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Evaluation of the acute toxicity and antidiabetic activity of *Coutoubea spicata* "nicolau" ethanolic extract and ethyl acetate fraction

[Evaluación de la toxicidad aguda y la actividad antidiabética del extracto etanólico y la fracción de acetato de etilo de *Contoubea spicata* "nicolau"]

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dos Santos JL, Silva AMO, Souza LMV, Pereira RO, Lima CA, Voltarelli FA, Abreu LS, Montalvao MM, Correa CB, Rabelo TK, Duarte MC, Aidar FJ, Tavares JF, Estevam CS, Marçal AC Evaluation of the acute toxicity and antidiabetic activity of Coutoubea spicata "nicolau" ethanolic extract and ethyl acetate fraction **Bol Latinoam Caribe Plant Med Aromat** 22 (5): 607 - 627 (2023). https://doi.org/10.37360/blacpma.23.22.5.44 **Abstract:** In order to understand antidiabetic potential and toxicity, this study aimed to evaluate the acute toxicity and antidiabetic activity of ethanolic extract and ethyl acetate fraction obtained from *Coutoubea spicata* "nicolau" shoots. Chemical constituents and acute toxicity were investigated. In alloxan-induced diabetic rats, extract and fraction were tested at dose of 100 mg/kg, p.o. Body weight gain, glucose, lipid profile and oxidative stress markers in serum and tissues were determined. *In vitro* antioxidant activity was performed. Swertiamarin, gentiopicrin, deoxyloganic acid, clovin and robinin, and their p-coumaric ester were identified. Extract and fraction were classified as safe (category 5). In diabetic rats, *Coutoubea spicata* reduced glycaemia, which was accompanied by body weight recovery gain and attenuation in oxidative stress markers. Fraction showed scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical (ABTS) radicals and reducing power higher than that of the extract. Extract and fraction of *Coutoubea spicata* didn't present significant toxicity and it can be investigated as a therapeutic alternative in diabetes.

Keywords: Coutoubea spicata; Acute toxicity; Diabetes; Redox profile; HPLC-ESI-MS/MS.

Resumen: Con el fin de conocer el potencial antidiabético y la toxicidad, este estudio tuvo como objetivo evaluar la toxicidad aguda y la actividad antidiabética del extracto etanólico y la fracción de acetato de etilo obtenidos de los brotes de *Coutoubea spicata* "Nicolau". Se investigaron los componentes químicos y la toxicidad aguda. En ratas diabéticas inducidas por alloxan, se probaron el extracto y la fracción en dosis de 100 mg/kg, p.o. Se determinó el aumento de peso corporal, la glucosa, el perfil lipídico y los marcadores de estrés oxidativo en suero y tejidos. Se realizó una actividad antioxidante *in vitro*. Se identificaron la suertiamarina, la gentiopicrina, el ácido desoxilogánico, la clovina y la robinina, así como su éster p-cumárico. El extracto y la fracción se clasificaron como seguros (categoría 5). En ratas diabéticas, *Coutoubea spicata* redujo la glicemia, lo que se acompañó de una recuperación del peso corporal y de la atenuación de los marcadores de estrés oxidativo. La fracción mostró una actividad e barrido contra los radicales 1,1-difenil-2-picrilhidrazilo (DPPH) y 2,2'-azino-bis (ácido 3-etilbenzotiazolina-6-sulfónico) y un poder reductor superior al del extracto. El extracto y la fracción de *Coutoubea spicata* no presentaron una toxicidad significativa y pueden ser investigados como alternativa terapéutica en la diabetes.

Palabras clave: Coutoubea spicata; Toxicidad aguda; Diabetes; Perfil redox; HPLC-ESI-MS/MS

INTRODUTION

Coutoubea spicata (*C. spicata*) belongs to the family Gentianaceae, third largest family of the order Gentianales. The genus Coutoubea includes five tropical species, among them *C. spicata*. It can be found in Central and South America, and it is popularly known as "nicolau" or "genciana". In popular medicine, *C. spicata* shoots are used as gastrointestinal stimulant and hypoglycemic agent (Guimarães & Klein, 1985; Schaufelberger *et al.*, 1987; Struwe *et al.*, 2002; Delgado *et al.*, 2009).

Some substances have been isolated from its shoots, including two flavonol glycosides, unusual for Gentianaceae, clovin (quercetin 3-0-robinobioside 7-0-rhamnoside) and quercetin [3-0-rhamnosyl-(1-,6) -(4'-[trans-p-coumaroyl) galactoside 7-0-rhamnoside (4"-trans-pcoumaroylclovin)]. Secoiridoid glycosides gentiopicrin and swertiamarin have been identified as the bitter principles of *C. spicata* (Schaufelberger et al., 1987). Flavonoids present antioxidant potential and can modulate the redox state of the cell and thus attenuate oxidative stress involved in several diseases (Xu *et al.*, 2017; Jucá *et al.*, 2018).

The great potential of *C. spicata* as medicinal herb is supported by applications described in literature for species of the same family, mainly for helping to control hyperglycemia (Mokashi *et al.*, 2017; Ghazanfar *et al.*, 2017; Anyanwu *et al.*, 2019). However, data about the hypoglycemic effects arising from the use of *C. spicata* have not yet been described.

