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Evaluation of the antigenotoxic and antioxidant activity induced by *Croton antisyphiliticus*

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Abstract: This study aimed to investigate antigenotoxicity and antioxidant potential of extract, fractions and vitexin from *C. antisyphiliticus*. Methanolic extract was fractionated through solvents of increasing polarity. The composition of extracts and fractions were evaluated through phytochemical screening. Micronucleus test was performed in mice to evaluate the antigenotoxicity. Antioxidant activity was measured using the assay 1,1-diphenyl-2-picrylhydrazyl (DPPH), iron ion chelating, thiobarbituric acid assay and nitric oxide scavenging. Treatment with extract, fractions and vitexin did not produce an increase in Micronucleus mean values. However, Micronucleus (MN) mean values decreased in relation to control. methanolic extract presented antioxidant potential for DPPH (81%), iron ion chelating (77.8%), Thiobarbituric Acid (TBARS) (32.49%) and Nitric Oxide (NO) (80.97%). Ethyl acetate fraction showed the highest antioxidant activity (65.46%). The vitexin showed a Inhibitory Concentration (IC50) of DPPH value smaller in relation to control. Vitexin flavonoid was detected by High Performance Liquid Chromatography (HPLC), infrared spectrometry and nuclear magnetic resonance. It can be inferred that methanolic extract, fraction ethyl acetate and vitexin isolated from *C. antisyphiliticus* is endowed with antigenotoxic and antioxidant potential.

Key words: Cytotoxicity, micronucleus, DPPH, phenolic content, flavonoids, mice.

INTRODUCTION

The *Croton* species is the second largest group of Euphorbiaceae family, comprising about 1,250 species, approximately 350 of which are found in Brazil (Braga et al. 2007). Pharmacological studies of extracts and compounds isolated from *C.* species had shown important therapeutic action with emphasis on their antilipidemic, antidiarrheic, immunomodulatory, antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant and antitumor activities (Júnior et al. 2014, Brito et al. 2018). Phytochemical studies have demonstrated which classes of compounds are present in this genus, for example volatile oil, alkaloid, proanthocyanidin, flavonoids and mainly diterpenoid esters such as phorbols, clerodanes, labdanes, kauranes and trachylobanes (Motta et al. 2013).

C. antisyphiliticus Mart. is a shrub found mostly in Brazilian's cerrado and it is known popularly as ''curraleira'' or ''pé-de-perdiz'' (Hirschmann & Arias 1990). It is used in folkloric medicine to treat syphilis, rheumatisms, ulcerative lesions, cancer and inflammatory disorders. Studies about therapeutic and pharmacological properties of *C. antisyphiliticus* have demonstrated antimicrobial, anti-inflammatory and cytotoxic activity in tumor cells (Fernandes et al. 2013).

The present study was designed to evaluate the antigenotoxicity and antioxidant potential of

C. antisyphiliticus for new compounds discovery with possible application in the prevention and treatment of diseases caused by oxidative stress and genome damage. The antigenotoxic effect of the *C. antisyphiliticus* methanolic extract, fractions and vitexin isolated were performed through micronucleus test in bone marrow cells of mice and mitotic index determination. The antioxidant potential was evaluated by the DPPH radical scavenging method, iron ion chelating, inhibition of lipid peroxidation by the Thiobarbituric Acid (TBARS) method and Nitric Oxide (NO) radical scavenging. The preliminary phytochemical screening and concentration of total phenolic compounds and flavonoids were also determined. The study subsequently investigated flavonoid compounds by High Performance Liquid Chromatography(HPLC) and carried out the fractionation, isolation and chemical structure determination of vitexin from *C. antisyphiliticus*.

MATERIALS AND METHODS

Sampling and preparation of the plant material

The vegetative parts (leaves, stem and barks) of *C. antisyphiliticus* were sampled from specimens present in the Universidade Estadual Paulista (UNESP-Brazil) (22°32'20''S and 50°22'60''W). A voucher specimen has been deposited in the *Herbarium* Assisense (HASSI – Assis, Brazil) under the number 760. After the samples were collected, leaves, stem and barks were selected, reunited and dried in forced-air oven at a temperature of 40°C for 24h. Immediately afterwards, they were ground and the resulting powder was stored in amber glass.

Preparation of methanolic extract

The methanolic extract was prepared by mechanic maceration of powdered plant material with methanol in PA (IMPEX, Brazil) at a concentration of 1:10 (p/v) for 24h at room temperature. Then, the extract was filtered at low pressure under a vacuum. The extraction was repeated three times with the same plant material. The resulting extracts were combined and concentrated in a rotary evaporator (model: MA120, Marconi, Brazil) at a mean temperature of 60°C; then the dried residue was used in the biological assays (Harborne 1973).

Fractioning of the methanolic extract

The methanolic extract of the *C. antisyphiliticus* was fractionated according to laboratory protocol (Hossain et al. 2014). For this purpose, a chromatographic column was fitted with approximately 75% silica and 25% Silica Gel 60 (Sigma-Aldrich®, USA) incorporated with 2.0g of extract. The sequence of solvents for the elution was n-hexane (100%), dichloromethane (100%), ethyl acetate (100%), ethyl acetate: methanol (70:30) and methanol (100%). Changes in solvents were performed whenever the fraction remained without evidence of separation. Filtered fractions were concentrated on a rotary evaporator at 40±2°C. Then, they were subjected to bioassays for both genotoxicity and antioxidant potential.

