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ORIGINAL ARTICLE

First phytochemical study and biological activity of the leaves ethanolic extract from *Cissus spinosa Cambess*

Primeiro estudo fitoquímico e atividade biológica do extrato etanólico de folhas de Cissus spinosa Cambess

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Abstract:

Aims: The objective of this study was to identify the phytochemical profile and to evaluate the biological effects of the crude ethanolic extract (EE) and the ethanolic fraction (EF) of leaves of the species *Cissus spinosa* Cambess, after oxidative stress induced by cyclophosphamide (CP) in mice.

Methods: Phytochemical profile was performed detecting functional groups and, analysis of total flavonoids and phenols concentration, as well as the antiradical activity in EE and EF. The phytochemical characterization was done for the identification of flavonoids present in the leaves of the plant. In the biochemical tests, hematological parameters, glucose, total cholesterol, creatinine, alkaline phosphatase and aminotransferases dosages in plasma, enzymatic and non-enzymatic antioxidants and lipid damage marker were evaluated in different tissues (liver, kidney and heart), besides genotoxic and immunological analyzes. The animals received 15 days of treatment, via gavage, with EE (50 mg kg^{-1}) or EF (50 mg kg^{-1}) and on the 15^{th} day, an intraperitoneal injection of CP (100 mg kg^{-1}) or saline (0.9%). After 24 h the last treatment, the animals were anesthetized for blood withdrawal, sacrificed and removal of the organs.

Results: In the phytochemical analyzes, the presence of alkaloids, flavonoids and phenols was identified, the latter presented a higher concentration for EF. Eight flavonoids were identified - Rutin, Quercetin-3-β-D-glucoside, Quercitrin, Taxifolin, Quercetin, Canferol, Luteolin and Apigenin. In the biochemical analyzes, in general, EE showed a better antioxidant action against oxidative damages, hypoglycemic and antitilipemic action when comparing with EF, probably due to the synergism caused by flavonoids. It was observed the reduction and an increase of micronucleated polychromatic erythrocytes, due to the action of antioxidant compounds and alkaloids present in the plant, also considering the question of the seasonal period that directly interferes in the production of these compounds. In the immunological analysis, the extracts did not stimulate the spontaneous production of oxygen peroxide (H_2O_2) and nitric oxide (NO•).

Conclusions: Other studies, such as the variation of the chemical composition of the plant by local seasonality, hypoglycemic and antilipemic action, should be carried out to better delineate the biological action present in this plant.

Keywords: antioxidant; Cissus; oxidative stress; flavonoids; genotoxicity; immunomodulation.

Abreviations: ALT, Alanine Aminotransferase; ALP, Alkaline Phosphatase; ANOVA, one-way analysis of variance; AST, Aspartate Aminotransferase; CAT, Catalase; CP, Cyclophosphamide; DNA, Deoxyribonucleic Acid; EC_{50}, effective concentration; EE, Ethanolic Extract; EF, Ethanolic Fraction; GSH, Reduced Glutathione; H_2O_2, Hydrogen Peroxide; m/z, Mass Charge; LC-MS/MS, sequencial mass spectrometry; MNPCE, Micronucleated Polychromatic Erythrocytes; NO•, Nitric Oxide; SOD, Superoxide Dismutase; TBARS, Thiobarbituric Acid Reactive Substances; TNF-α, Tumor NecrosisFactor-α.

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Resumo:

Objetivos: O objetivo deste estudo foi identificar o perfil fitoquímico e avaliar os efeitos biológicos do extrato etanólico bruto (EE) e da fração etanólica (EF) das folhas da espécie *Cissus spinosa* Cambess, após estresse oxidativo induzido pela ciclofosfamida. (CP) em camundongos.

Métodos: O perfil fitoquímico foi realizado detectando grupos funcionais e, análise da concentração de flavonoides e fenóis totais, bem como a atividade antirradical em EE e EF. A caracterização fitoquímica foi realizada para a identificação de flavonoides presentes nas folhas da planta. Nos testes bioquímicos, os parâmetros hematológicos, as dosagens de glicose, colesterol total, creatinina, fosfatase alcalina e aminotransferases no plasma, os antioxidantes enzimáticos e não enzimáticos e o marcador de dano lipídico foram avaliados em diferentes tecidos (fígado, rim e coração), além de análises genotóxicas e imunológicas. Os animais receberam 15 dias de tratamento, via gavagem, com EE (50 mg kg^{-1}) ou EF (50 mg kg^{-1}) e no 15º dia, injeção intraperitoneal de CP (100 mg kg^{-1}) ou soro fisiológico (0,9%) . Após 24 h do último tratamento, os animais foram anestesiados para retirada do sangue, sacrificados e após a retirada dos órgãos.

Resultados: Nas análises fitoquímicas, identificou-se a presença de alcaloides, flavonoides e fenóis, estes últimos apresentaram maior concentração para EF. Oito flavonoides foram identificados - rutina, quercetina-3-β-D-glicosídeo, quercitrina, taxifolina, quercetina, canferol, luteolina e apigenina. Nas análises bioquímicas, em geral, o EE apresentou melhor ação antioxidante contra os danos oxidativos, ação hipoglicemiante e antitilipêmica quando comparada à EF, provavelmente devido ao sinergismo causado pelos flavonoides. Observou-se a redução e um aumento de eritrócitos policromáticos micronucleados, devido à ação de compostos antioxidantes e alcaloides presentes na planta, considerando também a questão do período sazonal que interfere diretamente na produção desses compostos. Na análise imunológica, os extratos não estimularam a produção espontânea de peróxido de oxigênio (H_2O_2) e óxido nítrico (NO•).

Conclusões: Outros estudos, como a variação da composição química da planta por sazonalidade local, ação hipoglicemiante e antilipêmica, devem ser realizados para melhor delineamento da ação biológica presente nesta planta.

