ORIGINAL ARTICLE

Antioxidant potential of *Carica papaya Linn (Caricaceae)* leaf extract in mice with cyclophosphamide induced oxidative stress

Potencial antioxidante do extrato de folhas de Carica papaya Linn (Caricaceae) em camundongos com estresse oxidativo induzido por ciclofosfamida

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ABSTRACT

AIMS: This study aimed to investigate the effects of crude extract of *Carica papaya* leaves on oxidative stress in mice induced by cyclophosphamide, as well as phytochemical profile characterization of this extract.

METHODS: The male *Swiss* mice received 15 days of treatment with the extract (500 mg kg⁻¹, via gavage) and intraperitoneal injection of cyclophosphamide (75 mg kg⁻¹) or saline (0.9%) on the 15th day. After 24 h the last treatment, the animals were anesthetized for blood withdrawal, sacrificed and removal of the organs for analyses (liver, kidney and heart). In the biochemical tests were determined: hematological parameters in blood, aminotransferases, alkaline phosphatase, glucose and total cholesterol dosages in plasma, enzymatic and non-enzymatic antioxidants and lipid damage marker were evaluated in different tissues, besides genotoxic and histopathological analyzes.

RESULTS: In the extract of *Carica papaya* leaves, the flavonoids quercetin-3β-D--glucoside and rutin were identified, besides present positive results for alkaloids, saponins and tannins. This extract increased the activity of glutathione-S-transferase and catalase enzymes in the liver and reduced the levels of reduced glutathione in the kidneys and hematocrit levels, red cell count, and hemoglobin. It promoted the decrease of the reactive species of thiobarbituric acid (TBARS) in the kidneys and the activity of enzyme aspartate aminotransferase in the plasma and was antimutagenic in the micronucleus test.

CONCLUSIONS: The study showed that extract of *Carica papaya* was beneficial against oxidative events and prevented DNA damage. The extract also showed hepatotoxicity, therefore prolonged infusion of papaya leaves is not advisable.

Keywords: antimutagenicity; antioxidant defense; ethnobotany; secondary metabolites; erythropoiesis.

RESUMO

OBJETIVOS: O objetivo deste estudo foi investigar os efeitos do extrato bruto de folhas de *Carica papaya* sobre o estresse oxidativo em camundongos induzidos pela ciclofosfamida, bem como a caracterização do perfil fitoquímico deste extrato.

MÉTODOS: Os camundongos *Swiss* machos receberam 15 dias de tratamento com o extrato (500 mg kg⁻¹, via gavagem) e injeção intraperitoneal de ciclofosfamida (75 mg kg⁻¹) ou salina (0,9%) no 15° dia. Após 24 h do último tratamento, os animais foram anestesiados para retirada do sangue, sacrificados e retirada dos órgãos para análises (fígado, rim e coração). Nos testes bioquímicos foram determinados: parâmetros hematológicos em sangue, aminotransferases, fosfatase alcalina, dosagens de glicose e colesterol total no plasma, antioxidantes enzimáticos e não enzimáticos e marcador de dano lipídico foram avaliados em diferentes tecidos, além de análises genotóxicas e histopatológicas.

RESULTADOS: No extrato de folhas de *Carica papaya* foram identificados os flavonoides quercetina-3β-D-glicosídeo e rutina, além de resultados positivos para

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alcaloides, saponinas e taninos. Este extrato aumentou a atividade das enzimas glutationa-S-transferase e catalase no fígado e diminuiu os níveis de glutationa reduzida nos rins, a concentração do hematócrito, a contagem dos glóbulos vermelhos e a hemoglobina. Promoveu a diminuição das espécies reativas de ácido tiobarbitúrico (TBARS) nos rins, a atividade da enzima aspartato aminotransferase no plasma e foi antimuta-gênico no teste do micronúcleo.

CONCLUSÕES: O estudo mostrou que o extrato de *Carica papaya* foi benéfico contra eventos oxidativos e preveniu danos no DNA. O extrato também mostrou hepatotoxicidade, portanto, a infusão prolongada de folhas de mamão não é aconselhável.

Descritores: antimutagenicidade; defesa antioxidante; etnobotânica; metabólitos secundários; eritropoiese.

ABBREVIATIONS: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; CE, crude extract; CP, Cyclophosphamide; DPPH·, 1,1-diphenyl-2-picryl hydrazil; GST, glutathione-S-transferase; Hb, hemoglobin; HCT, Hematocrit; LC-MS/MS, liquid chromatography coupled to mass spectrometry; MDA, malondialdehyde; mg EQ g^{-1}, milligrams of quercetin equivalent per gram of extract; PCE, polychromatic erythrocytes; PCEMN, micronucleated polychromatic erythrocytes; RBC, red cell count; SOD, superoxide dismutase; TBARS, thiobarbituric acid; Tris, trisaminomethane; WBC, white cell count.

INTRODUCTION

Free radicals are molecules or molecular fragments containing one or more unpaired electrons, a condition that confers high reactivity and may present considerable interference in cellular integrity [1]. The adverse effects of free radicals occur when there is an overproduction of reactive species and a deficiency of antioxidant enzymes and non-enzymatic antioxidants (reduced glutathione, ascorbic acid, tocopherols, carotenoids, vitamins A and E) [2].