Phytochemical Screening

Quantification of total phenols and flavonoids

The total phenols and flavonoids were quantified with the methanolic extract diluted in ethanol at concentrations of 100, 250, 500, 1000, 1500 and 2000μg mL^{-1} and fractions (500 μg mL^{-1}). The *Folin-Ciocalteu* method was performed to determine total phenols. For each 0.5 mL of extract at different concentrations, 5mL of distilled water and 0.25 mL of *Folin-Ciocalteu* reagent were added. After 3 minutes, 1mL of saturated Na_2CO_3 solution at 10% was added and the mixture was

stored for 1 hour. The absorbance was measured at 725nm using a UV-Vis spectrophotometer (model: SP220, Biospectro, Brazil). All the tests were performed in triplicate and the results were expressed in mg gallic acid per gram of extract.

Quantification of the extract's total flavonoids was performed by UV-Vis spectrophotometer with the preparation of samples based on complexation of flavonoids with $AlCl_3$ (Zhishen et al. 1999). An aliquot of 250 µL of each different concentration of extract and fractions were mixed with 1.25 mL of distilled water and 75 µL of $NaNO_2$ solution at 5%. After 6 minutes, 150 µL of $AlCl_3/H_2O$ solution at 10% was added. After 5 minutes, 0.5 mL of NaOH 1M solution was added and then the total volume was completed by adding 2.5 mL of distilled water. The samples were shaken in a vortex mixer and the absorbance was measured at 510 nm. All the tests were performed in triplicate and the results were expressed in mg of rutin per gram of extract.

High performance liquid chromatography (HPLC)

Chromatographic separations were performed on high performance liquid chromatography (analytical, quaternary gradient) model PU-2089S Plus (Jasco), coupled to a diode array detector with photo scan range 200-900 nm, MD-2015 model Plus (Jasco), automatic injector model AS-2055 (Jasco) with 50 mL loop and column oven model CO-2060 Plus. Jasco Chrom Pass (version 1.8.1.6) was used during the acquisition and processing of chromatographic data. Reverse phase column immobilized with octadecylsilane was accomplished by the apparatus Luna C18 (2) 100A (Phenomenex®) of 250 x 4.6 mm i.d., with an average particle size of 5 µm with guard column (Phenomenex®) of 4 x 3 mm i.d. An aliquot of 10 mg from methanolic extract, ethyl acetate fraction and standard compounds (kaempferol, orientin, rutin, vitexin, quercitin and myricetin) was dissolved in 1 mL of acetonitrile (ACN) 100% and filtered through a syringe filter with pore size of 0.45 µm. Samples were monitored by PDA detector in a range of 200-600 nm. Chromatogram was obtained at 334 nm. Mobile phase: Acetonitrile + 0.1% Formic Acid (A) and Water + 0.1% Formic Acid (B). Gradient: 10-35% of A in B for 40 min (Valdés et al. 2017).

Fractionation and isolation of vitexin

The powder of the *C. antisyphiliticus* was subjected to extraction with an ethanol (99.2%) to cover the plant material (plant material/ethanol 1:10, w/v) at room temperature. After 7 days, the obtained extract was filtered and the solvent was completely removed using a rotary evaporator. This procedure was repeated three times to obtain maximum yield. Ethanolic extract was resuspended in 250 mL of ethanol/water (15:85) and fractionated by liquid–liquid extraction using solvents with increasing polarity to obtain hexane, ethyl acetate and aqueous fractions (Tanaka et al. 2005).

The fraction aqueous was adsorbed on silica gel and submitted to chromatographic fractionation on a deactivated silica gel 60 column and eluted with ethyl acetate (750 mL) and increasing amounts of ethanol (0–100%) and methanol (0–100%), resulting in 35 fractions of 100 mL. The fractions 10–22 (80:20, ethyl acetate: ethanol) were combined and purified in ethyl acetate to yield 145.0 mg of vitexin as a yellow powder; TLC Rf 0.8 (75:20:3:2 EtOAc–H2O–HCOOH–AcOOH); IR (KBr) tmax cm-1 3,380, 3,261, 2,961, 1,652, 1,612, 1,363. 1H NMR (Pyr, ppm) d 4.24 (m, H-300, H-400, H-500), 4.57 (m, H-200, H-600), 5.97 (d, H-100) J = 9.55 Hz, 6.73 (s, H-6), 6.84 (s, H-3), 7.30 (d, H-30, H-50) J = 8.30 Hz, 8.30 (d, H-20,

H-60) J = 8.30 Hz, 13.97 (s, chelated OH).13C NMR (Pyr, ppm) d 183.3 (C-4), 165.1 (C-5), 164.8 (C-7), 162.9 (C-40), 162.4 (C-2), 157.5 (C-9), 129.9 (C-20, C-60), 122.7 (C-10), 117.1 (C-30, C-50), 106.3 (C-8), 105.5 (C-10), 103.4 (C-3), 99.4 (C-6), 83.7 (C-500), 81.0 (C-300), 75.7 (C-100), 73.2 (C-200), 72.5 (C-400), 63.2 (C-600).

The chemical structure of the vitexin was determined based on their physical properties, spectral data from infrared (IR) analysis, nuclear magnetic resonance (1H and 13C NMR at 400 and 100 MHz, respectively) and melting point. The structure of the isolated compound was confirmed by comparing them with data previously reported in the literature (Tanaka et al. 2005), as well as by thin layer chromatography with authentic samples.