Palavras-chave: antioxidante; *Cissus*; estresse oxidativo; flavonoides; genotoxicidade; imunomodulação.

Introdução

Plants are used for thousands of years for ornamental, aesthetic, culinary and, mainly, medicinal purposes. In several regions of the world, medicinal plants are used as a resource for various diseases [1, 2]. This knowledge has been passed from one generation to another for many years, and it is from this ethnopharmacological information that most scientific research has been carried out to confirm the biological action on the individual's organism and its supposed toxicity [1, 3]. The biological effect promoted by these plants originates from the secondary metabolism, which is coming from the pathways of shikimic acid and acetyl coenzyme A, producing compounds that are important in protection, development and reproduction, as well as possessing actions beneficial to the organism [4, 5].

One of these actions is the antioxidant effect, which is able to inhibit or retard cell degradation, such as damage to lipids and membrane proteins and mutations in Deoxyribonucleic Acid (DNA), caused by molecules such as reactive oxygen species and reactive nitrogen species [6]. These substances are produced through physiological processes of the cell such as respiration and can also be produced after exposure to xenobiotics [7-9].

The genus *Cissus* is considered the most abundant within the Vitaceae family with approximately 350 species worldwide distributed in the tropical regions [10]. The extracts of plants belonging to this genus are used in the treatment of various pathologies [11-15]. Some examples are the anti-inflammatory action present in leaves of *C. pteroclada* due to the presence of bergenin derivatives, which inhibit the production of cytokines, modulating the expression of pro-inflammatory substances [16]. Bharti *et al.* showed that the high concentration of tannins in the leaves of *C. quadrangularis*, has an astringent action in which it can prevent bleeding and skin diseases [17]. The presence of substances derived from kaempferol and quercetin in the leaves extract of *C. sicyoides* have an antidiabetic, antilipemic, anti-inflammatory and gastroprotective activities [18]. The hypoglycemic and antilipemic effect of *C. verticillata* leaves extract may be due to the presence of the flavonoids kaempferol, quercetin and rutin [19].

The species *Cissus spinosa* Cambess (*C. spinosa*) is known as cipó-de-arraia, due to its popular use as a plaster for stinging stingrays. It belongs to the group of lianas, which is between 2 and 8 meters in length or extension, with a zigzag stem, prickly and producing flowers almost all year round. Its distribution in the Brazilian territory is concentrated in the central-western region, but also in the states of Bahia, Minas Gerais

and Paraná [20]. However, this species does not present a study in the literature about its chemical composition and its action in the organism, only study in the other species of the same genus.

Cyclophosphamide is a chemotherapeutic drug, belonging to the group of the nitrogen mustards, considered an alkylating agent. It is widely used in the treatment of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, and cancer, such as myeloma, lymphomas and leukemias [21, 22]. It is metabolized in the liver by cytochrome P-450 and converted to acrolein and phosphoramide mustard, which are its active metabolites [23-25]. These can also bind to DNA causing cross-linking and formation of micronucleus, cytotoxicity in important organs such as the heart, liver and kidneys, and reactive oxigen species production. The increase of these substances modifies the levels of molecules and important enzymes in the pro-oxidant-antioxidant balance, such as reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) [23, 26-28].

The lack of scientific study and the pharmacological activity of this species, of the genus *Cissus*, stimulated to identify the phytochemical profile of the *C. spinosa* Cambess species and to evaluate the biological, genotoxic and immunological effects on oxidative stress induced by the drug cyclophosphamide in mice.

MATERIAL AND METHODS

Botany Identification and Preparation of Extract and Fraction

C. spinosa was collected on the side of the Teles Pires River, in the geographical coordinate S 11° 43' 22" and W 55° 43' 43", Sinop, Mato Grosso, Brazil, in September, during the period of drought. An exsicta was deposited in the Herbarium Centro Norte Mato Grossense, Federal University of Mato Grosso, under the number of fall 6850 and identified by Professor Milton Omar Côrdova Neyra.

The plant was dried (40 °C) and crushed (261 g), and then extraction by maceration with absolute ethyl alcohol (2.5 L) occurred. The obtained extract was filtered, concentrated in a rotary evaporator, resuspended with methanol-water (1:1) to remove the chlorophyll, filtered and concentrated again, resulting in crude ethanolic extract (EE) without chlorophyll.

The EE was fractionated by Column Chromatography using as the silica gel stationary phase and as the mobile phase the hexane, ethyl acetate and ethanol solvents. The ethanol fraction (EF) was concentrated in a rotary evaporator and both EE and EF were used in the *in vitro* and *in vivo* studies in this research.

Phytochemical Screening

For the qualitative analyzes of the constituents of the *C. spinosa* EE, the methodologies described by Costa and Simões *et al.*, using colorimetric and/or precipitated chemical reactions characteristic of the following functional groups, steroids/triterpenes, flavonoids, tannins/phenols, saponins, alkaloids, polysaccharides, heterosides cyanogens, purines, coumarins and anthraquinones [29, 30].

Total Phenols, Total Flavonoids and the radical scavenging Activity in EE and EF

Analysis of total phenols was performed using the Folin-Ciocalteau method according to Sousa *et al.* with modifications [31]. The calibration curve with gallic acid was made at concentrations of 0.625 to 200 μg mL^{-1}, adding 5 mL of distilled water, 1 mL of Folin-Ciocalteau reagent and 1 mL of sodium carbonate (Na_2CO_3) 7.5%. EE and EF were solubilized in methanol to a final concentration of 200 μg mL^{-1}. Aliquots of 1 mL of EE and EF, 1 mL of Folin-Ciocalteau reagent, 5 mL of distilled water and 1 mL of Na_2CO_3 were homogenized and, after 1 hour, the absorbances was determined in a spectrophotometer at 750 nm. The analysis was done in triplicate, with the result expressed in milligrams of gallic acid equivalent per gram of extract or fraction (mg GAE g^{-1}).

