variance (ANOVA), followed by the *Tukey* test to verify the differences between the experimental groups. In all cases, a level of significance was established for rejection of the null hypothesis of 5% ($p < 0.05$). For the micronucleus frequency test, the Qui-square test (Pereira 1991).

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RESULTS

FLAVONOID CONTENT AND ANTIOXIDANT POTENTIAL IN VITRO

The *in vitro* antioxidant activity of the fractions was evaluated, where F1 had antioxidant activity better than the standards used the ascorbic acid and rutin, obtaining EC_{50} of $31.2 \mu\text{g mL}^{-1}$, whereas F2 failed to reach 50% elimination of radical DPPH[•]. The amount of flavonoids found in F1 was 40 mg EQ g^{-1} extract and F2 was 17 mg EQ g^{-1} extract.

PRESENCE OF COMPOUNDS BY PHYTOCHEMICAL SCREENING

In the phytochemical evaluation of the fractions, F1 showed positive results for the presence of saponins and tannins, while F2 was positive for the presence of alkaloids, saponins and tannins. For the other tests, such as coumarins, steroids, polysaccharides and purines, the results were negative for these compounds.

FLAVONOID IDENTIFIED IN LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY

(LC-MS/MS)

In the fractions analyzes, flavonoids, rutin (1), quercetin-3 β -D-glucoside (2) and luteolin (3) were identified (figure 2). The fragments, molecular ions and retention time of each flavonoid all data in Table 1. The flavonoids identified in F2 were the same found in F1, except the luteolin.

Table 01. Characterization by LC-MS/MS of the compounds identified in fractions.

Compound d	RT (min.)	Theory m/z	[m/z - 1]	MS ²	Compound identified	Molecular formula
1	10	610.52	609.52	300.20	Rutin	C ₂₇ H ₃₀ O ₁₆
2	10,3	464.38	463.38	300.00	Quercetin-3- β -D-glucoside	C ₂₁ H ₂₀ O ₁₂
3	15	286.24	285.24	133.00	Luteolin	C ₁₅ H ₁₀ O ₆

RT (minutes) retention time, MS² fragment identified.

Source: Author

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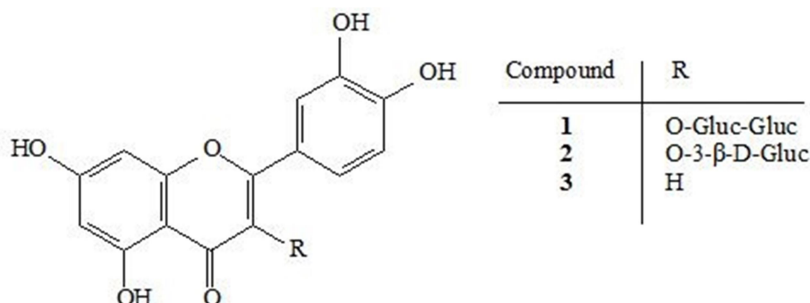


Figure 02. General structure of compounds identified. Source: Author

BIOMARKERS OF OXIDATIVE STRESS

The activities of glutathione-S-transferase (GST) and superoxide dismutase (SOD) were evaluated in hepatic tissue, according to Figure 3 (A and B, respectively). GST had a significant increase of 33% ($p < 0.05$) in the CP group compared to the control group. While SOD presented a significant reduction ($p < 0.05$) in the CP group and in the F1 group, both of 21% compared to the control group.