Micronucleus test

Mouse bone marrow micronucleus test was performed to evaluate genotoxic effects of *C. antisyphiliticus* methanolic extract, fractions and isolated compound on micronucleus in mice. The test was performed using twelve-week-old male Swiss albino mice (*Musmusculus* Rodentia, Muridae) weighting 25-30g obtained from an animal breeding center (Universidade do Estado de Minas Gerais, MG, Brazil) and acclimatized in cages at 24±1°C under 12h light periods for one week. The experimental protocol followed the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and was approved by the Ethical Committee for Animals Use (Permit number: 002/2010). During acclimatization and throughout the experiments the mice had free access to standard granulated chow and drinking water. Each cage contained five mice, which were randomly assigned to one of the following four groups: control group, which received distilled water by oral gavage; Cyclophosphamide treatment group, which received a single intraperitoneal injection with of cyclophosphamide (CASn.50-18-0; Endoxan, Baxter Oncology Gmb, Germany) 0.2mg for each 100g of body weight (bw) dissolved in distilled water; methanolic extract group, which received the equivalent of 1mg, 3mg and 6mg for each 100g bw, daily for 7 days by oral gavage; fractions group, which received the equivalent of 6mg for each 100g bw of different fractions and the equivalent of 1mg for each 100g bw of isolated vitexin (Tanaka et al. 2005).

All the mice were sacrificed by cervical dislocation after immobilization by anesthesia on day eight. Anesthesia was achieved by i.p. injection of 1.5mg/mouse of pentobarbital sodium; animals were comatose within 15min, exactly 10sec after cervical dislocation. This study conforms to the relevant Brazilian guidelines regarding ethical use of living animals. Genotoxic effects on mice were evaluated in their bone marrow by the micronucleus test. Immediately after sacrifice, both femurs were removed from each mouse and the bone marrow was flushed out into centrifuge tubes containing 2mL of fetal calf serum at 1000rpm for 10min, after which the supernatant was discarded and the pellet was resuspended in a drop of serum and a smear was made on a clean slide. The smear was air-dried, fixed with absolute methanol for 5min then air-dried and either stored at room temperature or directly stained for 5min with a freshly prepared working solution of Giemsa stain diluted 1:1 v/v in 0.06 M sodium phosphate buffer and 0.06 M potassium phosphate buffer (both at pH 6.8). After staining, the slides were rinsed in distilled water, dried at room temperature and scored for micronuclei (Krishna & Hayashi 2000) using 100x magnification and a Carl Zeiss optical microscope. It was scanned 2000 polychromatic erythrocytes (PCE) per mouse and recorded the number of micronucleated PCE (MNPCE). The mitotic index was determined by (total number

of dividing cells/total number of analyzed cells) 100x (Tanaka et al. 2005).

To compare the frequencies of MNPCE and normal PCE between treated and control groups, the results were expressed as mean ± standard deviation and analyzed statistically using the non-parametric Mann-Whitney U-test with the significance level set at α = 0.05. The statistical analysis was carried out using the statistical package SPSS 12.0 for PCs (SPSS, Chicago, IL) (Tanaka et al. 2005).

Test of the antioxidant activity

Scavenging DPPH radical test

The antioxidant activity of the methanolic extract, fractions and isolated compound was determined by the ability of the H^+ donor to stabilize radical 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma, USA). The experiment was performed in triplicate using a solution comprised of 1 mL of acetate buffer (pH 5.5 and 100 mM), 1.25 mL of ethanol P.A., 250 µL of DPPH solution and 50 mL of samples. The extract, fractions or isolated compound reacted with DPPH radical for a period of 30 min in the dark and were measured by UV-Vis spectrophotometer (Femto-600 Plus) at an absorbance of 517 nm (Brand-Williams et al. 1995). The calculation of antioxidant activity was performed according to the following formula: antioxidant activity (%) = [(control-sample)/control] x 100. The antioxidant activity of the extract, fractions or isolated compound can be determined by the degree of discoloration of the reagent after the 30 min required for the reaction to attain a plateau. The IC50 value signifies the ability of the extract to inhibit the radical oxidation of 50% (Di Mambro & Fonseca 2005) and was determined for isolated compound (vitexin). Gallic acid, rutin and caffeic acid (Vetec - Fine Chemicals, Brazil) were used as standard (Blois 1958).

Iron-ion-chelating activity

Six dilutions in methanol (100, 250, 500, 1000, 1500 and 2000 μgmL^{-1}) were prepared from the dried extract. Briefly, 0.05mL of each dilution was added to a 2.7mL TRIS buffer (pH7.4). Thereafter, 0.05 mL of 2 mM $FeCl_2$ was added and vortexed for 15 seconds. At 30 seconds, the reaction initiated by the addition of 5 mM ferrozine (0.2 mL), the mixture was shaken vigorously at Vortex (Velp Scientifica, UE) for 10 seconds. After 1 minute beyond addition of $FeCl_2$ solution, the absorbance was measured spectrophotometrically at 562 nm. The ability of extract to chelate the ferrous ion was calculated relative to the control (consisting of TRIS buffer, iron and ferrozine only) using the formula: chelating activity (%) = 100 x $[(A_C A_S)/A_C]$, where A_C is the absorbance of the control, and A_S is the absorbance of the sample (Dinis et al. 1994).

Nitric oxide scavenging

The nitric oxide (NO) scavenging assay was accomplished using sodium nitroprusside. This can be determined by the use of the Griess reaction. Two mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline PBS (pH 7.4) was mixed with 0.5 mL of methanolic extract at various concentrations (100, 250, 500, 1000, 1500 and 2000µg mL^{-1}) and incubated at 37°C for 150 minutes. After incubation the mixture was maintained for 60 minutes at room temperature. The absorbance was measured at 540 nm. The percentage of inhibition was calculated according to the following equation referent to the sodium nitrite calibration curve (y=0.0052x+0.0349) (Shahriar et al. 2013).