The determination of total flavonoids was performed by the colorimetric method with aluminum chloride, described by Rio with adaptations [32]. A calibration curve was made with quercetin at concentrations of 15 to 60 μg mL^{-1}. The analyzes were performed in triplicate, the result being expressed in milligrams of quercetin

equivalent per gram of extract or fraction (mg QE g^{-1}).

The radical scavenging activity, which is the ability of an antioxidant compound to reduce a stable radical, was determined following the methodology of Sousa *et al.*, using the radical 2,2-diphenyl-1-picrylhydrazyl and quercetin and ascorbic acid standards, expressed as Effective Concentration (EC_{50}) (concentration required to reduce 1,1-Diphenyl-2-picryl Hydrazil concentration by 50%) [31].

Identification of Flavonoids by sequencial mass spectrometry (LC-MS/MS)

The presence of flavonoids in EE and EF was confirmed by LC-MS/MS, in the Multiple Reaction Monitoring mode of acquisition and transition ions previously stablishhed by flavonoid pattern analysis, using an Ultra Hight Performance Liquid Chromatography 1290 Infinnity system coupled with 6460 Tripe Quad liquid chromatography coupled to mass spectrometry, both from Agilent Technologies [33, 34]. The analytical standards of Rutin, Quercetin-3-β-D-glucoside, Quercitrin, Taxifolin, Quercetin, Kaempferol, Luteolin and Apigenin were used for the identification of flavonoids present in EE and EF. The EE and EF samples were submitted to LC-MS/MS 20 µL of sample was injected and the separation of the components occurred on a C-18 (Zorbax Eclipse AAA 4.6 x 150 mm diameter, 3.5 µm particle size) column. The sample elution method used a mobile phase flow of 0.5 mL/min and a 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 as characteristic from 0 to 30 min, 5 - 95% B, 30 to 32 min, 95 - 100% B and from 32 to 33 min, 95 - 5% B (initial condition). Samples were detected by mass spectrometry using electrospray ionization in the negative mode. The gradient sample elution method had a source temperature of 300 °C and a desolvation temperature of 250 °C. The results were compared to the standards and to literature references.

Animals and *In Vivo* Treatment

Male *Swiss* mice, with an average weight of 30 to 40 g (40 days of age), remaining throughout the acclimation and experimental period under controlled temperature conditions (26 ± 2 °C), relative humidity (55 ± 10%), light cycle (12 hours light/dark), receiving commercial feed and filtered water, kept in polyethylene boxes with a diameter of 41 x 34 x 16 cm and stainless steel grid.

The Malone Hippocratic Test was done to investigate different doses of EE and EF (50, 100, 250, 500 and 1000 mg kg^{-1}, via gavage) to observe the behavioral parameters of the animals and the possible toxicity of the plant [35]. Thus, the established dose was 50 mg kg^{-1}, and it was administered orally, by gavage (0.3 mL), throughout the treatment period. Cyclophosphamide (CP) (100 mg kg^{-1}) was the drug used to promote oxidative damage [21]. The animals received oral treatments (water with Tween 80 0.1%, EE or EF) for 15 days and an intraperitoneal injection of CP or saline 0.9% on the 15^{th} day. After 24 h of drug administration, the mice were anesthetized via i.p. with ketamine (50 mg kg^{-1}), xylazine (20 mg kg^{-1}) and acepromazine (20 mg kg^{-1}), followed by cardiac puncture for the collection of blood with heparinized syringes, being euthanized for the removal of the liver, kidneys, heart and femur. For the *in vivo* tests, 6 groups were used with 8 animals. This research was approved by the Animal Ethics Commitee - Federal University of Mato Grosso under protocol number 23108.913311/2017-94.

Hematological and Biochemical Analysis

The analysis of whole blood for red blood cell count, white blood cells, platelets, hematocrit and hemoglobin, as well as analysis of biochemical parameters of blood plasma, such as glucose, cholesterol, creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using commercial Labtest® kits .

The biochemical techniques used to investigate oxidative stress were the evaluation of enzymatic antioxidants: SOD determined according to Misra and Fridovish, where the adrenochrome formation occurs by the oxidation of adrenaline, and its result is expressed as UI SOD mg $protein^{-1}$ using a wavelength of 480 nm [36]. Glutathione-*S*-Transferase determined

according to Habig *et al.*, where the formation of the S-2,4-dinitrophenyl glutathione compound occurs, with the result expressed as µmol S-2,4-Dinitrophenyl Glutatione min^{-1} mg $protein^{-1}$ using a wavelength of 340 nm [37]. Catalase (CAT) activity was determined according to Nelson and Kiesov, where the decomposition of H_2O_2 is observed and the result expressed in µmol H_2O_2 min^{-1} mg $protein^{-1}$, using a wavelength of 240 nm [38].

For the evaluation of the non-enzymatic antioxidant, the reduced glutathione (GSH) analysis was used and followed the method of Sedlack and Lindsay [39], with the formation of thiolate anilide. The result was presented as µmol GSH mg $protein^{-1}$, by using the wavelength of 412 nm.

Analysis of the lipidic damage (thiobarbituric acid reactive substances - TBARS) which measures the malonaldehyde content, a toxic product of lipid peroxidation, it was performed according to Buege and Aust with some modifications [40]. The absorbances were compared to a calibration curve of malondialdehyde (0.03 mM), the result being expressed in nmol malondialdehyde mg $protein^{-1}$, using a wavelength of 535 nm.