The use of medicinal plants plays a central role in primary health-care due to its low cost, and this has led the Brazilian Government to implement programs to guarantee safe and free access to medicinal plants (Carvalho *et al.*, 2011; Marmitt *et al.*, 2018). However, pharmacological and toxicological properties of plants still need to be studied through biocompatibility cytotoxicity tests, sensitization, acute systemic toxicity and other tests prior to clinical trials to better elucidate the efficacy and risks of their use by humans (Li *et al.*, 2015).

Literature reports that the ingestion of *C*. *spicata* by bovine leads to symptoms such as restlessness, rumem paralysis, in addition to increased respiratory and cardiac frequency (Guimarães & Klein, 1985).

Therefore, the present study aimed to investigate the hypoglycemic properties and evaluate the acute toxicity of the extract and ethyl acetate fraction of *C. spicatain in vivo* and *in vitro* experimental models and also to investigate its phytochemical and redox profile.

MATERIALS AND METHODS

Plant material: collection, processing and extraction C. spicata shoots were collected in March/2011 at the municipality of Japaratuba, state of Sergipe, Brazil (10°32'4.49" S and 36°53'57" W). The plant material was identified by PhD botanist Ana Paula do Nascimento Prata and a specimen was deposited under registration number ASE 25.136 (SISGEN A6AC079) at the Herbarium of the Federal University of Sergipe, São Cristóvão, Sergipe, Brazil.

C. spicata shoots were dried at room temperature, reduced to powder and submitted to with 95% ethanol for maceration 5 days. Subsequently, the material was filtered and concentrated in a rotatory evaporator under reduced pressure at 45°C to give the ethanolic extract (yield of 9.53%). A 100 g portion of the ethanolic extract was dissolved in 250 mL methanol and 333 mL water approximately (2:3 v/v) and subjected to liquid-liquid extraction with organic solvents to obtain hexane, chloroform, ethyl acetate and hydromethanol fractions.

The study of the phytochemical and redox profiles and toxicity was carried out with the extract and ethyl acetate fraction.

Quantification of total phenolics and flavonoids

Total phenolic content was analyzed by the Folin-Ciocalteau method with some modifications (Swain & Hills, 1959). A standard gallic acid curve was used and results were expressed as mg of gallic acid equivalents (GAE)/g of extract or fraction. Total flavonoid content was determined using AlCl3 method and was calculated by using calibration catechin curve. The content was expressed as milligram of Catechin Equivalents/g (mg CE/g extract or fraction) of total flavonoid content (Zhishen *et al.*, 1999).

Liquid chromatography–high-resolution mass spectrometry instrumentation and conditions

Shimadzu[®] (Kyoto, Japan) High-Performance Liquid Chromatography System, coupled to Amazon X or micrOTOF II (Bruker Daltonics, Billerica, MA, USA) with electrospray source ion (ESI) was used to perform ESI-IT-MS/MS and ESI-TOF-MS analyses,

respectively. The LC System consisted of LC-20AD solvent pump unit (flow rate of 600 µL.min⁻¹); DGU-20A₅ online degasser; CBM-20A system controller and SPD-M20A (190 - 800 nm) diode array detector. The LC separation was performed on Kromasil C-18 5 µm 100 Å, 250 x 4.6 mm (Kromasil, Bohus, Sweden) analytical column. Injections (20 µL) were performed using autosampler (SIL-20A). The mobile phase consisted of 0.1% formic acid in water (solvent A) and methanol (solvent B). Exploratory gradient was performed to eluition in 60 minutes. The analysis parameters are the following: capillary 4.5 kV, ESI in negative mode, final plate offset 500 V, 40 psi nebulizer, dry gas (N2) with flow rate of 8 mL/min and temperature of 300°C. CID fragmentation was achieved in auto MS/MS mode using advanced resolution mode for MS and MS/MS mode. The spectra (m/z 50-1000) were recorded every two seconds.

Cell viability assay

Fibroblasts (L929) were distributed into 96-well plates (2 x 10^4 cells/well) and incubated for 24 h in

5% CO₂ atmosphere at 37°C. After this period, the medium was removed and adhered cells were treated with C. spicata extract or fraction at concentrations of 25-200 µg mL⁻¹ for 24h under the same incubation conditions. Untreated cells were used as controls and considered with 100% cell viability. After the treatment period, cell viability was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay as described in ISO 10993-5 with modifications (ISO, 2009), For this, the cell monolayer was washed twice with PBS (pH 7.4) and then 200 µl MTT (0.5 mg mL-1 in PBS, Sigma-Aldrich, St. Louis, MO, USA) were added to each well. Plates were again incubated under the same conditions above for a period of three hours. After the incubation time, MTT was aspirated and formazan crystals were solubilized in 200 µL Dimethyl sulfoxide (DMSO) (Vetec by Sigma-Aldrich, USA). After 10 min, the optical density (OD) was measured on a microplate reader at wavelength of 570 nm. Results were expressed as viability percentage according to the following equation:

Viability = absorbance (treated cell) x 100

absorbance (control cell)

Each experiment was conducted in quadruplicate and repeated at least twice. Data were expressed as mean \pm standard deviation (\pm SD).