Lipid peroxidation assay (TBARS)

The thiobarbituric acid reactive species (TBARS) assay (Awah et al. 2010), using egg

yolk homogenates as lipid-rich media, was performed to measure the formation of lipid peroxide. Malondialdehyde (MDA), a secondary end-product of the oxidation of polyunsaturated fatty acids that reacts with two molecules of thiobarbituric acid (TBA) to yield a pinkish chromogenic with maximum absorption at 532 nm. Egg homogenate (500 µL) of 10%, v/v in phosphate buffered saline (pH 7.4) and 100 µL of sample (100, 250, 500, 1000, 1500 and 2000 µg mL^{-1}) were added to 1mL of the hydrochloride 2.2''Azobis (2-amidinopropane) dihydrochloride (AAPH) (0.12M) to induce lipid peroxidation. The mixture was incubated for 30 minutes at 37°C. After incubating and cooling at room temperature, 0.5 mL of trichloroacetic acid (TCA) (15%) and 0.5 mL of TBA (0.6%) were added. The mixture was incubated at 97ºC for 15 minutes. After incubation, the centrifugation was performed with 1mL of n-butane (2783 rpm for 10 minutes). Then, the reading of the supernatant was performed at 532 nm. Inhibition (%) of lipid peroxidation was calculated using the following equation: Inhibitory activity (%) = $[(A_C - A_S)/A_C]$ x100, where A_C is the absorbance of the control and A_S is the absorbance of the sample (Ruberto & Baratta 2000).

Statistical analysis for test of the antioxidant activity

The data were expressed in mean ± error standard by measuring three independent replicates. Analysis of variance using one-way ANOVA followed by Tukey's test was performed to test the significance of differences between means obtained among the treatments at the $\alpha \leq 0.05$ level of significance using the software Bio Estat version 5.0 (Callegari-Jacques 2003).

RESULTS

Analysis of the methanolic extract (Figure 1a) and ethyl acetate fraction (Figure 1b) obtained from HPLC analysis revealed the presence of six different flavonoids: kaempferol (1), orientin (2), rutin (3), vitexin (4), quercetin (5) and myricetin (6) when compared with flavonoid controls (Figure 1c). These compounds were identified when compared with scan spectra of flavonoid standards obtained by HPLC under the same conditions as the methanolic extract and ethyl acetate.

The fractionation of the aqueous fraction resulted by the isolation of vitexin as main constituent (Figure 2). This compound was analyzed using spectroscopic methods and in comparison, to data previously reported in the literature (Tanaka et al. 2005, Miyazawa & Hisama 2003). The ^{1}H NMR spectrum of vitexin showed a characteristic profile of 4',5,7-trihydroxyflavone. One singlet was observed at δ_H 13.97, corresponding to a chelated hydroxyl at C-5; another two singlets, one at δ_H 6.84, characteristic of H-3 of flavones, and another at δ_H 6.73, characteristic of H-6. The pair of doublets centered at δ_H 8.30 (H-2' and H-6') and δ_H 7.30 (H-3' and H-50') with a coupling constant for *ortho*-related hydrogens (*J*=8.30 Hz) establishes that the substitution pattern of the Bring is *para*-disubstituted. The doublet centered at δ_H 5.97 with *J* = 9:55 Hz, typical for *trans*-diaxial coupling, was attributed to H-1" from anomeric carbon. The signals observed between δ_H 3.00 and 5.00 combined with data from the literature suggest the structure of a glucose moiety (Bianco & Santos 2003). The positioning of the sugar at C-8 was confirmed by heteronuclear multiple bond correlation (HMBC) through the correlations between H-1" and the signal at δ_C 157.5 (C-9) and between H-6 and the carbons of ring A, especially the correlation with the signals at δ_C 164.8 (C-7), δ_C 165.1 (C-5), δ_C 105.4 (C-10). The 13C NMR spectrum showed 19 signals, equivalent to C-2' and C-6' with chemical shifts of δ_C 129.9, and C-3' and C-6' with chemical shifts