The protein content for all determinations was made by the Bradford method [41], using bovine serum albumin as the standard for construction of the calibration curve. The samples were read at 595 nm.

Micronucleus Analysis

The micronucleus test was performed according to the methodology of MacGregor *et al.*, where 500 cells per slide were analyzed in duplicate, thus totaling 1000 polychromatic erythrocytes per animal [42]. The observation was performed in a blind test using an optical microscope with magnification of 1000 times. The test aims to observe the frequency of micronucleus polychromatic erythrocytes (MNPCE), in which it indicates DNA damage.

According to Manoharan and Banerjee and Waters *et al.*, the verification of the percentage reduction of damages is calculated according to the following formula [43, 44]:

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

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

Immunological Analysis

The animals were divided into three groups (n=5) and treated daily by gavage with water and Tween 80 0.1%, EE or EF for 15 days and at the end of treatment, the animals were euthanized by cervical dislocation to evaluate the parameters of hydrogen peroxide (H_2O_2) release and nitric oxide production (NO) by the peritoneal macrophages.

After euthanasia of the animals, peritoneal macrophages were obtained by inoculating ice-cold buffered saline into the peritoneal cavity.

The determination of H_2O_2 was performed following the method developed by Pick and Mizel [45]. A calibration curve of H_2O_2 at concentrations of 0.625 to 8 nM was made. The absorbances of the samples were read on Enzyme-Linked Immunosorbent Assay reader at the wavelength of 620 nm and the result expressed in nmoles of H_2O_2 per $2x10^5$ cells.

The analysis of spontaneous NO production was performed using the colorimetric method based on the Griess reaction [46]. The calibration curve of nitrite was made at concentrations of 0.049 to 100 µM. Samples were read on Enzyme-Linked Immunosorbent Assay reader at wavelength of 540 nm and the result was expressed in µmoles of nitrite per $2x10^5$ cells.

Statistical Analysis

Statistical data (mean ± standard deviation) of the immunological, hematological and biochemical analyzes were analyzed by parametric and non-parametric tests, if necessary, as one-way analysis of variance (ANOVA), followed by *Tukey* test or Kruskal -Wallis followed by *Dunn's* test, respectively, to verify the differences between the

experimental groups. The results were considered significant when $p<0.05$. For the micronucleus frequency test, the chi-square test was used according to Pereira [47].

RESULTS

Phytochemical profile

The dried leaves yielded of approximately 261 g. After extraction by maceration with ethanol, the crude ethanolic was submeted extract chlorophyll, yielded 7.56 g (EE) without chlorophyll. A sample of the EE was fractionated, obtaining 0.2 g of hexane fraction and 2.0 g of ethanolic fraction (EF), approximately. It was not possible to quantify the ethyl acetate fraction due to loss during the drying process.

In the qualitative analysis, the presence of flavonoids, tannins/phenols and alkaloids was observed, and for the other trials, steroids/triterpenes, saponins, polysaccharides, heterosides cyanogens, purines, coumarins and anthraquinones, negative results were obtained.

The concentration of total phenols and total flavonoids were higher in EF when compared to EE (**Table 1**).

TABLE 1 – Analysis of the concentration of total phenols, total flavonoids and radical activity in Ethanol Extract and Ethanol fraction of *C. spinosa* leaves.

Samples or Analytical Standards	Total Phenols (mg GAE/g^{-1})	Total Flavonoids (mg QE/g^{-1})	EC_{50} (µg mL^{-1})
Ethanol Extract	22	34	> 250
Ethanol fraction	68	53	69
Ascorbic Acid	-	-	26
Rutin	-	-	50

mg QE g^{-1}: milligrams of quercetin equivalent per gram of extract or fraction.
mg GAE/g^{-1}: milligrams of gallic acid equivalent per gram of extract or fraction.

Eight flavonoids were identified: Rutin, Quercetin-3-β-D-glucoside, Quercitrin, Taxifolin, Quercetin, Kaempferol, Luteolin and Apigenin for EE, and only Rutin and Quercetin-3-β-D-glycoside for EF (**Figures 1 and 2**) (**Table 2**).

TABLE 2 – Characterization of the identified Ethanolic Extract and Ethanolic Fraction compounds of *C. spinosa* by LC-MS/MS.

Compound	Retention time (min)	Molecular mass	Ion Mass	Main ionized fragment	Compound Identified
1	10.0	610.52	609.52	300.20	Rutin
2	10.3	464.38	463.38	300.00	Quercetin-3-β--D-Glucoside
3	11.8	448.38	447.38	301.00	Quercitrin
4	12.0	304.25	303.25	285.00	Taxifolin
5	15.0	302.24	301.24	120.90	Quercetin
6	16.8	286.24	285.24	93.00	Kaempferol
7	15.0	286.24	285.24	133.00	Luteolin
8	16.2	270.24	269.24	116.80	Apigenin

LC-MS/MS, sequencial mass spectrometry.

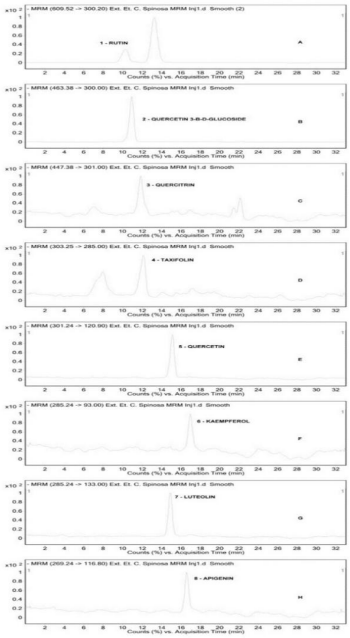
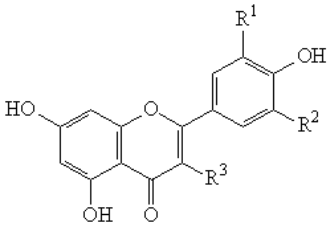


Figure 1 – Chromatograms and respective Multiple Raction Monitoring of the flavonoids identified in the extracts of *C. spinosa* leaves by sequencial mass spectrometry (LC-MS/MS).