Acute toxicity assay

Acute toxicity evaluation was conducted based on guidelines of the Organization for Economic Cooperation and Development (OECD) for acute oral toxicity toxicity (OECD, 2001).

Healthy young adult female Wistar rats (8 weeks old) were obtained from the Center for the Creation and Experimentation of the Nucleus of Research in Intracellular Signaling of the Federal University of Sergipe. Animals were kept at $22^{\circ}C$ (\pm 3°C) with free access to food and water under 12-hr light/dark cycle. All experiments were conducted in agreement with guidelines of the Brazilian College of Animal Experimentation and the National Institutes of Health Guidelines and were approved by the Ethics Research Committee for Animal Use of the Federal University of Sergipe (26/2018). At the end of experiments, animals were euthanized by

exsanguination under anesthesia.

Animals were fed with conventional laboratory Nuvilab chow and water *ad libitum*. Animals were group-caged by dose, randomly selected, marked to permit individual identification, and kept in cages for at least 5 days prior to dosing.

Fifteen rats were randomly divided into five groups (3 in each group); prior to administration, animals were fasted for 12 h and had free access to water. Extract and fraction were dissolved in distilled water (1 ml/100 g body weight [BW]) and orally administered to rats at doses of 300 mg/kg BW, and 2000 mg/kg BW; rats in control group were orally treated with equal amount of distilled water. After a single dose administration, mortality and clinical signs associated with toxicity were observed and daily recorded for consecutive two weeks; body weight changes were daily determined, calculated and recorded.

On day 14, after being weighed, animals were fasted for 12 h (free access to water) and intraperitoneally anesthetized with the combination

of ketamine (100 mg/kg BW) and xylazine (10 mg/kg BW). Blood samples were collected from the portal vein into Ethylenediamine tetraacetic acid (EDTA)-containing tubes for the determination of serum biochemical parameters including Aspartate aminotransferase (AST), Alanine Aminotransferase (ALT), urea and creatinine using commercial Labtest® kits (Lagoa Santa, Minas Gerais, Brazil). Blood samples were collected for hematological

analysis. Hematology parameters evaluated included blood cell counts (erythrocyte, platelets, total white blood cells and differential leukocyte), and hemoglobin.

Complete necropsy was performed. Some vital organs comprising liver, spleen, kidney, and heart were collected and weighed. Relative organ weight was calculated according to the following formula:

Relative organ weight % =organ weight x 100

body weight

Necropsy and histopathological findings were performed for all animals at all dose levels, including control group. Organs collected from animals were preserved in alcohol solution (70%) for histopathologic examination. Fixed tissues were trimmed, processed, embedded in paraffin, sectioned with microtome, placed on glass microscope slides, stained with hematoxylin and eosin, and examined by light microscopy.

Diabetes induction

Animals

Healthy young adult male Wistar rats (12 weeks old) were randomly divided into five groups (8 in each group): control, diabetic, and diabetic groups treated with extract, ethyl acetate fraction or metformin (100 mg/Kg, p.o) during four weeks, three times a week.

Diabetes was induced using alloxan (150 mg/kg body weight, i.p.). Animals with fasting blood glucose equal to or higher than 200 mg/dL were included in the study. Body weight gain and blood glucose were weekly monitored. At the end of experiments, animals were euthanized by exsanguination under anesthesia. Blood samples were collected and serum biochemical parameters including AST, ALT, triglycerides, total cholesterol and high density lipoproteins (HDL cholesterol) were analyzed using commercial kits. Liver, heart and pancreas were collected for analysis of oxidative stress markers (sulfhydryl groups [SH] and substances reactive to thiobarbituric acid- [MDA]).

Evaluation of the in vitro antioxidant activity

For the antioxidant activity determination, *in vitro* experiments described below were performed twice

and in triplicate. In these experiments, trolox was used as positive control and tests were performed using different concentrations $(10 - 1000 \ \mu g \ mL^{-1})$, with some modifications for 96-well visible/UV microplate kinetics reader.

The antioxidant capacity against the 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) was evaluated as previously reported with minor modifications (Brand-Williams *et al.*, 1995). Absorbance values were expressed as DPPH inhibition percentage [DPPH inhibition % = [(control - test)/control]x100].

The antioxidant capacity against the 2,2'azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical (ABTS) was determined as previously described with some modifications (Re *et al.*, 1999). The ABTS radical was initially formed from the reaction of 5 mL ABTS (7 mmol/L) with 88 μ L potassium persulfate (K₂S₂O₈, 2.45 mmol/L), which was incubated at room temperature in the absence of light for 16 h. Subsequently, ABTS solution was diluted in ethanol until it presenting absorbance of 0.70 ± 0.05 at 734 nm. Results were expressed as ABTS inhibition percentage [ABTS inhibition % = [(control - test)/control] x 100].

The reducing potential was determined by the ferric reducing/antioxidant power (FRAP) assay, as previously reported with modifications (Pulido *et al.*, 2000). Freshly FRAP solution was prepared (0.3 mmol/L acetate buffer pH 3.6, 2,4,6-tripyridyl-s-triazine (10 mmol/L) and ferric chloride (FeCl₃). Standard ferrous sulphate curve (FeSO₄) was performed and used to calculate the reducing power of extracts.