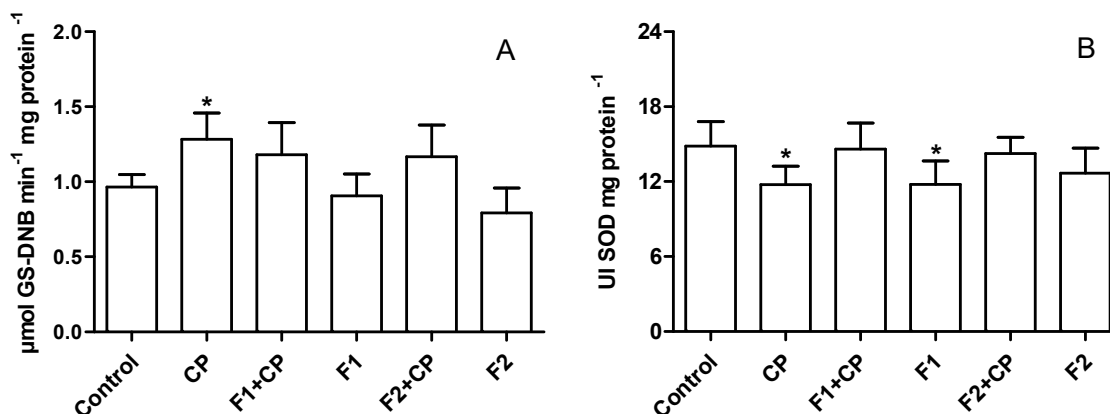


Figure 03. Effect of pre-treatment of F1 and F2 in mice (50 mg kg^{-1}) on CP-induced oxidative stress (75 mg kg^{-1}), evaluating the enzymatic biomarkers (A and B) of the hepatic tissue, $n = 8$. Anova followed by Tukey $*p < 0.05$ compared to the control. Source: Author.

Tests were performed to investigate the activity of the enzyme catalase (CAT) in the hepatic, renal and cardiac tissues (Table 2). There was a significant ($p < 0.05$) reduction in CAT in the CP group, 20% in hepatic tissue and 32% in cardiac tissue, compared to control. In the liver, the groups that received F1 and F2 with CP presented a significant increase of CAT, of 17% and 13%, respectively, compared to the CP group. In the heart, the groups pretreated with F1 and F2 who

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received CP significantly increased ($p < 0.05$) the CAT activity compared to CP group. F1 increased the CAT activity by 57% and F2 by 124%. F2 compared to the control group, the CAT activity in heart still increased 54%. Regarding renal tissue, there was no significant difference between the groups.

Table 02. Effect of pre-treatment with F1 and F2 in mice (50 mg kg⁻¹) on CP-induced oxidative stress (75 mg kg⁻¹) for evaluation of CAT activity in hepatic, renal and cardiac tissues..

Treatments	Catalase activity ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$)		
	Liver	Kidney	Heart
Control	34.57 \pm 1.86	37.55 \pm 7.71	8.93 \pm 1.05
CP	27.86 \pm 1.85*	40.04 \pm 7.23	6.15 \pm 0.94*
F1 + CP	32.63 \pm 2.54**	39.91 \pm 5.79	9.68 \pm 1.67**
F1	35.42 \pm 3.56	46.49 \pm 6.35	9.65 \pm 1.63
F2 + CP	31.51 \pm 1.27**	40.57 \pm 8.21	13.78 \pm 2.24***
F2	32.63 \pm 1.40	44.87 \pm 4.13	8.82 \pm 2.02

Anova followed by *Tukey* (n = 8). * $p < 0.05$ compared to control. ** $p < 0.05$ compared to the CP group. *** $p < 0.05$ compared to the control and CP groups.

In the three tissues evaluated, there was a significant decrease ($p < 0.05$) in GSH content in the CP group compared to control (Table 3). In the hepatic tissue the reduction was of 23%, in the kidney (34%) and 31% in the heart. In the liver, a significant decrease ($p < 0.05$) in GSH was observed in the group receiving F1 and F2 with CP and F1 alone, reductions were 29% (F1 + CP), 37% (F2 + CP) and 63% in the F1 group. On the other hand, in renal tissue, the groups that received F1 and F2 plus CP had a significant increase ($p < 0.05$) in GSH of 51% (F1 + CP) and 82% (F2 + CP) when compared to the CP group. In the cardiac tissue, F1 plus CP increased significantly ($p < 0.05$) GSH by 63% compared to the CP group, and F1 alone increased by 35% in relation to the control. F2 plus CP increased GSH by 217% compared to CP and F2 alone by 122% compared to control.