The organism has an antioxidant defense system, enabling the evaluation of oxidative stress by means of analysis of some antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, glutathione-S-transferase (GST) and others such as reduced such as reduced gluthatione (GSH) levels as even though it is a non-enzymatic antioxidant agent, GSH can act as a substrate in the reactions catalyzed by enzymes, for example GST, or can act directly in free radical scanning [3].An additional parameter useful in evaluating oxidative stress is the lipid peroxidation generated in the cellular membranes. This triggers several actions harmful to the cell, which can result in its death. As the free radical has a very short half-life, it is only possible to be measured by markers such as malondialdehyde through TBARS analysis (thiobarbituric acid reactive substances) [4].

Cyclophosphamide (CP) is a widely used drug for the treatment of chronic diseases, autoimmune diseases and cancer [5]. The antineoplastic activity of CP is due to phosphoramide mustard, which promotes the alkylation of DNA, in addition to the other metabolite, acrolein, which interferes with the antioxidant system producing reactive species, superoxide radical and hydrogen peroxide, leading to toxicity of various organs [6].

The *Carica papaya* Linn, known as *mamoeiro* (in Brazil) is a tree present in tropical and subtropical regions of the world, with its fruit known as papaya. The fruit stands out as having a pleasant taste and aroma and high nutritional value, being rich in sugars, calcium, carotenoids and vitamin C [7].

The fruits, leaves, flowers, roots, seeds and even latex are all widely used in traditional medicine to treat a variety of diseases. In particular, the leaves are used in healing, in the treatment of dengue, jaundice and malaria [8]. Some studies have investigated these medicinal properties of the leaves, for example the methanolic extract promoted inhibition of sickle hemoglobin formation and hemolysis *in vitro* tests [9] and antioxidant and cytoprotective action of the hydrometanolic extract in human liver cell lines oxidatively stressed with tert-butyl peroxide [10]. The ethanolic extract presented analgesic action compared to the aspirin action in an experimental model with mice [11]; antibacterial and antithrombocytopenic activity in *Wistar* rats using aqueous extract and lastly antiproliferative and antimetastatic activity of dry leaf extract on prostate cancer cell lines was observed [12].

The objective of the present study was to investigate the effects of raw aqueous extract of *C. papaya* leaves on biochemical, hematological and mutagenic parameters in mice induced by oxidative stress induced by cyclophosphamide, an experimental model adopted unpublished in the literature, besides the phytochemical characterization of the extract.

MATERIAL AND METHODS

Chemical products

CP from Baxter, amentoflavone, apigenin, canferol, luteolin, quercetin, quercetin-3β-D-glucoside, rutin, taxifoline, aluminum chloride, 1,1-diphenyl-2-picryl hydrazil (DPPH·), ascorbic acid, Triton X-100, hydrogen peroxide, reduced glutathione (GSH), 2-thiobarbituric acid, 5,5'-dithiobis (2-nitrobenzoic acid), Bradford's reagent, trichloroacetic acid, potassium phosphate monobasic, potassium phosphate dibasic, sodium phosphate, Ethylenediamine tetraacetic acid, trisaminomethane (Tris) and bovine serum albumin were all purchased from Sigma-Aldrich (St. Louis, USA). The solvents used for the tests were all from Merck. Glucose, cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) dosages used were from kits purchased from Labtest®, Diagnóstico S.A, Minas Gerais, Brazil.

Collection and botanical identification

The leaves of *C. papaya* were collected in the city of Sinop, Mato Grosso, Brazil, geographical coordinates S 11˚53'53.016" W 55˚30'18.828". Voucher specimens were deposited in the Herbarium Centro Norte Matogrossense, Federal University of Mato Grosso under registration number 7012 and identified by Professor Milton Omar Côrdova Neyra.

Preparation of *Carica papaya* Linn extract

For the preparation of the extract, the leaves of *C. papaya* were collected and cleaned with distilled water and exposed to the fan-forced oven drying process at an average temperature of 40 ˚C for seven days. After drying, the leaves were crushed, yielding a weight of 220 g. The crushed leaves were infused with distilled water (4.5 L) under a stable temperature of 70 ˚C for 1 hour in a water bath. After this time, the material was filtered and rotated with vacuum pump under reduced pressure and water bath at 60 ˚C. Subsequently, the samples were frozen and lyophilized to obtain the final crude extract (CE) of 68.47 g.

The aqueous extract was produced to resemble that which is used by the general population, in which it is commonly used to make tea or as an infusion. The selected dose was 500 mg kg⁻¹,in accordance with [13].

Determination of flavonoids and DPPH· test

The determination of the amount of total flavonoids was performed using quercetin as a standard curve in a reaction with aluminum chloride, in accordance with [14]. 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 based on the methodology of [15]. From the consumption of the DPPH· free radical (2,2-diphenyl-1-picrylhydrazine) in the samples, the absorbance of the solutions read at wavelength 515 nm using rutin and ascorbic acid as standards was measured.

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 following the methodology of [16].