Hematological Analysis

In the analysis of whole blood, the EE was able to increase the amount of red cells - red blood cells, and CP reduced the white cells - leukocytes. The other parameters, hemoglobin, hematocrit and platelets did not have significant differences between the study groups (**Table 3**).



Compound	R^1	R^2	R^3
1	H	OH	O-(raminosil-glicosídeo)
2	H	OH	O-glicosídeo
3	H	OH	O-ramnosideo
4	H	OH	OH
5	OH	H	OH
6	H	H	OH
7	H	OH	H
8	H	H	H

Figure 2 – Structure of the eight flavonoids identified in Ethanolic extract and/or Ethanolic fraction in leaves of *C. spinosa*.

TABLE 3 – Effect of the pre-treatment with Ethanolic Extract and Ethanolic Fraction (50 mg kg^{-1}) of *C. spinosa* leaves on Cyclophosphamide-induced oxidative stress (100 mg kg^{-1}) in hematological parameters in whole blood of the mice: hemoglobin, hematocrit, red blood cell count, platelet count, and white blood cell count.

Treatments	Hemoglobin (g dL^{-1})	Hematocrit (%)	Red blood cell count (10^6/mm^3)	Platelet count (10^3/mm^3)	White blood cell count (mm^3)
Control	14.3±0.5	44.5±3.2	4.9±0.4	453.0±64.5	4102±962.7
CP	14.3±0.7	43.0±2.0	4.7±0.2	545.0±86.7	2460±434.6*
EE+CP	14.0±0.3	40.6±2.0	4.8±0.5	378.5±75.5	2325±579.4*
EE	15.1±0.5	44.3±4.0	6.1±0.2*	526.1±114.4	3483±670.5
EF+CP	15.0±0.7	47.1±3.1	5.6±0.5	401.7±64.6	3297±564.5
EF	14.3±1.0	41.6±4.8	5.2±0.6	368.0±62.6	3789±690.0

CP = Cyclophosphamide; EE = Ethanolic Extract; EF = Ethanolic Fraction; ANOVA followed by *Tukey* Test. * $p<0.05$ compared to control.

In plasma analysis, EE decreased glucose and cholesterol levels (**Figure 3A and 3B**), and EF only cholesterol. For the markers of renal and hepatic damage, CP increased the concentration of both creatinine (renal damage) (Figure 3C) and ALP activity (liver damage) (Figure 4A), but EE for creatinine and EF, for creatinine and ALP, were able to prevent the alterations caused by CP. EE was also able to reduce ALT and AST activities (Figure 4B and 4C) when comparing with the respective control groups.

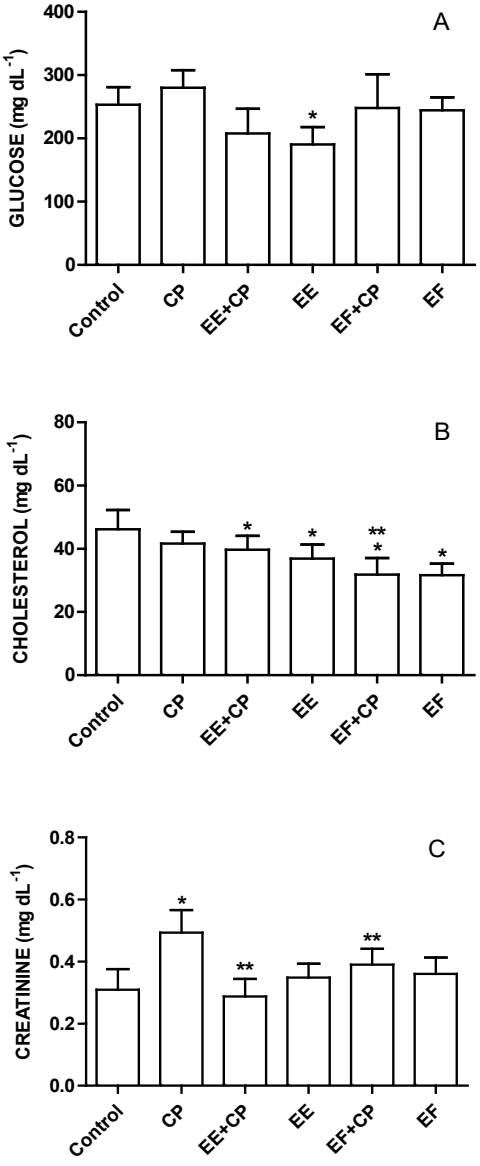


Figure 3 – Effect of pre-treatment with EE and EF (50 mg kg^{-1}) of *C. spinosa* leaves on CP-induced oxidative stress (100 mg kg^{-1}) in plasma biochemical parameters: (A) glucose, (B) cholesterol, (C) creatinine.
ANOVA followed by *Tukey* Test. Kruskal-Wallis followed by the Dunn's Test. * p <0.05 compared to control; ** compared to CP.
CP, Cyclophosphamide; EE, Ethanolic extract; EF, Ethanolic fraction.