Lipid peroxidation was evaluated by observing the malondialdehyde production (MDA) by thiobarbituric acid test (TBARS) according to Table 4. A significant increase ($p < 0.05$) in TBARS was observed in the CP treated group, 124% in the liver and 39% in the kidney, when compared to the control group. Fractions increased significantly ($p < 0.05$) TBARS in the liver, the first one alone increased by 62% compared to control. F1 plus CP decreased TBARS by 27% in relation to the CP group, but in comparison with the control increased by 64%. The F2 plus CP increased TBARS by

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313% when compared to control group. A significant increase ($p < 0.05$) of 62% of TBARS in the F2 plus CP was observed in the heart.

Table 03. Effect of pre-treatment with F1 and F2 in mice (50 mg kg⁻¹) on the oxidative stress induced with CP (75 mg kg⁻¹), for evaluation of GSH in hepatic, renal and cardiac tissues.

Treatments	Reduced glutathione – GSH ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$)		
	Liver	Kidney	Heart
Control	10.83 \pm 1.75	5.54 \pm 0.92	32.68 \pm 5.27
CP	8.40 \pm 1.90*	3.70 \pm 0.82*	22.69 \pm 3.83*
F1 + CP	7.76 \pm 1.47*	5.61 \pm 0.95**	37.02 \pm 3.45**
F1	4.01 \pm 0.65*	4.72 \pm 0.38	44.01 \pm 7.16*
F2 + CP	6.89 \pm 1.37*	6.75 \pm 1.36**	72.00 \pm 6.14***
F2	10.49 \pm 0.68	6.73 \pm 1.18	72.62 \pm 3.89***

Anova followed by *Tukey* (n = 8). * $p < 0.05$ compared to control. ** $p < 0.05$ compared to the CP group. *** $p < 0.05$ compared to the control and CP groups.

Table 04. Effect of pretreatment with F1 and F2 in mice (50 mg kg⁻¹) on the oxidative stress induced with CP (75 mg kg⁻¹) in TBARS dosages in hepatic, renal and cardiac tissue.

Treatments	Thiobarbituric acid reactive species – TBARS ($\mu\text{mol MDA mg protein}^{-1}$)		
	Liver	Kidney	Heart
Control	0.37 \pm 0.06	2.25 \pm 0.46	4.00 \pm 1.06
CP	0.83 \pm 0.16*	3.13 \pm 0.63*	3.38 \pm 0.62
F1 + CP	0.61 \pm 0.09**	2.70 \pm 0.52	5.05 \pm 0.78
F1	0.60 \pm 0.05*	2.69 \pm 0.41	5.24 \pm 1.21
F2 + CP	1.53 \pm 0.13***	2.65 \pm 0.27	6.51 \pm 1.43*
F2	0.54 \pm 0.10	2.33 \pm 0.55	5.17 \pm 1.03

Anova followed by *Tukey* (n = 8). * $p < 0.05$ compared to control. ** $p < 0.05$ compared to the CP group. *** $p < 0.05$ compared to the control and CP groups.

BIOCHEMICAL AND HEMATOLOGICAL PARAMETERS

The AST presented a significant increase ($p < 0.05$) in the CP group (50%) and in the F2 plus CP and F2 alone groups, of 71 and 80%, respectively. Only the group that received pre-treatment with F1 was able to reduce AST, a reduction of 53% compared to the CP group. Significant increase ($p < 0.05$) of the ALP (52%) was observed in the group that received CP; in the group treated with F1 plus CP (80%); in group F2 plus CP (55%) and in the group that received only F2 (38%), all in comparison

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to control. Glucose was significantly decreased ($p < 0.05$) in the group treated with F1 only, in 43% when compared to the control group. Cholesterol did not present significant difference between the groups. All results are given in Table 5.

Table 05. Effect of pre-treatment with F1 and F2 in mice (50 mg kg^{-1}) on the oxidative stress induced with CP (75 mg kg^{-1}) for the evaluation of biochemical parameters of plasma.