β-carotene/linoleic acid model system is

based on the de-coloration of β -carotene by peroxides generated during the oxidation of linoleic acid at elevated temperature (Miller, 1971). In brief, solution containing β -carotene, linoleic acid and Tween 20 was prepared and oxygenated water was added. The plate was incubated at 470°C. Readings were immediately performed and after 120 min. The antioxidant activity of extract and fraction was expressed as % oxidation.

Statistical analysis

Results are expressed as means \pm standard deviation (SD). Statistical evaluation of data was performed using t-Student test or one-way analysis of variance (ANOVA) followed by Tukey's test. *p* values lower than 0.05 were considered significant. For this, the GraphPad Prism statistical software version 7.0 was used.

RESULTS

Total phenolic and flavonoid contents and chemical characterization of C. spicata by HPLC-ESI-MS/MS

This study evaluated the constituents of ethanolic extract and ethyl acetate fraction from C. *spicata* shoots.

The total phenolic content of ethanolic extract and ethyl acetate fraction was 43.73 ± 1.65 and 47.73 ± 1.09 mg GAE/g, and 1.25 ± 0.05 and 1.66 ± 0.01 mg CE/g for flavonoid content, respectively. As shown above, the fraction contains the highest amount of these compounds (p<0.05).

The chemical analysis of extract and ethyl acetate fraction of *C. spicata* shoots is presented (Figure No. 1 and Table No. 1).



Figure No. 1

Base peak chromatograms (BPC) in negative ion mode of ethanolic extract (A) and ethyl acetate fraction (B) of aerial parts of *Coutoubea spicata* by HPLC-ESI-MS/MS. Legend: (1) Deoxyloganic acid, (2) Swertiamarin, (3) Gentiopicrin, (4) Clovin, (5) Robinin, (6) 4"-trans-pcoumaroylclovin I, (7) 4" -trans-pcoumaroyl robinin I, (8) 4"-trans-pcoumaroylclovin II and (9) 4" -trans-pcoumaroyl robinin II

dos Santos et al.

- HFLC-ESI-MIS/MIS analysis of constituents of <i>Coutoubea spicata</i> (Gentianaceae)							
Peak No.	t _R (min.)	[M-H] ⁻ / [M+HCOOH-H] ⁻	Molecular formula	MS/MS	Tentative assignment	Reference	
1	18.6	-/405.1417	$C_{16}H_{24}O_9$	MS ² [405]: 359; 225; 179; 162	Deoxyloganic Acid	Miettinen et al. (2014)	
2	24.8	-/419.1192	$C_{16}H_{22}O_{10}$	MS ² [419]: 357; 355; 179; 161	Swertiamarin	Feng et al. (2014)	
3	26.5	-/401.1112	$C_{16}H_{20}O_9$	MS ² [401]: 355; 225;179; 162	Gentiopicrin	Feng et al. (2014)	
4	32.2	755.2025/-	$C_{33}H_{40}O_{20}$	MS ² [755]: 609; 343; 301; 271	Clovin	Schaufelberger et al. (1987)	
5	33.7	739.2052/-	$C_{33}H_{40}O_{19}$	MS ² [739]: 593; 327; 285; 255	Robinin	Silva <i>et al.</i> (2018)	
6	35.3	901.2381/-	$C_{42}H_{46}O_{22}$	MS ² [901]: 755; 609; 301	4"-trans- pcoumaroyl clovin I	Schaufelberger et al. (1987)	
7	37.5	885.2442/-	$C_{42}H_{46}O_{21}$	MS ² [885]: 739; 593; 285	4"-trans- pcoumaroyl robinin I	Sugimoto et al. (2014)	
8	39.0	901.2386/-	$C_{42}H_{46}O_{22}$	MS ² [901]: 755; 609; 301	4"-trans- pcoumaroyl clovin II	Schaufelberger et al. (1987)	
9	40.1	885.2431/-	$C_{42}H_{46}O_{21}$	MS ² [885]: 739; 593; 285	4" -trans- pcoumaroyl robinin II	Sugimoto et al. (2014)	

Table No. 1 IPLC-ESI-MS/MS analysis of constituents of *Coutoubea spicata* (Gentianaceae

I and II Numbers used to discriminate putative individual isomers.

Extract and ethyl acetate fraction were analyzed in elution gradient (5-100% B) in 60 min. From the Extract Ion Chromatogram (EIC) analysis, it was possible to identify the presence of ions with m/z 401 [M + HCOOH-H]⁻ ($t_{\rm R}$ = 26.5), 419 [M + HCOOH-H]⁻ ($t_{\rm R} = 35.3$) and 901 [M-H]⁻ ($t_{\rm R} = 37.5$ and 40.1) being gentiopicrin, swertiamarin and 4 "trans-pcoumaroylclovin isomers, respectively, already reported (Schaufelberger et al., 1987), were identified in our study by HPLC-ESI-MS/MS, confirming the main compounds in family Gentianaceae. Ion m/z 405 [M+HCOOH-H], which produces in MS² ion m/z 179 [M-H-Agly]⁻ was identified as deoxyloganic acid (Miettinen et al., 2014). Ion m/z 885 [M-H]⁻ corresponding to 16 Da less than ion m/z 901 was also observed. Both have similar fragments corresponding to two losses of 146, one representing a p-coumaroyl and deoxyhexose unit, in addition to that with 308, one representing the exit of the robinobiose group (6-rhamnosyl galactose) leaving ion m/z 285 representative of aglycone

kaempferol. This substance was identified as 4"trans-pcoumaroyl robinin in *Ixora undulate* (Sugimoto *et al.*, 2014). Corroborating these results, ions related to robinin (m/z 739) and clovin (m/z 755) were also identified.