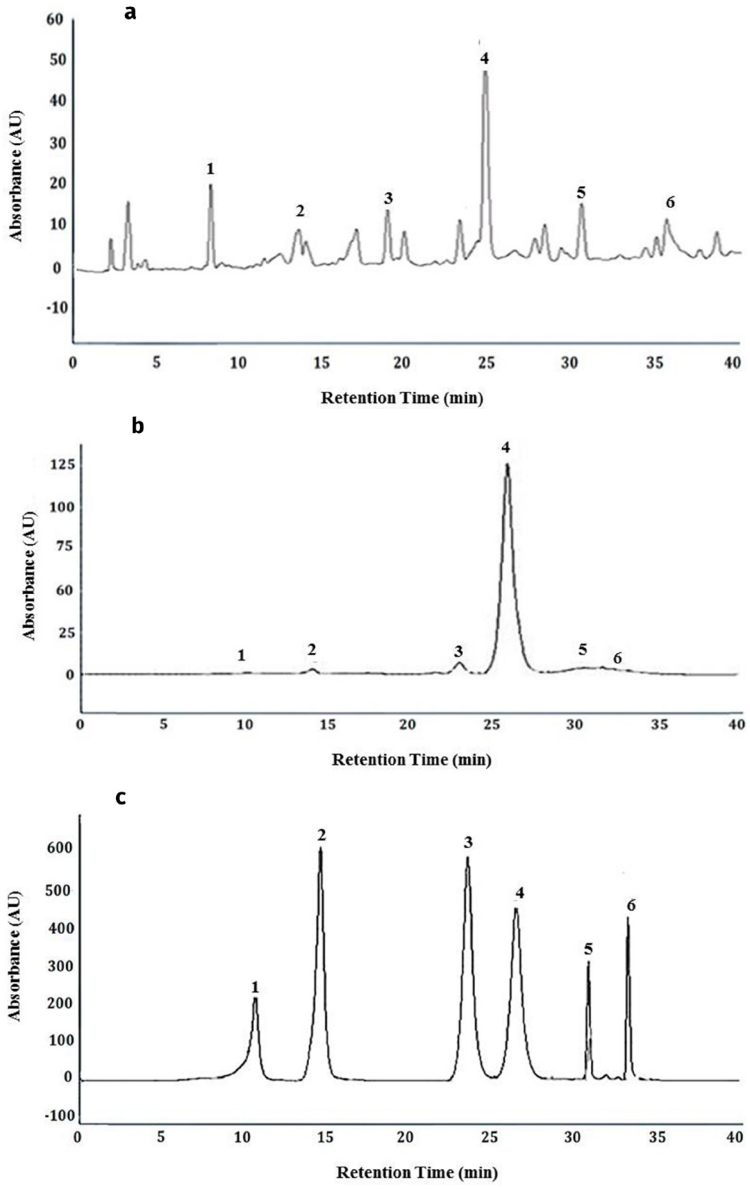

Figure 1. HPLC fingerprints obtained by methanolic extract (a), ethyl acetate fractions (b) and standard compounds (c): 1-kaempferol, 2-orientin, 3-rutin, 4-vitexin, 5-quercitin and 6-myricetin.

of δ_C 117.1, consistent with the literature (Tanaka et al. 2005).

Micronucleated polychromatic erythrocytes frequency (%MNPCE±SD) was 0.38±0.05 for the control group, while for the mice from groups treated with methanolic extract (1,3 or 6mg for each 100 g^{-1} bw) the frequencies were 0.27±0.06, 0.23±0.05 and 0.18±0.02, respectively. The latter had the lowest rate between the groups treated with extract. The control group and the treated groups were significantly different. For the groups treated with different fractions of the methanolic extract, there was significant difference between all studied groups and in-between. The ethyl acetate (AcOEt) fraction showed the lowest frequency (0.04±0.02) between the groups treated with the different fractions tested with a significant difference with the control group. The group treated with isolated vitexin showed the lowest frequency (0.02±0.001) among all treatments and did not differ significantly of the AcOEt fraction butit

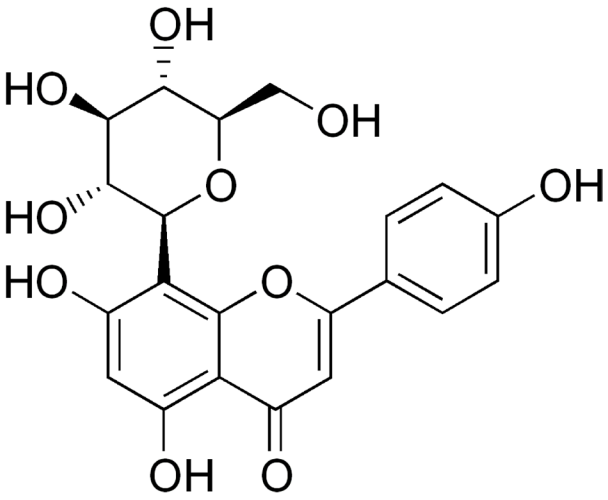

Figure 2. Chemical structure the vitexin of *Croton antisyphiliticus* Mart.

was significantly different from control group (NC) and Cyclophosphamide group (Table I). In relation to the mitotic index determination, the groups treated with methanolic extract and those treated with fractions and vitexin (isolated and standard) did not differ from each other or from the control group, but were significantly different from the Cyclophosphamide treatment group.

Results of MNPCE frequency analysis for groups treated with methanolic extract, fraction (Ethyl Acetate), vitexin isolated from *C. antisyphiliticus* and standard vitexin in association with Cyclophosphamide to evaluate antigenotoxic activity. The micronucleated polychromatic erythrocytes frequency (%MNPCE±SD) was 0.34±0.09 for the control group, while for the mice from groups treated with methanolic extract associated with Cyclophosphamide (1,3 or 6mg for each 100g of body weight) the frequencies were 1.78±0.23, 1.03±0.13 and 0.98±0.17, respectively. The control group and the treated groups were significantly different. The ethyl acetate (AcOEt) fraction associated with Cyclophosphamide showed the frequency of 0.31±0.08 and it did not present significant difference in comparison to control group (CG), but showed significant difference when compared to treated groups with methanolic extract. For the group treated with vitexin isolated from *C. antisyphiliticus* the frequency was 0.16±0.01, the lowest observed between treatments. The groups treated with vitexin showed a significant flavonoids difference in comparison to group (CP). In relation to the mitotic index determination, the groups treated with ethyl acetate (AcOEt) fraction and groups treated with vitexin isolated from *C. antisyphiliticus* did not differ from the control group (CG), but were significantly different from the Cyclophosphamide treatment group (Table II).