Treatments	AST (U L ⁻¹)	ALP (U L ⁻¹)	GLU (mg dL ⁻¹)	CHO (mg dL ⁻¹)
Control	88.7 ± 9.5	63.7 ± 13.3	263 ± 22.6	69.4 ± 11.7
CP	133.4 ± 28.3*	96.9 ± 17.8*	260 ± 23.3	68.2 ± 14.5
F1 + CP	62.7 ± 14.5**	115.0 ± 8.0*	268 ± 31.9	76.2 ± 11.4
F1	88.8 ± 14.1	78.6 ± 8.6	152 ± 24.0*	81.1 ± 5.8
F2 + CP	152.0 ± 26.4*	99.2 ± 10.5*	245 ± 23.1	60.2 ± 11.0
F2	160.7 ± 27.5*	88.4 ± 14.7*	236 ± 23.8	66.0 ± 7.3

Anova followed by *Tukey* (n = 8). *p < 0.05 compared to control. **p < 0.05 compared to the CP group.

There was a significant increase ($p < 0.05$) in the HCT levels in the CP group (24%), the F1 group plus CP (17 %), the F1 group (38%), F2 with and without CP 29% and 35%, respectively. The WBC levels were significantly decreased ($p < 0.05$) in 43% in the CP group, 45% in the F1 plus CP group, F1 alone decreased by 26% and F2 plus CP by 55%. Successive increase in RBC levels, 12% in the CP group, 13% in the F2 plus CP group, in the groups that received only F1 and F2, increases of 21 % and 16%, respectively. Hb also increased in the groups CP (31%), F1 plus CP (24%), F1 only (45%), F2 plus CP (37%) and F2 alone (41%). Platelets (PLT) had a significant ($p < 0.05$) increase of 44% compared to the control in the F1-only group.

MICRONUCLEUS TEST

In order to evaluate the antimutagenic and mutagenic activity of the F1 and F2 fractions, the PCEMN frequency was observed, according to Table 7. The CP received group showed an increase in the micronucleus frequency of 80% when compared to the control, showing the genotoxic effect of compound. F1 did not prevent the increase in the PCEMN frequency induced by CP. F2, in turn, significantly reduced PCEMN frequency, and indicating antimutagenic activity by promoting a reduction of 37.3% ($p < 0.01$).

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Table 06. Effect of pre-treatment with F1 and F2 in mice (50 mg kg⁻¹) on the oxidative stress induced with CP (75 mg kg⁻¹) for the evaluation of biochemical parameters of whole blood (hematocrit (HCT), white cell count (WBC), red cell count (RBC), hemoglobin (Hb) and platelet counts (PLT).

Treatments	HCT (%)	WBC (10 ⁹ L ⁻¹)	RBC (10 ¹² L ⁻¹)	Hb (g L ⁻¹)	PLT (10 ⁹ L ⁻¹)
Control	34.3 ± 3.3	12.5 ± 1.7	8.3 ± 0.7	10.2 ± 1.7	820.7 ± 73.8
CP	42.8 ± 2.5*	7.2 ± 1.3*	9.3 ± 0.6*	13.4 ± 0.9*	828.7 ± 132.2
F1 + CP	40.3 ± 0.8*	6.9 ± 1.6*	9.1 ± 0.1	12.7 ± 0.5*	893.3 ± 119.3
F1	47.4 ± 1.2*	9.3 ± 1.3*	10.1 ± 0.6*	14.8 ± 0.4*	1184 ± 121.5*
F2 + CP	44.3 ± 2.0*	5.6 ± 1.2*	9.4 ± 0.3*	14.0 ± 0.6*	843.8 ± 127.7
F2	46.6 ± 2.8*	12.6 ± 2.1	9.7 ± 0.4*	14.4 ± 0.8*	903.0 ± 139.2

Anova followed by *Tukey* (n = 8). *p < 0.05 compared to control.

Table 07. Effect of pre-treatment with F1 and F2 in mice (50 mg kg⁻¹) on the oxidative stress induced with CP (75 mg kg⁻¹), to evaluate antimutagenic and mutagenic activity of fractions, on PCEMN cell frequency of bone marrow.