Two compounds of the flavonols group, robinin and its ester 4'-trans-pcoumaroyl robinin, also identified in *Ixora undulate* (Sugimoto *et al.*, 2014), were identified in *C. spicata*, although the main characteristic of Gentianaceae is not to produce C-glycosyl flavones instead of flavonoid glycosides (Kaouadji *et al.*, 1990).

MTT assay

In general, ethanolic extract and ethyl acetate fraction did not show cytotoxicity on L929 fibroblasts at the concentrations evaluated (25-200 μ g mL-1), with the exception of the fraction in the highest dose that presented low cytotoxicity (55.42 ± 16.89%). The results of the tests are expressed as a percentage of viability (Figure No. 2).

An intensity scale based on the methods of Rodrigues *et al.* (2014), was used to classify the cytotoxicity in low cytotoxicity (viability between >50% and $<\!80\%$), moderate cytotoxicity (viability between $>\!30\%$ and $<\!50\%$) and non-cytotoxic (viability $>\!80\%$).



Figure No. 2

Cell viability evaluated by MTT assay. (A) Effect of extract of *C. spicata* on cell viability of L929 cell line and (B) effect of ethyl acetate fraction of *C. spicata* on cell viability of L929 cell line. Cells were pre-incubated in the presence or absence of extract or fraction for 24 hours. Data are expressed as mean ± SD of three independent experiments (Student's T-Test: **p*<0.05 *vs* in medium alone)

Acute toxicity of ethanolic extract and ethyl acetate fraction

The acute toxicity test through a single administration of 300 or 2000 mg/kg of *C. spicata* did not show any signs of morbidity and mortality in treated female rats during 14 days. As shown in Table No. 2, no significant differences in body weight changes were observed. However, effects of ethyl acetate fraction on relative weight were demonstrated in heart, liver, and kidney.

Regarding the biochemical parameters, AST increased in the fraction 2000-treated group when compared to the control group. The values of ALT, urea and creatinine did not show significant changes. Alterations were also not observed for hematological parameters (Table No. 3).

Histopathological analysis

Figure No. 3 shows that the extract and ethyl acetate fraction did not induce any hepatocellular alteration. All groups presented preserved liver parenchyma without vacuolation, cholestasis and fibrosis. In addition, no regions with necrosis or multiple clusters of inflammatory cells were visualized. As shown in Figure No. 4, the extract and fraction did not alter the morphology of the glomeruli and did not induce tubulointestitial fibrosis. The extract and fraction did not alter the architecture of the spleen. All groups presented lymphoid follicles, and preserved white and red pulp (Figure No. 5). No changes were observed in the heart of the animals that received extract or fraction of *C. spicata* (Figure No. 6).

Table No. 2						
Effects of oral administration of the extract and ethyl acetate fraction of Coutoubea spicata						
Groups Control		Extract	Extract	Fraction	Fraction	
Dose (mg/Kg)	-	300 mg/Kg	2000 mg/Kg	300 mg/Kg	2000 mg/Kg	
Weight						
Initial weight (g)	189.00 ± 9.29	183.00 ± 14.18	173.00 ± 2.08	193.00 ± 6.43	175.00 ± 3.61	
Final weight (g)	214.00 ± 4.24^{ab}	213.00 ± 15.50 a	210.00 ± 4.04^{b}	233.00 ± 8.14^{a}	208.00 ± 2.65 ^b	
Weight gain (g)	24.00 ± 2.83	31.33 ± 1.53	37.00 ± 5.57	33.67 ± 9.07	33.00 ± 6.00	
Relative weight of						
tissues						
Spleen	0.30 ± 0.06	0.31 ± 0.05	0.36 ± 0.09	0.29 ± 0.03	0.41 ± 0.02	
Heart	$0.33\pm0.03^{\rm b}$	0.30 ± 0.03^{b}	$0.30\pm0.01^{\rm b}$	$0.33\pm0.02^{\rm b}$	$0.42\pm0.02^{\rm a}$	
Liver	3.48 ± 0.36^{b}	3.38 ± 0.14 ^b	3.44 ± 0.21 ^b	3.57 ± 0.19^{b}	4.72 ± 0.41 $^{\rm a}$	
Kidney	$0.71\pm0.03^{\text{ b}}$	0.68 ± 0.04^{b}	0.74 ± 0.06^{b}	$0.69\pm0.02^{\text{ b}}$	0.97 ± 0.04 $^{\mathrm{a}}$	

Data were presented as mean ± SD of 3 animals. Different letters same line indicate statistically significant differences, *p*<0.05 (Anova One-Way followed by Tukey's test)