Phytochemical identification of *C. papaya* leaves by liquid chromatography coupled to mass spectrometry

The analytical standards for amentoflavone, apigenin, canferol, luteolin, quercetin, quercetin-3β-d-glucoside, rutin and taxifoline were used to identify flavonoids present in the leaves of *C. papaya.* The extract was 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 4.6 x 150 nm diameter, 3.5 μm particle size). The sample elution method used a flow rate of 0.5 mL min⁻¹ and an elution gradient composed of Solvent A (water : formic acid; 99.9: 0.1% (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 (m/z-1). The identification of flavonoids occurred in the mode of acquisition by negative ionizationaccording to [17], source temperature 300 ˚C, and desolvation temperature 250 ˚C.

Animals and experimental design

This research was approved by the Animal Use Ethics Committed under protocol number 28108.722412 / 2017-58. Male *Swiss* mice, with a mean weight of 30-40 g, were used. Throughout the experiment's entirety, the animals were kept under controlled conditions of temperature (22 ± 2 ˚C), relative humidity (55 ± 10%), light cycle (12 hours light/dark), commercial diet and filtered water kept in boxes of polyethylene and stainless-steel grid. The acclimatization period was two weeks. The animals received oral treatments (water with vehicle or extract, by gavage (0.3 mL) for 15 days and an intraperitoneal injection of CP or saline on the 15th day. The following are the groups and their treatments (**Figure 1**). CP at a concentration of 75 mg kg^{-1} following the protocol of [18].
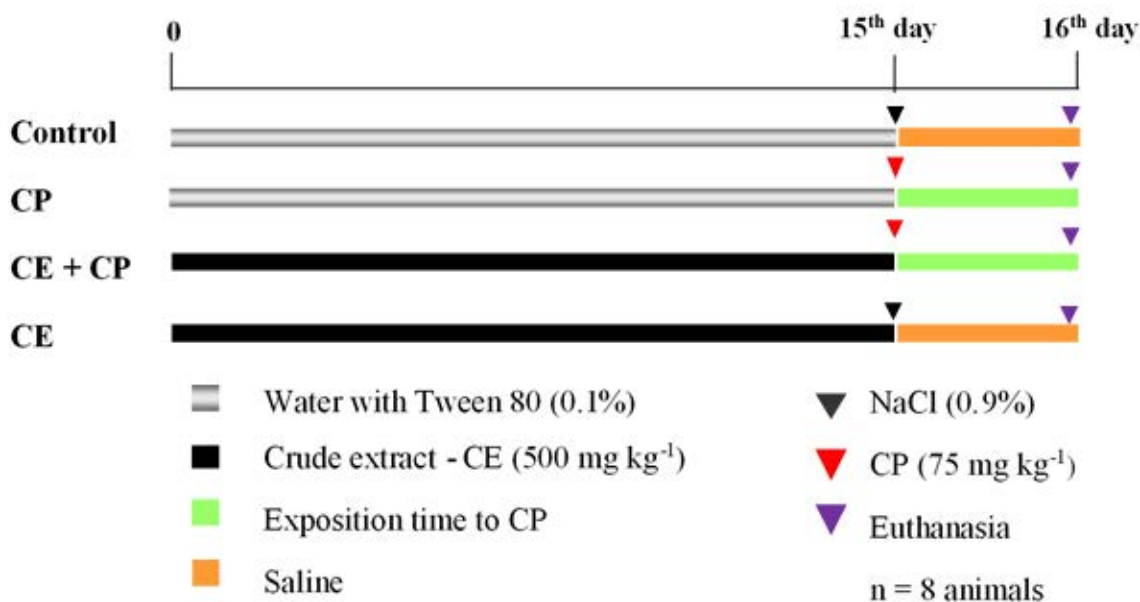

Figure 1 – Experimental design for the evaluation of antioxidant and antimutagenic effects of the CE of *C. papaya.* CE: Crude extract; CP: cyclophosphamide

Biochemical and hematological analyzes

Twenty-four hours after the last treatment dose and 8 hours of fasting, the animals were anesthetized intraperitoneally with ketamine 50 mg kg^{-1}, xylaxine 20 mg kg^{-1}and acepromazine 20 mg kg^{-1}. Blood was withdrawn via cardiac puncture with 5,000 IU mL^{-1} sodium heparin-containing syringes and the animals were subsequently sacrificed for liver, kidney and heart samples, which were frozen at -80 ˚C.

Biochemical analyzes were performed on superoxide dismutase (SOD) based on [19], the result being expressed as UI SOD mg protein^{-1}. Catalase activity (CAT) was measured according to [20] and the results expressed in μmol min^{-1}mg protein^{-1}. GST analysis followed the methodology [21], with the GST activity expressed in μmol GS-DNB min^{-1}mg protein^{-1}. Reduced glutathione (GSH) was quantified according to [22], with thiolate anion formation evaluated and compared to a standard GSH curve. The result was expressed in μmol GSH mg protein^{-1}. The thiobarbituric acid reactive substances (TBARS) followed the method

described by [23].The results were compared with a calibration curve of increasing concentration of 0.75, 1.5, 3.0, 6.0, 12.0 mM MDA (malondialdehyde) and the amount of lipid peroxidation was expressed in nmol MDA mg protein^{-1}. Analysis of the protein content of the samples of all tissues is necessary to obtain the results of the tests in mg protein^{-1}, followed by the method of [24].