Results from the different antioxidant tests and the determination of total phenols and flavonoids in methanol extract. These results revealed a dose-dependent activity and at the concentration of 2000 µg mL^{-1} presented the highest antioxidant activity by the tests for DPPH (81.0±3.23%), iron ion chelating (77.80±3.93%) and NO radical scavenging (80.97±0.81%). For the thiobarbituric acid (TBARS) assay, the highest lipidanti-peroxidation activity was shown at the concentration of 1500 µgml^{-1} (45.77±2.65). The latter was the only test of antioxidant activity that did not show a significant difference among the concentrations tested. Similarly, the total phenols and flavonoids contents were the highest at the 2000 µg mL^{-1}concentration (Table III).

The results on antioxidant activity as well as the presence of phenols and flavonoids in the fractions obtained from the methanolic extract are displayed in Table IV. All the fractions significantly differed from each other, and the ethyl acetate fraction showed the highest antioxidant activity (DPPH = 65.46±1.22) and highest total content of phenols (66.40±1.98) and flavonoids (59.42±1.49).

Table I. Mitotic Index and Micronucleated polychromatic erythrocyte (MNPCE) frequency in Swiss albino mice (n=5) treated with different concentrations of methanolic extract (ME) (1, 3 and 6 mg 100g^{-1}bw) and fractions (N-Hexane=Hex, Dichloromethane = CH_2Cl_2, Ethyl Acetate = AcOEt, Methanol = MeOH) (6.0 mg g^{-1}bw), vitexin isolated from *C. antisyphiliticus* (Vitexin$_{ca}$) (1.0 mg g^{-1}bw), standard sample vitexin (Vitexin$_{ss}$) (1.0 mg g^{-1}bw), control group (CG) and group (CP) treated with Cyclophosphamide® (2mg 100g^{-1}bw). 2000 Polychromatic Erythrocytes (PCE) per mouse.

Groups	mg 100g^{-1}bw	%MNPCE±DP	Mitotic Index
CG	0.0	0.38 ± 0.05a	10.38±0.52a
Extract ME			
	1,0	0.27 ± 0.06b	9.72±0.23a
	3.0	0.23 ± 0.05b	9.01±0.39a
	6.0	0.18 ± 0.02b	9.23±0.87a
Fractions			
Hex	6.0	0.35±0.05a	10.33±1.09a
CH_2Cl_2	6.0	0.27±0.09b	10.21±1.17a
AcOEt	6.0	0.04±0.02c	9.98±0.98a
AcOEt:MeOH (70:30)	6.0	0.18±0.04b	10.78±1.09a
MeOH	6.0	0.41±0.04a	10.17±1.12a
Vitexin$_{ca}$	1.0	0.02±0.001c	10.08±0.18a
Vitexin$_{ss}$	1.0	0.31±0.09b	9.78±0.79a
CP	2.0	2.85 ± 0.38d	4.82±0.81b

Means sharing the same letter in a column do not differ significantly by Mann-Whitney and U-test (0.05).

Results of DPPH Radical Scavenging Activity IC50 (µg/mL)of vitexin isolated from *C. antisyphiliticus* (vitexin$_{ca}$), standard sample vitexin (vitexin$_{ss}$) and positive control (rutin and caffeic acid). The antioxidant activity wasthe highest for vitexin$_{ca}$ (10.49±0.11 µg/mL), which did not show significant difference compared to the positive control (rutin) (Table V).

DISCUSSION

Recent studies on the *C. antisyphiliticus* have reported pharmacological actions that are related to reports of its use in traditional Brazilian medicine that provides evidence of the anti-inflammatory properties of this species, by the inhibition pro-inflammatory enzymes and cytokines, however its chemopreventive activity has not been reported (Braga et al. 2007, Fernandes et al. 2013). The present study correlated for the first time the anti-genotoxicity and antioxidant activity of extracts, fractions and isolated compound from *C. antisyphiliticus*, providing a scientific basis for the use of this plant in folk medicine.

The phytochemical screening carried out on the methanolic extracts and fractions in HPLC-PDA detected a major presence of flavonoid compounds. Salatino et al. (2007) and Zou et al. (2010) demonstrated the presence of different flavonoids in extracts and fractions of *Croton* species. In absence of genotoxicity and the possible antigenotoxic action, added to the presence of flavonoid compounds in methanolic

Table II. Mitotic Index and Micronucleated polychromatic erythrocyte (MNPCE) frequency in Swiss albino mice (n=5) treated with different concentrations of methanolic extract (ME) (1, 3 and 6 mg $100g^{-1}$bw) and fraction (Ethyl Acetate = AcOEt) (6.0 mg g^{-1}bw), vitexin isolated from *C. antisyphiliticus* ($Vitexin_{ca}$) (1.0 mg g^{-1}bw), standard sample vitexin ($Vitexin_{ss}$) (1.0 mg g^{-1}bw) in association with Cyclophosphamide® (2mg $100g^{-1}$bw), control group (CG) and group (CP) treated with Cyclophosphamide® (2mg $100g^{-1}$bw). 2000 Polychromatic Erythrocytes (PCE) per mouse.

Groups	mg $100g^{-1}$bw		%MNPCE±DP	Mitotic Index
	Treatments	Cyclophosphamide		
CG	0.0	0.0	0.34 ± 0.09a	11.17±0.61a
ME+CP	1.0	2.0	1.78 ± 0.23b	7.24±0.19b
	3.0	2.0	1.03 ± 0.13b	8.01±0.11b
	6.0	2.0	0.98 ± 0.17b	8.73±0.27b
AcOEt+CP	6.0	2.0	0.31 ± 0.08a	10.18±0.44a
$Vitexin_{ca}$+CP	1.0	2.0	0.16 ± 0.01c	10.89±0.31a
$Vitexin_{ss}$+CP	1.0	2.0	1.77 ± 0.19b	9.17±0.43b
CP	0.0	2.0	3.17 ± 0.56d	5.42±0.32c

Means sharing the same letter in a column do not differ significantly by Mann-Whitney and U-test (0.05).