Treatments	Observed PCE	PCEMN	% of reduction MN
Control	16.000	300	-
CP	16.000	540	-
F1 + CP	16.000	531	#
F1	16.000	320	-
F2 + CP	16.000	468**	37.2
F2	16.000	209	-

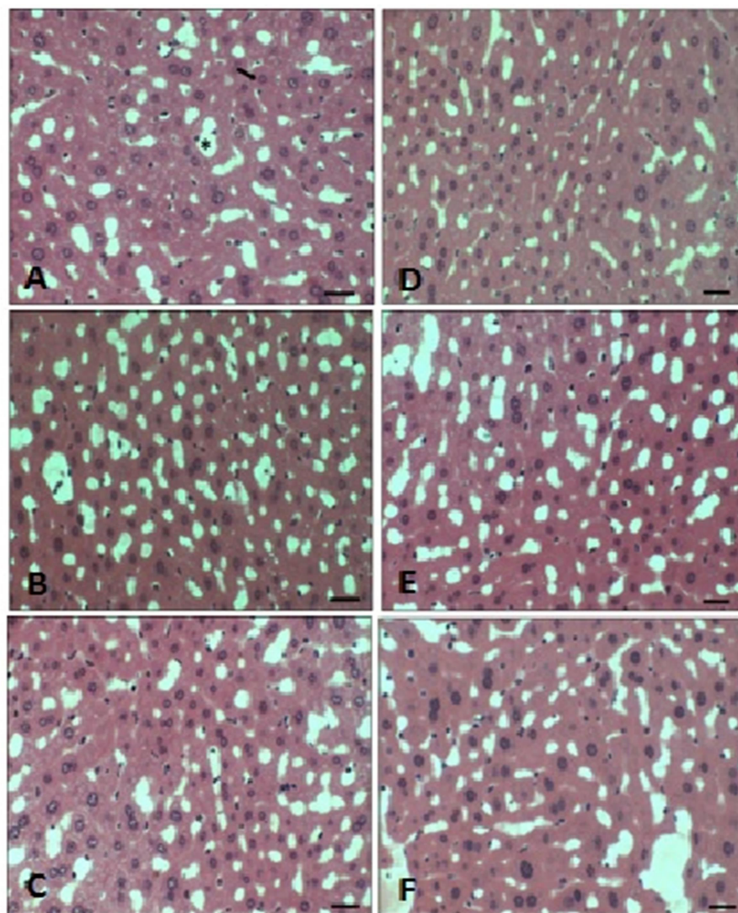
Qui-square test. **p < 0.01 compared to CP group. # There was not significant reduction.

HISTOLOGICAL ANALYSIS OF THE LIVER

No significant histological differences were observed between the treated groups, as observed in Fig. 4 (A, B, C, D, E and F).

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Source: Author

Figure 04. Photomicrograph of the hepatic tissue of mice Swiss; A = control, B = CP (75 mg kg⁻¹), C = F1 + CP, D = F1 (50 mg kg⁻¹), E = F2 + CP and F = F2 (50 mg kg⁻¹). Hepatocyte (arrow). Sinusoid (asterisk). Stained HE. Bar = 50µm.

DISCUSSION

This is the first study involving antioxidant activity this species and had the objective of evaluating the antioxidant action of methanolic fractions obtained from leaves of *C. distachya*, a plant known as embaúba. There are other species of the same genus, which have a number of uses in popular medicine, such as used for treat heart and lung problems, diuretic, antihypertensive and anti-inflammatory drugs (Lorenzi and Matos 2008). In the phytochemical studies using LC-MS/MS, the flavonoids, quercetin-3β-D-glucoside, rutin and luteolin were detected in the fractions. It is possible to observe that Compound 1 presented molecular ion 609.52 [m/z-H] and fragment 300.20 [m/z - 309.32], which corresponds to the loss of two glycols being similar to that obtained by (Zhou et al. 2016). Compound 2 presented molecular ion of 463.38 [m/z -H] and fragment 300.00 [m/z -163.38]

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indicating the breakdown of the glycoside grouping of this glycans (Souza et al. 2016). In relation to Compound 3, which presented molecular ion of 285.24 [m/z -H] and fragment 133.00 [m/z -152.24], representing the C-ring breaking, benzene diol and described similar fragmentation in negative ionization mode analysis by (Brahim et al. 2017). Regarding the phytochemical screening, among other compounds, saponins and tannins were also observed in the species *C. pachystachya* ethanolic extract (Sousa et al. 2015). Flavonoids are viewed by the scientific community as a class of molecules that can be exploited for many pharmacological effects, including antioxidants, cytoprotectors, vasoprotectants, anticancer and cardioprotective agents (Ganeshpurkar & Saluja 2017).