Glucose, cholesterol, aspartate aminotransferase (AST), alanine aminotransaminase (ALT) and alkaline phosphatase (ALP) assays were all assayed using commercial (Labtest®) kits. Hematocrit (HCT), white cell count (WBC), red cell count (RBC), hemoglobin (Hb) and platelets were determined using the biochemical analyzer (XT-18000 Sysmex, Roche, Hitachi Ltd, Tokyo, Japan).

Micronucleus test

The micronucleus test was performed in accordance with the methodology of [25] where 1000 cells per slide (two slides) and 2000 polychromatic erythrocytes (PCE) per animal were analyzed. The observation was performed under blind test using a light microscope with 1000 times magnification. The objective of this test is to observe the frequency of micronucleated polychromatic erythrocytes (PCEMN) indicating DNA damage.

A formula was used to verify the percent of harm reduction as the mean frequency decrease of micronucleated cells according to [26]and [27] using the formula:

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

Where A corresponds to the CP group (positive control); B to the analysis group (group receiving the extract and CP) and C to the negative control group.

Histology of the liver

The livers of the mice 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. Paraffin sections of approximately 4 μm

were assembled and stained with Hematoxylin and Eosin. The evaluation criteria for histological analysis were the observation of the sinusoids and central vein if there were dilation, infiltrations in the hepatic tissue by inflammatory cells and vacuolization.

STATISTICAL ANALYSIS

In order to compare the differences in the biochemical variables between pretreatment (water with vehicle or extract) and treatment (CP or saline), statistical analyses were performed using one-way or two-way analysis of variance (ANOVA) followed by the *Tukey*'s test. *Bartlett's* test was performed to compare the homogeneity of variances among the groups. Data were expressed as mean ± standard deviation. For the micronucleus frequency test, the chi-square test was used according to [28].

RESULTS

Flavonoid content and antioxidant activity *in vitro*

The CE extract presented low antioxidant activity *in vitro* when compared to ascorbic acid and rutin standards, not being able to reach 50% elimination of the DPPH· radical. On the total flavonoids, the value of 21.7 mg EQ g^{-1} was obtained in the CE (data not shown).

Presence of compounds by phytochemical screening

In the phytochemical screening of CE, the tests showed positive results only for the presence of alkaloids, saponins and tannins (data not shown).

Flavonoids identified in liquid chromatography coupled to mass spectrometry (LC-MS/MS)

In the LC-MS/MS analysis of the CE, flavonoids rutin and quercetin-3β-D-glycoside were identified (**Figure 2**). All information on the retention time, molecular ion and fragmentation of the compounds are in **Table 1**.
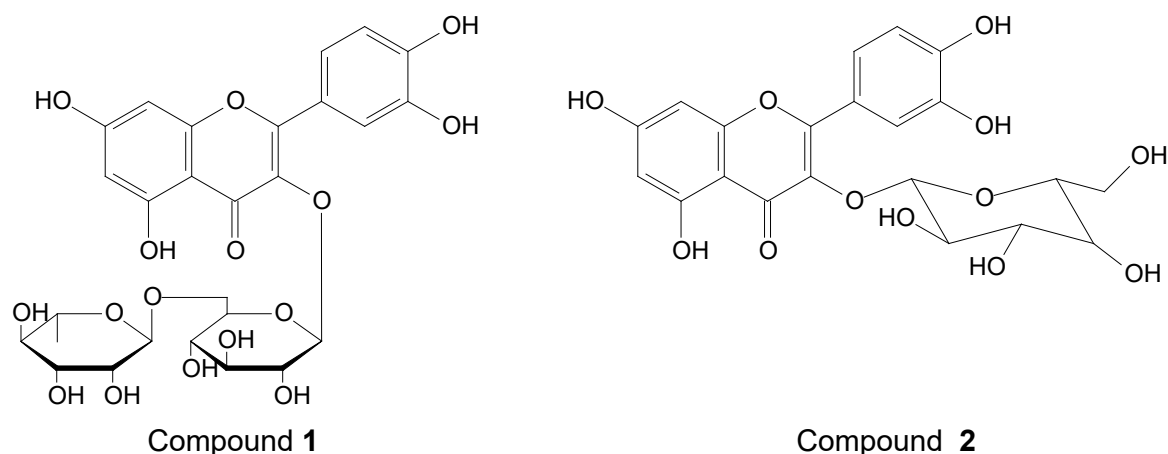
Compound **1**   Compound **2**

Figure 2 – Structure of the flavonoids found in the crude extract.