Table III. DPPH radical scavenging activity, iron ion chelating power, inhibition of lipid peroxidation (TBARS), NO radical scavenging and determination of total phenols and flavonoids at different concentration of methanolic extract (ME) of *C. antisyphiliticus*.

ME concentrations (µg mL^{-1})	% DPPH radical scavenging[a]	% Iron ion chelate	TBARS-Inhibitory activity (%)	% NO radical scavenging	Total phenol contents[b]	Flavonoid contents[c]
100	22.1±1.22a	31.40±1.01a	38.65±7.05a	51.81±3.54a	0.98±0.01	1.71±0.02
250	26.2±1.03a	40.30±2.33a	39.02±9.90a	43.67±2.18a	2.25±0.17	1.89±0.03
500	71.8±2.09b	53.00±2.89b	39.88±7.33a	53.78±0.36a	4.05±0.09	2.33±0.11
1000	73.0±2.78b	64.23±1.98b	37.76±10.6a	70.91±0.99b	7.06±0.98	3.24±0.05
1500	79.4±3.09bc	72.15±4.09bc	45.77±2.65a	74.20±0.60b	10.32±1.11	3.89±0.97
2000	81.0±3.23c	77.80±3.93c	32.49±7.17a	80.97±0.81b	14.54±1.27	4.01±0.83

Values are expressed as mean ±SE. Same letters within the same column indicate no significant differences among samples by Tukey test (α≤0.05). [a]Percentage values of DPPH radical scavenging activity; [b]Values of total phenol levels (mg of gallic acid equivalent/g of extract); [c]Total flavonoid levels in quercetin equivalent per mg/g of extract.

Table IV. DPPH radical scavenging activity and determination of total content of phenols and flavonoids in different fractions (N-Hexane=Hex, Dichloromethane = CH_2Cl_2, Ethyl Acetate = AcOEt, Methanol = MeOH) of methanolic extract of *C. antisyphiliticus* at concentration of 500 µg mL^{-1}).

Fractions (500 µg mL^{-1})	% DPPH radical scavenging[a]	Total phenol contents[b]	Flavonoid contents[c]
Hex	-	-	-
CH_2Cl_2	17.14±0.21a	9.09±0.99a	5.44±0.18a
AcOEt	65.46±1.22b	66.40±1.98b	59.42±1.49b
AcOEt:MeOH (70:30)	49.17±2.09c	43.34±1.79c	40.26±1.17c
MeOH	34.25±1.32d	23.28±1.33d	19.10±0.98d

Values are expressed as mean ±SD. Same letters within the same column indicates no significant differences among samples by Tukey test (α≤0.05). [a]Percentage values of DPPH radical scavenging activity; [b]Values of total phenol levels (mg of gallic acid equivalent/g of extract); [c]Total flavonoid levels in quercetin equivalent per mg/g of extract.

Table V. DPPH radical scavenging activity of vitexin isolated from *C. antisyphiliticus* ($Vitexin_{ca}$), standard sample vitexin ($Vitexin_{ss}$) and positive control (Rutin and Caffeic acid).

Compound	DPPH - IC_{50} (µg/mL)
$Vitexin_{ca}$	10.49±0.11a
$Vitexin_{ss}$	16.12±0.14b
Rutin	11.23±0.22a
Caffeic acid	15.27±0.21b

Values are expressed as mean ±SD. Same letters within the same column indicates no significant differences among samples by Tukey test (α≤0.05).

extract and fractions of *C. antisyphiliticus*, different antioxidant assays were performed, due to determination of this compounds. The possible antigenotoxic action besides

the presence of flavonoid compounds in methanolic extract, fractions and isolated vitexin from *C. antisyphiliticus* presented different antioxidant activity from other *Croton* species (Esmaeili et al. 2015).

Evaluation of micronucleus induction is the primary *in vivo* test in a battery of genotoxicity tests and is recommended by regulatory agencies around the globe as part of product safety assessment. The assay, when performed correctly, detects both clastogenic and eugenic effects (Krishna & Hayashi 2000, Tagliati et al. 2008). The experiments performed in this study show that the MNPCE frequency in the treated groups was significantly different from the frequency seen in the control groups. However, all tested concentrations showed lower MNPCE frequency compared to the control group. Highlighting that ethyl acetate fraction showed the lowest rate among the treatments (De Bona et al. 2012).

The mitotic index is another parameter that contributes to recognizing the genotoxic effects that quantify differences in cell division when an environmental parameter is changed (Fenech 2000, Pacheco & Hackel 2002). The present study

focused on the effects of *C. antisyphiliticus* on the mitotic index division rate in mice, and found no significant difference at all concentrations of the extract and fractions compared with control, suggesting an absence of genotoxic or cytotoxic activity. These results are consistent with Singleton & Rossi (1980), Camparoto et al. (2010), Munary et al. (2012), and Gamal-Eldeen et al. (2013), who evaluated the extracts and fractions of different plant species, demonstrating the absence of genotoxic or cytotoxic activity in combination with the absence of interference in the mitotic index or MNPCE frequency. Various studies conducted by Santos et al. (2006), Gupta et al. (2008) showed that different species of the *Croton* species have no genotoxic and/or mutagenic activity in *in vivo* assays.