The antioxidant potential of the fractions was evaluated in an in vitro test, where the ability to sequester the radical DPPH[•] was verified. F1 was able to sequester 98% of the DPPH[•] radical with EC₅₀ of 31.2 µg mL⁻¹, higher than those found in the standards tests, whereas the rutin sequestered 92% of the DPPH[•] radical of its EC₅₀ of 40.7 µg mL⁻¹ and ascorbic acid 96% with EC₅₀ of 32.8 µg mL⁻¹. F2 presented a lower in vitro antioxidant capacity, sequestering 45% of the DPPH[•] radical. Previously it was also observed high antioxidant capacity in the DPPH[•] test, the methanolic extract of *C. pachystachya* presented EC₅₀ of 3.1 µg mL⁻¹ (Aragão et al. 2010).

In vivo tests, CP impaired the antioxidant system, significantly reducing SOD (liver), CAT (liver and heart) and GSH (liver, kidney and heart) (Bhatt et al. 2017, Jnaneshwari et al. 2013). The decrease in SOD activity suggests that CP can induce superoxide radical formation (Ceribasi et al. 2010) and this enzyme is important because it dissociates the superoxide radical into hydrogen peroxide (Chakraborty et al. 2009). The reduction of another antioxidant enzyme, CAT, is due to the oxidative stress caused by CP, which also contributes to increased levels of hydrogen peroxide (Bhatt et al. 2017). In addition, acrolein (metabolite produced by CP) has other mechanisms to reduce enzymes, being able to induce irreversible inactivation of SOD activity, since it attacks the amino acid residue of this enzyme, histidine (essential for its functioning), leading later to the loss of the histidine and the copper of this metalloprotein, the copper becomes free of this enzyme that was oxidatively modified taking the loss of its activity. Acrolein is incorporated into the proteins and generates carbonyl derivatives. The superoxide radical in turn attack the CAT enzyme this process (Moghe et al. 2015). The GSH depletion caused by the same drug is due to the production of the metabolite acrolein, which is able to form conjugation with GSH, reducing its cellular level (Nafees et al. 2015). The decrease in CAT was observed in the liver and heart; however, in these tissues the F1 and F2 fractions attenuated the action of CP, preventing a significant decrease in CAT activity. The restored CAT levels are in accordance

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with the study (Ortmann et al. 2016), where they used a fraction of the leaves of the species *C. pachyschya* at the concentration of 50 mg kg⁻¹ in Wistar rats to attenuate the oxidative stress. F1 and F2 also account for the significant increase in GSH of the heart, higher than the control, consists of data found using ethanolic extract *C. obtusifolia* in in vitro tests, with oxidative stress induced by UV radiation, in addition to having also elevated CAT activity (Alves et al. 2016). The increase in cardiac GSH higher than those found in controls indicates that the fractions have a strong cardioprotection effect. Liver GST showed an increase after CP treatment. This may be a physiological response to the increase of free radicals (Yousefipour et al. 2005). This enzyme participates in the detoxification process of xenobiotics by conjugating the GSH with these compounds (Amien et al. 2015).