Effects of oral administration of the extract and ethyl acetate fraction of Coutoubea spicata						
Groups	Control	Extract	Extract	Fraction	Fraction	
Dose (mg/Kg)	-	300 mg/Kg	2000 mg/Kg	300 mg/Kg	2000 mg/Kg	
Biochemical markers						
ALT (U/L)	23.57 ± 4.94	23.28 ± 11.39	26.19 ± 2.31	25.32 ± 3.49	33.17 ± 5.45	
AST (U/L)	$37.25 \pm 3.07^{\circ}$	40.16 ± 7.56^{c}	62.86 ± 3.81^{b}	$42.78\pm9.07^{\rm c}$	$84.68\pm7.15^{\mathrm{a}}$	
Urea (mg/dL)	48.00 ± 1.43	51.53 ± 7.69	55.83 ± 7.64	55.86 ± 2.42	52.55 ± 5.66	
Creatinine (mg/dL)	$1.07\pm0.06^{\rm b}$	1.52 ± 0.20^{ab}	1.59 ± 0.29^{a}	1.41 ± 0.12^{ab}	1.41 ± 0.06^{ab}	
Hematological						
analysis						
RBC (10 ³ /uL)	7.32 ± 0.25	7.18 ± 0.00	7.36 ± 0.08	7.20 ± 0.15	7.09 ± 0.45	
Haemoglobin (g/dL)	14.90 ± 0.89	14.50 ± 0.61	14.60 ± 0.20	14.27 ± 0.31	14.77 ± 0.12	
Platelet $(10^3/uL)$	1143.33 ± 85.33	1102.67 ± 54.28	1078.67 ± 167.62	1188.33 ± 77.53	1129.00 ± 227.09	
WBC (10 ³ /uL)	7.31 ± 1.15	4.08 ± 1.99	5.93 ± 3.67	6.38 ± 0.46	12.22 ± 12.71	
Neutrophils (%)	40.27 ± 13.99	48.77 ± 25.51	40.57 ± 30.54	34.77 ± 8.52	45.37 ± 27.89	
Lymphocytes (%)	55.93 ± 14.42	46.90 ± 23.57	56.13 ± 29.49	58.40 ± 10.58	21.37 ± 26.41	
Monocytes (%)	1.14 ± 1.61	2.17 ± 2.55	0.75 ± 0.62	1.92 ± 2.67	8.87 ± 6.88	
Eosinophils (%)	1.74 ± 1.69	0.32 ± 0.43	0.90 ± 0.72	3.32 ± 2.94	23.75 ± 31.15	
Basophils (%)	0.90 ± 0.49	1.85 ± 1.79	1.68 ± 1.47	1.60 ± 0.65	0.66 ± 0.87	

Table No. 3

Data were presented as mean ± SD of 3 animals. Different letters same line indicate statistically significant differences, *p*<0.05 (Anova One-Way followed by Tukey's test)



Figure No. 3

Effects of oral administration of the extract and ethyl acetate fraction of *Coutoubea spicata* in liver histological analysis. Legend: VP = portal vein, a = artery, tip of the arrow = duct and P = hepatic parenchyma



Figure No. 4

Effects of oral administration of the extract and ethyl acetate fraction of *Coutoubea spicata* in kidney histological analysis. Legend: * = sub-capsular space, arrow with double point = Glomerulo, Circle = artery and arrow with open tip = proximal tubule



Figure No. 5

Effects of oral administration of the extract and ethyl acetate fraction of *Coutoubea spicata* in spleen histological analysis. Legend: Double arrow = white pulp and Cycle = red pulp



Effects of oral administration of the extract and ethyl acetate fraction of *Coutoubea spicata* in heart histological analysis. Legend: Cycle = fibers

Andiabetic and antioxidant activities in vivo

Figure No. 7 shows that extract and ethyl acetate fraction as well as metformin reduced the glycemia, which was accompanied by recovery of body weight gain. Diabetes condition increased MDA in the serum, liver, pancreas and heart. In addition, increased SH was also observed in these same tissues with the exception of the pancreas when compared to the control group. However, treatment with *C. spicata* decreased MDA concentrations (Table No. 4)



Figure No. 7

Effects of oral administration of the extract and ethyl acetate fraction of *Coutoubea spicata* in the glucose level and body weight of alloxan-induced diabetic rats. Data were presented as mean ± SEM of 7-8 animals. *p<0.05 vs diabetic in the same time (Two-way ANOVA followed by Tukey's test)

 Table No. 4

 Effects of oral administration of the extract and ethyl acetate fraction of *Coutoubea spicata*in alloxaninduced diabetic retr.