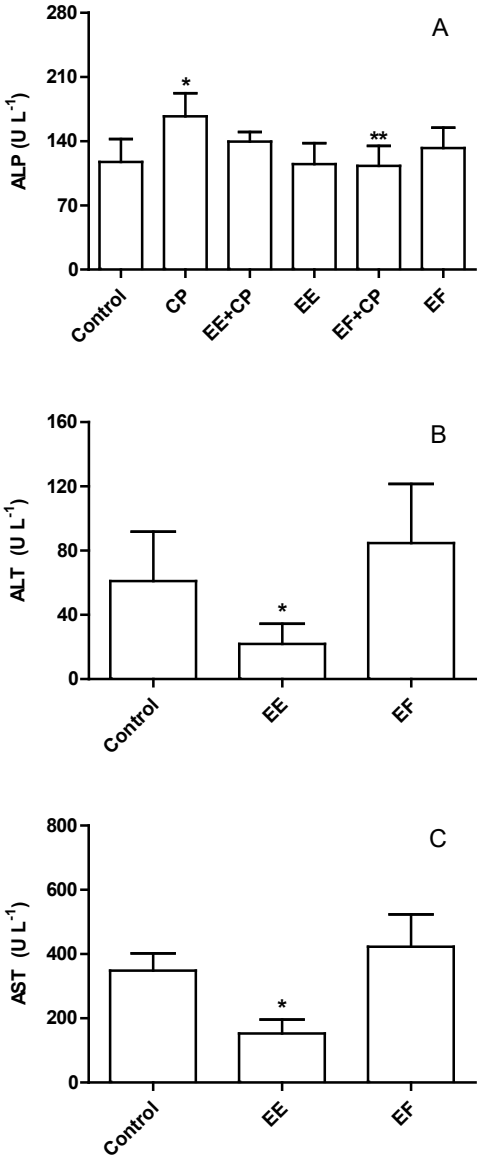


Figure 4 – Effect of pre-treatment with EE and EF (50 mg kg^{-1}) of leaves of *C. spinosa* on CP-induced oxidative stress (100 mg kg^{-1}) in plasma biochemical parameters: (A) ALP, (B) ALT and (C) AST.
ANOVA followed by *Tukey* Test. * p <0.05 compared to control; ** compared to CP.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CP, Cyclophosphamide; EE, Ethanolic extract; EF, Ethanolic fraction.

Biochemical Analysis of Oxidative Stress Markers

In the hepatic tissue, the SOD and glutathione-S-transferase enzymes had their activities decreased in the CP group when compared to the control group and EE and EF didn't alter these antioxidants (**Figure 5A and 5B**).

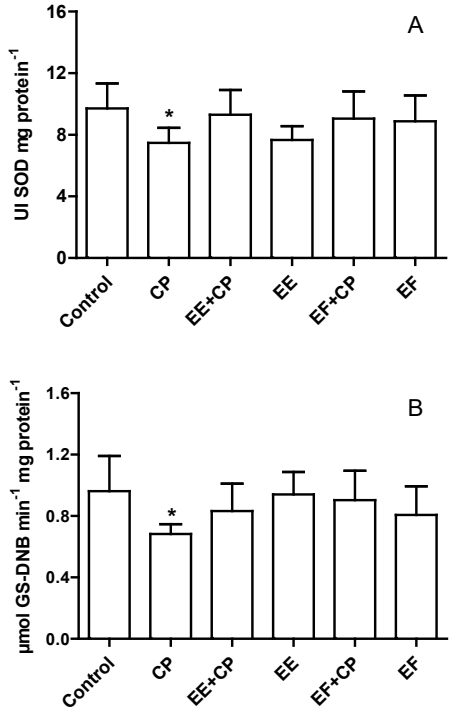


Figure 5 – Effect of pre-treatment with EE and EF (50 mg kg^{-1}) of leaves of *C. spinosa* on the oxidative stress induced by CP (100 mg kg^{-1}) in the enzymatic activities of SOD (A) and glutathione-S-transferase (B) in hepatic tissue.
ANOVA followed by *Tukey* Test. * p <0.05 compared to control.
CP, Cyclophosphamide; EE, Ethanolic extract; EF, Ethanolic fraction; SOD, superoxide dismutase.

The EE increased the enzymatic activity of CAT in the liver, but also prevented its decrease before CP administration, in the heart. On the other hand, EF reduced the activity of the enzyme in the same tissue. There was no significant difference in renal tissue (**Table 4**). EE contributed to the increase in GSH levels in the hepatic and renal tissues. However, EF and CP reduced GSH in the liver and heart, when compared to the control group. In the hepatic tissue there was an increase of TBARS in the groups pre-treated with EF and also with the inducer of the damage. In the cardiac tissue, both the extract and the fraction increased this biomarker. In renal tissue, there was no significant difference between groups (**Table 4**).

TABLE 4 – Effect of pre-treatment with Ethanolic Extract and Ethanolic Fraction (50 mg kg^{-1}) of *C. spinosa* leaves on Cyclophosphamide-induced oxidative stress (100 mg kg^{-1}) in Catalase activity (µmol H_2O_2 min^{-1} mg protein^{-1}), in the concentration of GSH (µmol GSH mg protein^{-1}) and in lipoperoxidation (thiobarbituric acid reactive substances - TBARS) (nmol malondialdehyde mg protein^{-1}) in hepatic, renal and cardiac tissues.

	Control	CP	EE + CP	EE	EF + CP	EF
Hepatic						
CAT	2.9±0.7	2.7±0.7	4.3±0.8*	4.5±0.9*	3.8±0.7	3.4±0.8
GSH	241.2±44.6	175.8±26.0*	214.7±27.2	303.6±45.3*	185.5±45.9*	181.8±25.3*
TBARS	0.26±0.03	0.37±0.06*	0.30±0.06	0.34±0.04	0.74±0.08$^{*, **}$	0.50±0.08*
Kidneys						
CAT	9.0±1.0	9.1±0.8	9.0±1.1	8.5±1.5	9.1±1.5	8.2±1.6
GSH	0.18±0.02	0.13±0.02*	0.19±0.04**	0.20±0.04	0.17±0.04	0.16±0.03
TBARS†	2.3±0.9	2.8±1.8	3.6±1.7	2.0±1.5	1.3±0.9	2.2±1.4
Heart						
CAT	5.1±0.8	3.4±0.4*	4.6±0.8**	4.2±0.6	4.3±0.4	4.1±0.8*
GSH	191.8±14.9	165.5±13.7*	184.0±18.3	201.2±11.2	162.5±24.0*	175.5±14.2
TBARS†	3.6±1.2	6.8±2.9	5.7±1.2	9.2±2.0*	9.3±3.6*	6.7±1.7

CAT = Catalase; CP = Cyclophosphamide; EE = Ethanolic Extract; EF = Ethanolic Fraction; GSH = Reduced Glutathione; TBARS, Thiobarbituric Acid Reactive Substances.