In the lesion protocol we observed increases in TBARS, AST, ALP (Basu et al. 2015, Jnaneshwari et al. 2013). The increase in these enzymes may be related to liver damage, although ALP is not specific to assess this type of damage, usually related to the condition of biliary obstruction together with the evaluation of bile salt concentration, as AST can be considered more specific for evaluate hepatocellular damage, however, it has a great tissue distribution and evidence of this type of damage must also be related to other complementary tests (Marshall et al. 2016). Since AST is also found in cardiac tissue, its increase may also be related to damage to this tissue, in this case it is possible to make a comparison with the effects observed in the GSH that was reduced by CP, together cardiac GSH and AST, may indicate damage to cardiac tissue. The levels of TBARS were evaluated in the three tissues (hepatic, renal and cardiac), but only in the CP heart there was no increase in lipid peroxidation, even cardiotoxicity being a characteristic of CP. However, it is reported in the literature that uncommon cardiac damage is associated with the administration of high doses of cyclophosphamide, usually occurring at doses of 4.5 g or more (Kufe et al. 2003). Levels of TBARS were significantly reduced ($p < 0.05$) with pre-treatment of F1 (liver). In previous data, the methanolic fraction of leaves of *C. pachyschya* reverted to lipoperoxidation in rats (Ortmann et al. 2016). As well with mice using the same species, but with aqueous extract, results are in agreement with that found with F1 treatment (Gazal et al. 2014). ALT and AST activities have also increased due to oxidative stress and are related to liver damage (Shanmugarajan et al. 2008). F1 decreased significantly ($p < 0.05$) AST activity, according to data found in which Wistar rats received the hydroethanolic extract from the leaves of *C. glaziovii* reversed the oxidative stress caused by carbon tetrachloride (Petrinilho et al. 2012).

In relation to the increase of HCT, RBC, Hb caused by CP, a reduction in these parameters generally occurs as observed at doses of 25 mg kg⁻¹ and 200 mg kg⁻¹ of this drug (Zhang et al. 2017,

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Song et al. 2016). However, as expected CP decreased WBC levels in the group treated with CP only (Roy et al. 2014). The decrease in WBC can be explained, because CP has immunosuppressive activity, a result that is in agreement with one of the characteristics of the drug (Gupta et al. 2001). Fractions promoted a significant increase in HCT, RBC and Hb. It is possibly because there is the presence of rutin in both fractions, since it has already been identified as a substance that can increase hematopoietic production (Han et al. 2017). The group pre-treated with F1 that received CP and group treated with F1 only showed a marked decrease in WBC levels, a fact that can be interpreted as a possible immunosuppressive effect attributed to the plant. In this perspective, verified immunosuppressive action of the roots of the Colombian embaúba, *Cecropia telenitida*, in tests performed on dendritic cell culture from mouse bone marrow cells (Pelaéz et al. 2013). It is possible that compounds found in *C. telenitida* are also found in *C. distachya*, but further studies are necessary for this confirmation.

F1 caused a significant reduction in the glycemia of the mice, showing the hypoglycemic potential of the plant, in addition, a significant increase in the number of platelets was observed. In previous studies, extracts of three species of the *Cecropia* family, *C. obtusifolia*, *C. pachystachya* and *C. glaziovii*, presented a hypoglycemic effect in rats (Andrade-Cetto et al. 2001, Arend et al. 2011, Arend et al. 2015). Even in tests in humans with diabetes mellitus type 2, the hypoglycemic effect with aqueous extract *C. obtusifolia* was observed (Revilla-Monsalve et al. 2007). This hypoglycemic effect of F1 can be attributed in part to the flavonoid present in this fraction, luteolin because tests performed with luteolin showed a hypoglycemic effect in mice (Zang et al. 2016).

The micronucleus test is widely used for the detection of clastogenic agents and aneugenic agents, which causes chromosome breakage or changes in the number of chromosomes (Ribeiro et al. 2003). In the present work, the results provided evidence of CP genotoxicity, since it significantly increased the PCEMN frequency by 80% compared to control, results already observed using doses of 25 and 50 mg kg⁻¹ of CP (Basu et al. 2015, Lin et al. 2017). This is due to the fact that, when metabolized, CP generates metabotes that alkylate DNA, provoking this genotoxic effect (Khan et al. 2014). F2 was antimutagenic by decreasing the frequency of PCEMN induced by CP ($p < 0.01$). The anti-mutagenic effect linked to F2 is possibly related to the flavonoids present, once studies reported the ability of flavonoids to act in chemoprotective (Iriti et al. 2017), including intervening in stages of carcinogenesis (George et al. 2017). In this sense, aqueous extracts of *C. obtusifolia* leaves in cultured human lymphocytes tests were used to verify the micronucleus frequency and somatic mutation, in