Cyclophosphamide has been widely used as a positive control in micronucleus test for induction of MNPCE. For the investigation of anti-genotoxic action of *C. antisyphiliticus,* this study evaluated the MNPCE frequency with different concentrations of methanolic extract, fraction (AcOEt), vitexin isolated from *C. antisyphiliticus* and standard sample vitexin in association with Cyclophosphamide (Krishna & Hayashi 2000).

In this experiment, the group cyclophosphamide (CP) showed the highest MNPCE frequency and lowest mitotic index, while treated groups that received CP in combination with methanolic extract, presented a decrease in the MNPCEs frequency in dose-dependent. However, the groups treated with CP associated to isolated vitexin from *C. antysiphiliticus* showed the lowest frequency among all treatments. The same can be observed in the determination of mitotic index, where the groups treated with the ethyl acetate fraction and isolated vitexin did not differ from the control group (CG), but differed from the CP group. These results suggest a possible anti-genotoxic action and this effect could also be attributed vitexin (Wang et al. 2008, Farsi et al. 2013).

Increasing evidence underscores the fact that oxygen-derived free radicals and nitrogen may provoke a variety of adverse pathological effects (e.g. DNA damage, carcinogenesis and cellular degeneration) and induce many diseases including cancer, atherosclerosis, diabetes and rheumatoid arthritis (Jomova et al. 2010). In the present study, the antioxidant properties of *C. antisyphiliticus* methanolic extract were evaluated by DPPH radical, iron ion chelating, TBARS and NO scavenging. The experiments performed in this study, showed that raising the concentration the radical scavenging activity, chelate effect and inhibitory lipid peroxidation activity increased. These data are consistent with studies (Moretti et al. 2013, Pascoal et al. 2014), who demonstrated an important relationship between the action of eliminating free radicals and the antigenotoxic action of different active compounds in plants. Furthermore, studies conducted by Fernandes et al. (2013) and Tauchen et al. (2015) showed that bioactive chemical compounds of different *Croton* species have antioxidant and anti-proliferative activity.

It is well known that plants contain many phenolic compounds, mainly flavonoids, which contain a hydroxyl group on an aromatic ring. These compounds interrupt chain oxidation reactions by donation of a hydrogen atom or chelating metals. So, they act as reducing agents and antioxidants (Bursal & Köksal 2011, Ly et al. 2015). In the present study, there were variations in total phenolic and flavonoid contents at all the concentrations evaluated. Similar data were reported by Lavor et al. (2014). In the same way Serra et al. (2005) showed the presence of different flavonoids in the species *C. antisyphiliticus* with possible antihypertensive and anti-inflammatory action.

The DPPH antioxidant test and quantification of total phenols and flavonoids in methanolic extract and fractions showed that ethyl acetate fraction had the highest antioxidant potential and the highest content of phenols and flavonoids. In view of these results, methanol extract and ethyl acetate fraction were analyzed in HPLC and presented a range of different flavonoid compounds that could be observed both in the extract and in the fraction, and mainly the flavonoid vitexin compared to the standard spectrum of the compound. The concentration required for 50% scavenging of the DPPH free radical (IC50) was determined and the isolated vitexin showed the lowest IC50 value, compared to the values presented by standards vitexin, rutin and caffeic acid (Brito et al. 2014).

Faced with the major presence of vitexin in both methanolic extract and in its ethyl acetate fraction, beyond the isolation this compound and your IC50 value presented, it may be inferred that the expressive antioxidant potential is mainly due to this flavonoid compound. Studies performed by Gökbulut et al. (2010) and Tsai et al. (2011) showed antioxidant action of vitexin in different *in vitro* tests. Added to these studies, An et al. (2012) showed vitexin antioxidant activity in animal models also presented results of antioxidant activity of this compound in cultured human dermal fibroblasts. The presence of vitexin in *C. antisyphiliticus*, also recently reported by Tsai et al. (2011), suggests that an anti-inflamatory effect is associated with the presence of vitexin and quinic acid in this species.

However, the results of *in vitro* antioxidant activity tests cannot fully elucidate the action *in vivo* due the physiological complexity of different organisms. But Huang et al. (2005) and Alam et al. (2013) reported that *in vitro* tests are valuable tools for clinical studies with combined data and bioavailability biomarkers of oxidative stress tests.

The presence of vitexin in the extract and fraction evaluated in this study can also be correlated with the absence of genotoxic activity and with anti-genotoxic effect. Studies performed by Wang et al. (2008) and Farsi et al. (2013) showed a lack of genotoxic activity andanti-carcinogenic action of vitexin.

CONCLUSION

In conclusion, it can be inferred that a methanolic extract, fraction ethyl acetate and vitexin isolated from *C. antisyphiliticus* is endowed with antigenotoxic property the data obtained showed no genotoxic activity under the evaluated experimental conditions. Furthermore, it could be observed an antioxidant potential in vitro of extracts, fractions, and vitexin from *C. antisyphiliticus*. Additional studies are needed to evaluate and identify the molecular mechanism behind these biological activities, especially regarding to the presence of flavonoid vitexin in the studied species.

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R.M.G. Silva performed all the phytochemical analysis, fractionation and isolation of the compounds active. C.C.M. Figueiredo, A.C. Gomes and P.S. Santiago performed antioxidants tests. F.O. Granero performed collecting the plant material and help in writing the manuscript. P.C. Ferreira performed the antigenotoxic assays. This work was done under the supervision of L.P. Silva and R.M.G. Silva. The authors declare that they have no conflict of interest.