TABLE 1 – Characterization by LC-MS/MS of the compounds identified in CE of *C. papaya.*

Compound	RT (min)	MW (g mol^{-1})	[M-H]	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-glycoside	$C_{21}H_{20}O_{12}$

CE, crude extract; LC-MS/MS, liquid chromatography mass spectrometry; [M-H], Less Hydrogen Molecule; MS^2; fragment identified; MW, Molecular weight; RT, retention time in minutes

Biomarkers of oxidative stress

The activity of the SOD and GST enzymes of the hepatic tissue was evaluated, according to **Figure 3** (A and B, respectively). There was a significant reduction in SOD activity ($p < 0.05$) in the CP group (27%) and in the group receiving CE plus CP (29%) compared to the control group. The CE could not prevent the decrease of SOD by CP. GST activity showed a significant increase ($p < 0.05$) in the CP group of 37%, and in the groups that received CE plus CP (41%) and CE alone (36%), compared to control.
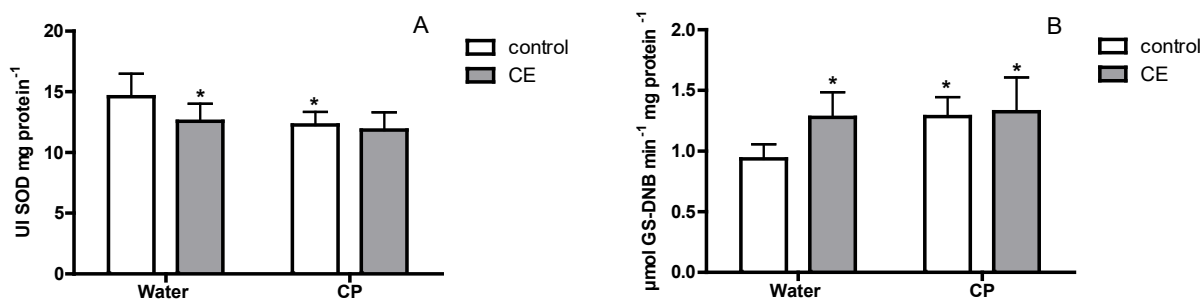

Figure 3 (A, B) – Effect of CE pre-treatment (500 mg kg^{-1}) on CP-induced oxidative stress (75 mg kg^{-1}) for the evaluation of enzymatic biomarkers SOD and GST in hepatic tissue (n = 8). Anova followed by *Tukey* test.

*p < 0.05 compared to control. CE: crude extract; CP: cyclophosphamide; GST: glutathione-S-transferase; SOD: superoxide dismutase.

Table 2 shows CAT activity in the hepatic, renal and cardiac tissues. In the hepatic tissue a significant reduction of the CAT ($p < 0.05$) in the CP group was observed, a reduction of 24% in comparison to the control. However, CE significantly restarted CAT activity ($p < 0.05$), the action of CP. The other tissues did not present significant differences.

TABLE 2 – Effect of CE pre-treatment (500 mg kg^{-1}) on CP-induced oxidative stress (75 mg kg^{-1}) for evaluation of catalase activity in hepatic, renal and cardiac tissues.

Catalase (µmol min^{-1} mg protein^{-1})

Treatments	Liver	Kidney	Heart
Control	31.81±4.01	44.83±10.76	8.06±1.60
CP	24.30±3.66*	44.67±7.23	6.73±1.49
CE + CP	31.89±6.73**	50.15±9.70	7.41±1.50
CE	30.44±3.60	48.78±7.76	9.92±2.14

*$p < 0.05$ compared to control. **$p < 0.05$ compared to the CP group. CE, crude extract; CP cyclophosphamide; (n = 8).

The GSH levels (**Table 3**) saw a significant decrease in GSH content ($p < 0.05$) in liver (32%), kidney (35%) and heart (41%) in the CP group. CE significantly increased GSH ($p < 0.05$), preventing CP action in renal tissue, and an increase of 72% when compared with CP group.

TABLE 3 – Effect of pre-treatment with CE (500 mg kg^{-1}) on CP-induced oxidative stress (75 mg kg^{-1}) for evaluation of GSH in hepatic, renal and cardiac tissues.

Reduced glutathione – GSH (µmol GSH mg protein^{-1})

Treatments	Liver	Kidney	Heart
Control	10.83±1.62	5.47±0.88	36.44±7.00
CP	7.43±0.87*	3.58±0.83*	21.80±5.46*
CE + CP	9.63±2.89	6.17±1.54**	25.32±4.13*
CE	9.82±1.68	6.22±1.52	34.45±8.07

*$p < 0.05$ compared to control. **$p < 0.05$ compared to the CP group. CE, crude extract; CP, cyclophosphamide; (n = 8).

To verify lipid peroxidation, the levels of malondialdehyde (MDA) were evaluated by means of the TBARS test in all tissues, according to **Table 4**. There was a significant increase of TBARS ($p < 0.05$) in hepatic (124%) and renal (32%) tissues in the CP group compared to control. While CE significantly decreased TBARS levels ($p < 0.05$) even after receiving CP, a 39% decrease in hepatic tissue and 46% decrease in renal tissue when compared to the CP group. The CE-only group induced a significant increase of 50% of TBARS ($p < 0.05$) in the liver, comparing with control. No significant differences were observed in cardiac tissue.

TABLE 4 – Effect of CE pre-treatment (500 mg kg^{-1}) on CP-induced oxidative stress (75 mg kg^{-1}) to assess the biomarker of lipid damage in hepatic, renal and cardiac tissues.