ANOVA followed by *Tukey* Test. ǂ Kruskal-Wallis followed by *Dunn's* Test. * p <0.05 compared to control. ** p <0.05 compared to CP.

Genotoxic Analysis

Table 5 shows the frequency of MNPCE, where the groups EE + CP and EF + CP showed a reduction of MNPCE of 36% and 14%, respectively, when compared to the positive control (CP). However, the EE and EF groups demonstrated an increase in MNPCE when compared to the negative control (Control).

TABLE 5 – Effect of the Ethanolic Extract and Ethanolic Fraction (50 mg kg^{-1}) pre-treatment of *C. spinosa* leaves on the Cyclophosphamide-induced oxidative stress (100 mg kg^{-1}) to evaluate the genotoxicity of *C. spinosa*, observing the frequency of micronucleated polychromatic erythrocytes in the bone marrow of mice.

Treatments	N° of Polychromatic Erythrocytes analyzed	MNPCE	% MN reduction
Control	8000	176	-
CP	8000	2060	-
EE+CP	8000	1378	36*
EE	8000	550*	-
EF+CP	8000	1797	14*
EF	8000	1570*	-

CP = Cyclophosphamide; EE = Ethanolic extract; EF = Ethanolic fraction. MNPCE, Micronucleated polychromatic erythrocytes. * p <0.001.

Immunological Analysis

In the immunological analysis, both EE and EF did not stimulate the spontaneous production of H_2O_2 and NO in treated animals when compared to the control (**Table 6**).

TABLE 6 – Effect of pre-treatment with Ethanolic Extract and Ethanolic Fraction (50 mg kg^{-1}) of *C. spinosa* leaves on the release of Hydrogen Peroxide and Nitric Oxide by peritoneal macrophages.

Treatments	Hydrogen Peroxide (nmoles per $2x10^5$)	Nitric Oxide. (µmoles per $2x10^5$)
Control	0.063±0.036	96.7±14.2
Ethanolic extract	0.024±0.015	91.8±16.0
Ethanolic fraction	0.045±0.031	90.8±4.8

DISCUSSION

The present work investigated the antioxidant effects of the compounds contained in the extract and in the fraction of leaves of *C. spinosa*, for the prevention of oxidative damage induced by cyclophosphamide in mice.

In the phytochemical screening the presence of flavonoids, tannins/phenols and alkaloids was verified, which is in accordance with the work on *C. quadrangularis* described by [48]. In anti-radical activity, because the EE has several components, such as oils, proteins, minerals, in addition to antioxidants, it has made its EC_{50} higher than the standards. However, EF, because it was able to concentrate the antioxidant compounds, resulted in an EC_{50} that was closer to the standards, being more potent than the extract. It is important to consider that the seasonality of the environment directly influences the production and concentration of secondary metabolites in the plant. This variation was seen in previous studies where the increase in total phenol content during the seasonal period of the Pantanal Mato-Grossense was observed [49].

The compounds identified by LC-MS/MS showed the respective molecular ions and fragmentation patterns which are in accordance with other works already described in the literature. Compound **1**, Rutin, presented the molecular ion 609.52 [*m/z* - H] and fragment 300.20 [*m/z* – 309.32], corresponding to the loss of two units of sugars, rhamnoside and glycoside, study is in accordance with the work done by Simirgiotis *et al*. [50]. Compound **2**, Quercetin-3-β-D-glucoside, showed a molecular ion of 463.38 [*m/z* - H] and fragment 300.00 [*m/z* – 163.38], which indicates the breakage of the group glycoside, this study was based on the work done by Pandit *et al*. [51]. Compound **4**, Taxifolin, on the other hand, had a molecular ion of 303.25 [*m/z* - H] and fragment 285.00 [*m/*z – 18.25] representing the loss of one unit of water, possibly a hydroxyl with a hydrogen of another hydroxyl, a result similar to that obtained by Yang *et al*. [52]. The compound **5**, Quercetin, with molecular ion of 301.24 [*m/z* - H] and fragment 120.90 [*m/z* – 180.34], the same pattern as that obtained by Chen *et al*. [53]. In relation to compound **6**, Kaempferol, was identified by the molecular ion 285.24 [*m/z* - H] and fragment 93.00 [*m/z* – 192.24], which indicates the loss of a phenol group with fragmentation similar to that described by Çelik *et al*. [54]. Compound **3**, Quercitrin, was identified by molecular ion 447.38 [*m/z* - H] and fragment 301.00 [*m/z* – 146.38], which corresponds to cleavage of the glycol. For compound **7**, Luteolin, molecular ion was identified as 285.24 [*m/z* - H] fragment and 133.00 [*m/z* – 152.24], coinciding with the loss of the B ring. Compounds **3** and **7** are in accordance with the work done by Barbosa *et al*. and Dai *et al*. [55, 56]. Compound **8**, Apigenin, presented molecular ion of 269.24 [*m/z* - H] and fragment 116.80 [*m/z* – 152.44], which represents C-ring cleavage between carbons 2 and 3 [55].