induced diabetic fats							
Groups Dose	Control	Diabetes	Diabetes +	Diabetes +	Diabetes +		
(mg/Kg)	-	-	Extract 100	Fraction 100	Metformin 100		
Biochemical							
markers							
TG	156.74 ± 8.26^{b}	239.99 ± 36.77^{a}	202.02 ± 20.73 ^a	198.13 ± 6.75 ^a	204.56 ± 25.81 a		
Total cholesterol	246.26 ± 34.24	228.48 ± 28.53	210.20 ± 33.37	200.91 ± 34.63	251.49 ± 33.48		
HDL cholesterol	44.13 ± 10.50	44.43 ± 4.20	42.71 ± 14.62	34.10 ± 11.01	33.13 ± 14.44		
AST	61.11 ± 12.07^{b}	135.42 ± 28.13^{a}	90.80 ± 14.89^{bc}	85.37 ± 19.36 bc	110.31 ± 29.10^{ac}		
ALT	$40.38\pm11.04^{\rm c}$	119.36 ± 24.48^{a}	70.96 ± 11.90^{bd}	63.18 ± 18.79 ^{cd}	106.70 ± 12.64 ^a		
Oxidatives							
stress marker							
TBARS							
Serum	83.38 ± 16.36^{b}	300.75 ± 53.04^{a}	157.69 ± 37.35°	233.91 ± 41.79^{d}	$126.91 \pm 35.19^{\text{ bc}}$		
Liver	30.36 ± 3.12^{b}	75.63 ± 17.81 a	28.36 ± 4.48 ^b	42.30 ± 7.53 ^b	30.79 ± 8.23 ^b		
Pancreas	7.62 ± 1.4^{b}	16.32 ± 3.95 ^a	$9.13 \pm 1.68^{\ b}$	13.21 ± 1.54^{a}	7.12 ± 1.66^{b}		
Heart	$11.92 \pm 1.7^{\mathrm{bc}}$	25.93 ± 6.55 a	17.58 ± 4.14 ^b	17.51 ± 2.93 ^b	$10.66 \pm 2.35^{\circ}$		
SH							
Serum	68.69 ± 20.71^{b}	130.19 ± 36.58 a	109.38 ± 42.47 ab	93.26 ±34.44 ^{ab}	97.08 ± 33.78^{ab}		
Liver	127.98 ± 56.07^{b}	210.11 ± 40.55 a	130.84 ± 51.44^{b}	149.19 ± 32.10^{ab}	152.27 ± 34.33 ^{ab}		
Pancreas	152.68 ± 14.43	225.10 ± 113.74	164.02 ± 22.47	150.33 ± 17.45	149.53 ± 39.44		
Heart	$81.84\pm43.06^{\text{b}}$	182.59 ± 92.56^{a}	131.29 ± 41.82 ab	129.33 ± 54.65^{ab}	$86.35 \pm 45.29^{\text{ b}}$		

Data were presented as mean ± SD of 7-8 animals. Different letters indicate statistically significant differences, *p*<0.05 (ANOVA followed by Tukey's test)

Antioxidant activity in vitro

The extract and fraction displayed significant free radical scavenging (DPPH and ABTS) (Figures No. 8A and No. 8B), and reducing power activities (Figure No. 8C). The same antioxidant performance was not observed in the β -carotene/linoleic acid system (Figure No. 8D). DPPH and ABTS radicals

scavenging activity increased with concentration, but extract and fraction remained lower than Trolox at the same concentration. The reducing power of the extract and fraction increased considerably with increasing concentrations. The fraction displayed better activity than extract.



Figure No. 8

Antioxidant activity of the extract and fraction of *C. spicata in vitro*. Extract and fraction were tested in the DPPH radical assay (A), ABTS radical assay (B), ferric reducing antioxidant power (FRAP) (C) and β -carotene/linoleic acid system (D). The results represent the mean ± SD of the values of percentual of inhibition; n = 2 experiments performed in duplicate. Trolox was used as standard antioxidant. **p*<0.05 *vs* System (reactional medium without antioxidants), **p*<0.05 *vs* doses the same sample, **p*<0.05 *vs* same doses but different samples (ANOVA followed by Tukey's test).



Figure No. 9 Chemical characterization of *Coutoubea spicata* by HPLC-ESI-MS/MS

DISCUSSION

This study evaluated the constituents of ethanolic extract and ethyl acetate fraction from C. spicata shoots. The compounds gentiopicrin, swerteamarin, clovin and its ester (4 "-trans-pcoumaroyl clovin), already reported (Schaufelberger et al., 1987), were identified in our study by HPLC-ESI-MS/MS, confirming the mainly compounds in the Gentianaceae family. Two compounds of the flavonols group, robinin and its ester 4'-transpcoumaroyl robinin, also identified in the Ixora undulate (Sugimoto et al., 2014), were identified in C. spicata, although it is not the main characteristic of Gentianaceae is not to produce C-glycosyl flavones instead flavonoids glycosides (Kaouadji et al., 1990).

Medicinal plants are rich in phytochemical compounds and have been extensively used in the treatment of many diseases, such as diabetes *mellitus*, cancer, cardiovascular, inflammatory disease, among others. However, risks and side effects can be presented at high doses, showing toxicological effects, whereas lower doses can also be health promoting (Guldiken *et al.*, 2018). In this sense, the cytotoxicity test is one of the most important indicators of the *in vitro* biological evaluation system and allows evaluating alterations in the cell growth and morphological effects caused by extracts and isolated compounds (Li *et al.*, 2015).

Cytotoxicity evaluation was performed in culture of fibroblasts exposed to treatment with extract or ethyl acetate fraction for 24 h. Fraction showed low toxicity Rodrigues et al. (2014). We suggest that fraction at high dose (200 µg/mL) under the test conditions has antiproliferative effects associated with its constituents. Compounds deoxyloganic acid, swertiamarin and gentiopicroside, also present in C. spicata, considered as the main iridoids of the Gentiana species, including G. Kurroo, can induce apoptosis of cancer cells via decreased mitochondrial membrane potential (Wani et al., 2013). Similar mechanisms were observed when fractions from Swertiamussotii extract were used. Fractions showed anti-proliferative effect on gastric cancer cell lines and induced apoptosis via depolymerization of cytoskeletal filaments, increased cytoplasmic ROS and Ca²⁺ levels and disrupted the mitochondrial transmembrane potential (Wang et al., 2018).