Thiobarbituric acid reactive species – TBARS (nmol MDA mg protein^{-1})

Treatments	Liver	Kidney	Heart
Control	0.37±0.06	2.37±0.50	5.41±1.00
CP	0.84±0.15*	3.13±0.61*	4.50±1.14
CE + CP	0.51±0.11**	1.71±0.29**	5.34±0.57
CE	0.56±0.12*	2.29±0.24	5.93±1.55

*$p < 0.05$ compared to control. **$p < 0.05$ compared to the CP group. CE, crude extract; CP, cyclophosphamide; MDA, Malondialdehyde; (n = 8).

Blood parameters

Biochemical parameters of the plasma of the treated animals were evaluated (**Table 5**). A significant increase ($p < 0.05$) of AST and ALP enzymes was observed, of 26% and 64% in the CP group compared to the control, respectively. Animals pretreated with CE had a significant decrease ($p < 0.05$) in AST. On the other hand, an increase in ALP was verified in CE plus CP when compared to control and also it was observed that CE alone also increased significantly ($p < 0.05$) the ALT and ALP enzymes, in 89% and 74%, respectively, when compared with control. No significant differences were observed in glucose and cholesterol among the groups tested.

TABLE 5 – Effect of CE pre-treatment (500 mg kg^{-1}) on CP-induced oxidative stress (75 mg kg^{-1}) for the evaluation of plasma biochemical parameters (AST, ALT, ALP, glucose and cholesterol).

Treatments	AST (U L^{-1})	ALT (U L^{-1})	ALP (U L^{-1})	Glucose (mg dL^{-1})	Cholesterol (mg dL^{-1})
Control	103.5±20.2	47.1±10.6	63.7±12.3	246.2±44.6	69.4±11.7
CP	130.8±27.2*	44.0±10.3	104.5±11.2*	271.4±36.9	68.2±14.5
CE + CP	83.7±11.9**	66.1±13.8*	89.6±8.5*	286.8±46.4	80.7±8.3
CE	94.0±16.9	89.0±7.0*	111.2±11.2*	288.6±65.4	76.0±13.3

*p < 0.05 compared to control. **p < 0.05 compared to the CP group. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CE, crude extract; CP, cyclophosphamide; (n = 7).

When estimating HCT levels, a significant increase (p < 0.05) was observed in the CP group of 16%, in the group that received CE plus the CP and CE group, both with a 17% increase compared to control. In WBC, a significant reduction (p < 0.05) of 50% was observed in the CP group and in the group receiving CE plus CP, with a reduction of 65% compared with the control group. The levels of RBC and Hb presented a significant increase (p < 0.05) of 20% and 21%, respectively, in the CP group compared to the control group. Likewise, a significant increase in RBC was observed in the group receiving CE plus CP (19%), and in the CE group (18%). Hb also increased significantly (p < 0.05) in the CE plus CP (22%) and CE (19%) groups. No significant difference was observed in platelets. All data on whole blood biochemical parameters are shown in **Table 6**.

TABLE 6 – Effect of CE pre-treatment (500 mg kg^{-1}) on CP-induced oxidative stress (75 mg kg^{-1}) for the evaluation of blood biochemical parameters.

Treatments	HCT (%)	WBC (10^9 L^{-1})	RBC (10^{12} L^{-1})	Hb (g L^{-1})	Platelets (10^9 L^{-1})
Control	36.6±5.2	14.3±3.0	7.7±1.2	11.1±1.9	802.3±83.1
CP	42.8±2.3*	7.2±1.2*	9.3±0.6*	13.4±0.8*	828.7±120.7
CE + CP	43.1±2.6*	6.5±1.5*	9.2±0.6*	13.5±0.8*	641.5±137.9
CE	42.9±3.3*	11.6±2.7	9.1±0.6*	13.2±0.9*	868.0±124.3

*p < 0.05 compared to control. CE (crude extract), CP (cyclophosphamide); Hb, hemoglobin; HCT, hematocrit; RBC, red cell count; WBC, white cell count; (n = 7).

Micronucleus test

Table 7 shows the frequency of PCEMN, where the group receiving CE plus CP showed a significant reduction of 28% (p < 0.05) micronucleus frequency in relation to the positive control, showing the antimutagenic potential of CE.

TABLE 7 – Effect of CE pre-treatment (500 mg kg^{-1}) on CP-induced oxidative stress (75 mg kg^{-1}) to evaluate antimutagenic and mutagenic CE activity, on PCEMN frequency of bone marrow cells.

Treatments	PCE observed	PCEMN	% reduction MN
Control	16.000	315	-
CP	16.000	559	-
CE + CP	16.000	491[*]	28
CE	16.000	245	-

*p < 0.05 compared to control. CP, cyclophosphamide; CE, crude extract; PCE, polychromatic erythrocytes; PCEMN, micronucleated polychromatic erythrocytes; (n = 8).