The active metabolites from the CP biotransformation are responsible for the pharmacological action, as well as for its cytotoxic action such as leukocyte decrease, gonadal atrophy, nausea, vomiting, liver, renal and cardiac lesions [21, 24, 57, 58]. We observed a significant reduction in total leukocyte count caused by CP and increased creatinine and ALP activity, thus confirming renal and hepatic damage also seen by other authors [23, 59, 60]. On the other hand, EF prevented alteration in ALP caused in animals treated with CP. Already the EE reduced the activity of ALT and AST in blood plasma, as well as increased CAT and GSH in the liver tissue. Li *et al*. showed hepatoprotective effect of *C. pteroclada* as well as other studies showed that the presence of flavonoids luteolin, quercetin-3-β-D-glucoside and rutin had the same hepatoprotective effect as antioxidant [15, 61-64]. Considering that this the first study using this plant, our findings suggest similar protection effect. It can also be observed the decrease in creatinine in plasma and an increase of the GSH in the renal tissue and increase of the activity of the CAT in the cardiac tissue in the animals that received EE. On the other hand, EF was not effective against the analysis of antioxidants and lipoperoxidation marker. Based on the previous studies, the antioxidant action of quercetin, quercetrin and apigenin, protected against damage caused by oxidative stress, as well as taxifolin and kaempferol had cardioprotective effect, as well as those found in EE [65-71].

Among other positive EE effects, there is also a hypoglycemic and antitilipemic action, as well as, EF showed to reduce cholesterol in the blood plasma. Although the analyzes were not done in a very specific experimental model, as in diabetic animals or that they received a hyperlipidic diet, we can still observe these results and could also be observed by Drobnik and Oliveira and Bieski *et al*. with the plants *Cissus verticillata* and *Cissus quadrangularis*. Another plant that also has such effects is *Cissus sicyoides*, popularly known in the northeastern region of Brazil as "vegetal insulin" [11, 72]. It is also used as antilipemic, anti-inflammatory, antirheumatic, in the treatment of gastric ulcers, epilepsy and in the cerebrovascular accident [18, 73, 74].

Because of the alkylating action in DNA, cyclophosphamide can cause damage and mutations in the animal's genome, and this change was confirmed in this study because of the increase in the number of MNPCE in the

group receiving the drug alone. For the animals pre-treated with EE and EF, a reduction in the number of MNPCE was observed (36% and 14%, respectively). Considering a study using a plant of the genus *Rhoicissus* (Vitaceae), it showed strong antimutagenic activity against aflatoxin B_1 [75]. However, EE and EF also showed an increase in mean MNPCE frequency. According to the authors Guterres *et al*. and Léon-Gonzalez *et al*., the observed change in our results may be associated with the presence of some groups of secondary metabolites, such as alkaloids and flavonoids, the latter being extremely sensitive under conditions of high pH and presence of active redox transition metals [76, 77].

Another evaluated biological effect was the immunomodulatory action of the plant on the function of the peritoneal macrophages of the treated animals. Macrophages are phagocytic cells that play an essential role in the inflammatory process, releasing proinflammatory cytokines such as interleukin-l, tumor necrosis factor alpha (TNF-α) and chemokines that "attract" other immune cells, as well as its microbicidal action with the production of lytic enzymes, reactive oxigen species and nitrogen reactive intermediates, such as NO˙ [78, 79]. Inflammation is an important defense mechanism used by the body, whose main function is to restore the structure and normal function of the affected tissue, either by injury or by the presence of microorganisms [80]. This action occurs through the cells of the immune system, such as neutrophils, dendritic cells, Natural Killer cells and macrophages, which are considered more efficient due to the fact that it remains viable in the tissue for months or years [81].

In the present study, the plant did not interfere in the spontaneous production of H_2O_2 and NO . In other studies such effects have also been observed. Evaluations conducted with the species *C. quadrangularis* showed decreased levels of pro-inflammatory cytokines, cyclooxygenases 1 and 2, lipoxygenase (5-Lipoxygenase) and interleukin-1β, but also the enzyme nitric oxide synthase-2 activity and produção de TNF-α [82, 83]. Srisook *et al*. used the ethyl acetate extract of *C. quadrangularis* in RAW 264.7 macrophages, resulting in the inhibition of NO production and also the expression of the nitric oxide synthase enzyme in these cells, showing the anti-inflammatory potential of this plant [84]. The *Vitis thunbergii* Sieb. & Zucc species attenuated the inflammation signaling pathways, N-terminal c-Jun Kinase, Extracellular Signal Regulated Kinase and nuclear factor kappa B (NF- κB) in rat messenger cells [85]. Besides, Salazar *et al*. showed in their studies, the anti-inflammatory action of *C. sicyoides* focal cerebral ischemia in *Wistar* rats [86].

CONCLUSION

This is the first work that investigates the phytochemical composition and biological action of *C. spinosa*, and it was verified the presence of eight flavonoids in the crude extract, being Rutin, Quercetin-3-β-D-glucoside, Quercitrin, Taxifolin, Quercetin, Kaempferol, Luteolin and Apigenin, and two in ethanolic fraction, Rutin and Quercetin-3-β-D-glucoside. In biochemical analysis, *C. spinosa* demonstrated a possible hypoglycemic and antilipemic effect. The EE had a better effect against oxidative damage, when compared to EF probably due to the synergism caused by flavonoids. EF may have concentrated substances of the alkaloid class, resulting in undesirable effects, such as those found in micronucleus analysis. In the macrophages analysis, the treatments did not stimulate the spontaneous production of H_2O_2 and NO . Other studies, such as the variation of the chemical composition of the plant by local seasonality, hypoglycemic and antilipemic action, should be carried out to better delineate the biological action present in this plant.

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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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