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which they pointed out that the extract, did not produce any genotoxic effect (Toledo et al. 2008). In addition, Mendonça et al. (2016) tested the genotoxic activity of *C. pachystachya* aqueous extract in a micronucleus and comet test. In the micronucleus test, peripheral blood of mice treated with 500-2000 mg kg⁻¹ was used; the increased frequency of micronuclei was not verified. In the comet assay a reduction of 60% damage to the DNA induced by H₂O₂ was observed (Mendonça et al. 2016). These works corroborate with results obtained in this research in which it points out an antimutagenic action of the F2 fraction. Even flavonoids have already been reported in DNA protection, the rutin (flavonoid present in the fractions); in low concentrations the rutin was able to protect DNA from mitomycin C-induced damage in human lymphocytes (Üngeder et al. 2004); and did not present mutagenic activity in the bone marrow of treated mice (Silva et al. 2002).

As for histology, no significant differences were observed between the groups, showing that the extract did not induce cellular damage in the hepatic tissue. In the same way, Pettrinilho et al. (2012) did not observe liver damage of rats receiving *C. glaziovii* extract at the concentration of 40 mg kg⁻¹.

Despite the several benefits obtained by the fractions, negative factors were observed, as F1 alone decreased SOD and GSH, both of the liver. F2 potentiated an increase in TBARS (liver and heart), as well as increasing the levels of AST and ALP alone, data not yet observed in the literature, which suggests a certain toxicity from the fractions.

CONCLUSION

The results of the present study demonstrate that the fractions present biologically relevant activities, stimulating the antioxidant system in order to avoid oxidative lesions, being still hypoglycemic (F1) and antimutagenic (F2) in protecting DNA from possible damages. F1 is also attributed possible immunosuppressive effect, something that can be further explored in new studies. Finally, the harmful effects attributed to the fractions can be associated to other compounds acting in synergism and not elucidated and isolated in this work. In this context, we see the need for other ethnobotanical studies that will ensure the safety in the use of natural products in relation to toxicological issues.

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Efeito quimioprotetor de extratos foliares de *Cecropia distachya* Huber (Urticaceae) em camundongos submetidos a estresse oxidativo induzido por ciclofosfamida

RESUMO

O uso de plantas medicinais ocorre há muitos anos em consequência de informações que são repassadas por sucessivas gerações. O interesse pela descoberta de novos e seguros fármacos antioxidantes tem aumentado, uma vez que as plantas são ricas em metabólitos secundários. Com esse propósito, o objetivo deste trabalho foi avaliar os efeitos quimioprotetores das folhas da embaúba *Cecropia distachya* Huber usando duas frações metanólicas (F1 e F2) sobre o estresse oxidativo induzido pela ciclofosfamida (75 mg kg⁻¹) em camundongos, bem como algumas análises fitoquímicas tais como LC-MS/MS. Os flavonoides quercetina-3β-D-glucosídeo, rutina e luteolina foram identificados na F1, mas apenas quercetina-3β-D-glucosídeo e rutina na F2. No modelo experimental, as frações aumentaram a catalase hepática e cardíaca (CAT), a glutatona reduzida (GSH) do rim e do coração. Além disso, ambas as frações aumentaram os níveis de glóbulos vermelhos (RBC), hematócrito (HCT)

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e hemoglobina (Hb) e diminuíram glóbulos brancos (WBC). Os efeitos atribuídos somente a F1 foram à redução na lipoperoxidação hepática (TBARS) e na atividade de aspartato aminotransferase (AST), aumento na contagem de plaquetas (PLT) e diminuição da concentração de glicose (GLU). Quanto a F2, apenas diminuiu a frequência de micronúcleos nas células da medula óssea. Em alguns casos, as frações também foram hepatotóxicas. Estes resultados demonstraram que ambas as frações estimulam as defesas antioxidantes, sendo hipoglicemiante (F1) e antimutagênico (F2), e, os efeitos nocivos atribuídos às frações podem ser a associação de compostos que não foram elucidados neste trabalho.

Palavras-Chave: Histologia, lesões oxidativas, hipoglicemia, imunossupressão.

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