Histological analysis of the liver

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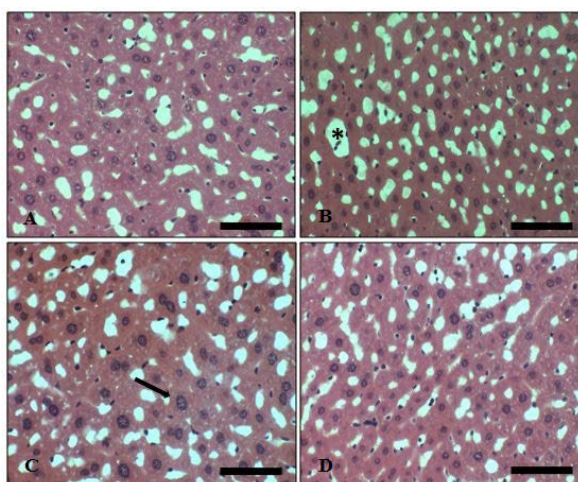

Figure 4 – Photomicrograph of the hepatic tissue of mice *Swiss*;

A = control; B = CP (75 mg kg^{-1}); C = CE + CP; D = CE (500 mg kg^{-1}). Hepatocyte (arrow). Sinusoid (asterisk). Stained HE. Bar = 50μm. CE (crude extract), CP (cyclophosphamide).

DISCUSSION

Plants are sources of remarkable active molecules that can modulate oxidative stress [29]. Currently, this mechanism has been extensively studied. Particularly, the leaves of *C. papaya* have aroused interest for these studies. In the present work, we investigated the effect of the crude extract (CE) of the leaves of *C. papaya* on the induction of oxidative stress using cyclophosphamide (CP) as experimental model adopted.

When performing the phytochemical analysis of CE, flavonoids, quercetin-3β-D-glucoside and rutin were identified. The quercetin-3β-D-glucoside was identified from the molecular ion of 463.38 (m/z-1) and fragment 300.00 (m/z-163.38) retention time of 10 minutes and rutin, it presented molecular ion 609.52 (m/z-1) and fragment 300.20 (m/z-309.32) retention time 10.3 minutes. The retention time and fragmentation profile of these flavonoids are similar to those of [30] and [31] where the fragmentary ion of the rutin corresponds to the loss of two glycans [32], and the quercetin-3β-D-glycoside fragment comprises the cleavage of the glycoside group. As regards phytochemical screening, the tests were positive for the presence of alkaloids, saponins and tannins as previously described in the literature by [33] also with aqueous extract.

In biological tests, CP caused a significant reduction (p < 0.05) in both SOD and CAT activity of liver tissue.In addition, we observed a decrease in the GSH for all tissues, as described by [34] and [35]. This occurs because during the metabolism of CP ROS is generated that lead to the depletion of antioxidant enzymes in different tissues [36-39]. In his studies [37] found that acrolein induces the irreversible inactivation of SOD activity by attacking its amino acid residues, histidine for example, which is pointed out as more susceptible since it is an essential amino acid for SOD activity and for increasing protein carbonylation. Other amino acids are susceptible to attack by acrolein, lysine, cysteine, serine, arginine, and threonine. Acrolein may have its production triggered by various conditions; some are metabolized by cyclophosphamide or oxidation of metal-

catalyzed polyunsaturated fatty acids. Point to the inactivation process of SOD, the superoxide radical induces the inactivation of the CAT enzyme [38]. The reduction of GSH after CP exposure occurs because GSH conjugates with acrolein to form mercapturic acid making it less likely to exert its toxic effects on the body and facilitates its elimination through the urine [39].

In contrast, CE restored liver CAT activity, helped decrease lipid peroxidation in liver and kidney, increased liver GST and kidney GSH. In a similar study, using *papaya* epicarp in human cells that had oxidative stress induced by hydrogen peroxide, the extract increased CAT activity and GSH levels, in addition to minimizing lipid peroxidation [40]. In other work [41], rats that had the oxidative stress induced by Fe^{2+} ions and received aqueous extract of the green fruit, saw a decrease in TBARS. Another study showed that fruit extract was also used against acrylamide-induced oxidative stress, resulting in a decrease in TBARS in the liver and kidney, as well as an increase in GSH and CAT in the tissues mentioned [42]. GST showed a significant increase in its activity, showing that the enzyme was not depleted by CP, similarly to [43]. This may be a response of the body of animals treated to combat the effects of CP. The role of GST is to protect against oxidants by catalyzing the conjugation of the sulfur atom of glutathione to an electrolytic center of toxic xenobiotics in order to produce compounds that facilitate its metabolism and excretion [44]. CE also induced an increase in GST activity, such as has been observed by [45] which identified a similar effect of fruit investigations, found that ethyl acetate extract from the fruits of *C. papaya* on GST increase rat liver cell lines at the 25 mg mL^{-1} concentration. Considering that the extract of the present study contains rutin and quercetin-3-β-d-glucoside, we suggest that these flavonoids may be interfering with these findings regarding the various oxidative stress parameters, since there are studies that confirm their antioxidant effects [48-50].

Although CP is widely used in clinical practice, its use produces several side effects in the organism, among them the elevation of liver enzymes [51]. Increased serum levels of AST and ALT are clinical markers used to assess hepatocellular toxicity [52]. CP treatment induced a significant increase of AST and ALP enzymes, as well as increased TBARS in liver and kidney, indicating hepatic and renal damage by CP administration, results already obtained in the literature [53-55]. This increase in TBARS levels is due to the fact that the production of free radicals mediated by CP metabolites stimulates the lipid peroxidation process [56].

CE resulted in a significant increase in enzymes ALT and ALP besides TBARS in the livers of the group receiving only the extract, suggesting toxicity. The increase in ALP has already been observed by [57] in *Wistar* rats using aqueous extract of *C. papaya* leaves for 7 days of administration. This liver toxicity caused by CE may be due to the presence of other substances that may exhibit toxic effects to the body or its prolonged use is not advised. In the phytochemical screening of CE, the tests were positive for the presence of tannins and alkaloids; in low doses these substances have a positive effect, but their excess can lead to hepatotoxicity as already reported by [58]. In addition [59], verified an abundance of calcium oxalate in leaves of *C. papaya* by micromorphology and chemical tests. The presence of these compounds may have led to the toxic event.

Although hepatotoxic action of the extract on hepatic enzymes was observed, no lesions were observed in the hepatocytes or any structure of the liver in the histological analysis of the treated animals. The same was observed by Ismail *et al.* [60] where rats treated with aqueous extract of leaves *C. papaya* at a concentration of 140 mg kg^{-1} for 13 weeks showed no histopathological differences in hepatic tissue. In that study the increase of the ALP enzyme was also observed.

In the histological analysis no damage was observed in the hepatic tissue by CP, although it is common to find works that show that this drug causes liver damage. Observed in mice receiving doses of 25 mg kg^{-1} for 10 days had dilation of the central and sinusoidal vein, in addition to leukocyte infiltration in histological analysis of the liver [53]. However, cyclophosphamide has been reported as hepatotoxic under unusual conditions, since only

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hepatocellular necrosis is observed at high doses or in conjunction with busulfan or BCNU [61]. In addition, the dose administrated of CP in our work was 75 mg kg^{-1} for 24 hours, so it is probably that was a short time exposure to cause histological alterations.

A significant increase in the levels of HCT, Hb, and RBC was observed with decrease of the counts of WBC in the groups CP and CE + CP. The number of WBC from peripheral blood can directly reflect the degree of myelosuppression of chemotherapeutic agents because myelosuppression often first manifests as a decline in white blood cells, followed by a series of hematopoietic impairments [62]. The number of WBC changes most obviously because of its short life cycle [63]. CE also caused a significant increase (p < 0.05) in HCT, Hb and RBC levels. Data similar has been observed in the work of Song *et al.* [64], where treatment with aqueous extract of leaves *C. papaya* during a period of 5 days (twice a day) was administered to a dengue patient, increasing, among other parameters, the HCT and RBC indexes. In this context, the work of Ahmad *et al.* [65] demonstrated that the leaf extract of papaya saw healthy increased levels of RBC in mice, indicating strong eritropoietic activation. In the studies of Dharmarathna *et al.* [66] it has also been observed that the ethanolic extract of the leaves of *C. papaya* promotes an eritropietic stimulation when analyzing cells of the bone marrow of mice. Increases in blood components may be related to the presence of rutin in CE, since it has already been associated with the ability to attenuate myelosuppression and increase eritropoietic production [67]. In addition, the rutin is attributed to the ability to ameliorate ROS action [55].

In the micronucleus test the CP induced an increase of PCEMN in comparison with control, results already observed in the works done with mouse bone marrow cells using a dose of 50 mg kg^{-1} [68-70]. The results show that CP induced chromosomal damage, because the drug does not specifically act on tumor cells, binds covalently to DNA and interferes with the cell cycle [71].

The CE showed antimugenic activity, decreasing significantly the PCEMN frequency by 28%, proving this effect. There are no papers in the literature using leaves of *C. papaya* with antimugenic action; on the other hand [72], verified that the aqueous extract of roots of *C. papaya* was antimutagenic in the micronucleus test with bone marrow of *Wistar* rats. Another study points to antiproliferative and anti-metastatic activity of papaya leaf extract on prostate cancer cell lines [12].The rutin has already been attributed to the ability to repair DNA damage, *Wistar* rats that were supplemented for two weeks with rutin (10 mg/100g) prior to induction of carcinogenic damage had reduced damage[73] and in our study rutin is one of the flavonoids present in CE used in the treatment of animals.

CONCLUSION

The present study showed that the crude extract of leaves of *C. papaya* has benefits against oxidative events, helping to increase antioxidant enzymes, besides inhibiting lipoperoxidation, preventing damage to DNA and showing signs of eritropoietic stimulation. In the phytochemical characterization two flavonoids, quercetin-3β-D-glucoside and rutin were found, which we can attribute part of the benefits to this plant. On the other hand, the extract increased ALT and ALP, suggesting toxicity, a fact that may have occurred due to prolonged treatment, causing subchronic intoxication and transient elevation of these enzymes. Therefore, the dose used and in this model of exposure was not considered totally safe. Therefore, the prolonged use of the infusion of papaya leaves is not advisable.

Conflicts of interest disclosure

The authors declare no competing interests relevant to the content of this study.

Authors' contributions.

All the authors declare to have made substantial contributions to the conception, or design, or acquisition, or analysis, or interpretation of data; and drafting the work or revising it critically for important intellectual content; and to approve the version to be published.

Availability of data and responsibility for the results

All the authors declare to have had full access to the available data and they assume full responsibility for the integrity of these results.

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