To test the high-safety properties of C.

spicata, single-dose extract or fraction (300 and 2000 mg/Kg, p.o) was orally administered in female rats. Animals were observed in the 14-day interval to evaluate the safety of *C. spicata* at doses recommended according to Acute Toxic Class Method (OECD, 2001). During the observation period, no death and noticeable clinical signs associated with toxicity were found in control and *C. spicata*-treated groups. As shown in Table No. 2, no significant differences in body weight changes were observed. However, effects of ethyl acetate fraction on relative weight were demonstrated in heart, liver, and kidney. Data suggest that the maximum tolerated *C. spicata* dose may be higher than 2 g/kg in female rats.

In order to verify possible toxic effects on the hepatic and renal function, biochemical evaluations were performed. Liver is the main organ responsible for drug metabolism, whereas AST and ALT serum enzymes are considered to be sensitive markers of hepatocellular toxicity; any increase in the levels of these markers indicates liver disease (Hussein *et al.*, 2013) as well as changes in creatinine and urea concentrations may indicate damage in kidneys (Khan *et al.*, 2016).

The administration of high dose of ethyl acetate fraction (2000 mg/kg) increased AST (confirmed by acute toxicity test), as well as relative heart, liver and kidney weights, without changing hematological and histological parameters. In the biochemical evaluation, the significant increase of AST does not suggest hepatic dysfunction, as it is not accompanied by alterations in ALT levels, since all groups presented preserved liver parenchyma without vacuolation, cholestasis and fibrosis (Figure No. 3). In the present study, creatinine and urea levels were similar to control, suggesting that kidney function was not affected by the administration of C. spicata, corroborating the histological analysis with intact glomerular morphology (Figure No. 4). Thus, extract and fraction presented low toxicity and may be included in category 5 with LD₅₀ estimated at 2000-5000 mg/Kg (OECD, 2001).

The present study was conducted in order to find scientific evidence to justify the use of *C. spicata* in the diabetes treatment, and it is pioneer to demonstrate the hypoglycemic effects of *C. spicata* in diabetic animals.

Figure No. 7 shows that extract and ethyl acetate fraction as well as metformin reduced blood

glucose, which was accompanied by body weight gain recovery. Animals treated with *C. spicata* extract or fraction significantly reduced glucose concentrations and reestablished body weight. In fact, there are reports of empirical use of *C. spicata* for diabetes treatment. This effect has been described for several species of the family Gentianaceae and attributed to the present compounds that were also identified in the present study, such as swertiamarin, gentiopicrin, and other already described iridoids, as well as synergism among other constituents, such as flavonoids. (Srivastava *et al.*, 2016; Ghazanfar *et al.*, 2017; Mokashi *et al.*, 2017).

The exact mechanism involved in the actions of *C. spicata* observed in the present study is not fully elucidated and further studies are needed. However, we hypothesize that the beneficial actions of *C. spicata* include inhibition in the activities of α glucosidase and α -amylase, improvement in the insulin signaling pathway and proliferation of pancreatic beta cells, leading to increased serum insulin levels. These mechanisms have been previously suggested by other recent studies (Leong *et al*, 2016; Patel *et al.*, 2018; Anyanwu *et al.*, 2019).

Diabetes condition increased MDA in serum, liver, pancreas and heart. In addition, increased SH was also observed in the same tissues with exception of pancreas when compared to control group. However, treatment with C. spicata decreased MDA concentrations (Table No. 4). Antioxidant activity was also confirmed by in vitro assays. Extract and fraction showed significant free radical scavenging (DPPH and ABTS) (Figures No. 8A and No. 8B) and reducing power activities (Figure No. 8C). The same antioxidant performance was not observed in the \beta-carotene/linoleic acid system (Figure No. 8D). The scavenging activity of DPPH and ABTS radicals increased with concentration, but extract and fraction remained lower than Trolox at the same concentration. Extract and fraction reducing power increased considerably with increasing concentrations. Fraction activity was higher than that of extract.

In the antioxidant assay, results showed that extract and ethyl acetate fraction exhibited certain scavenging capacities against DPPH, ABTS, as well as certain reducing power to ferric ion at different concentrations. However, when compared to standard antioxidant (Trolox), its performance was lower, and it could be observed that the antioxidant activity is dependent on concentration levels (Figure No. 8). No significant activity was observed in the oxidation inhibition in the β -carotene/linoleic acid model system. The free radical scavenging activity of *C*. *spicata* may be attributed to the presence of flavonoids clovin and robinin, and their esters.

Although species of the family Gentianaceae are not highlighted by their flavonoid content, which corroborates our results for total flavonoids (1.25 ± 0.05 and 1.66 ± 0.01 mg CE/g for flavonoid content, ethanolic extract and ethyl acetate fraction, respectively).

In fact, flavonoids can directly or indirectly exert antioxidant action (Jucá et al., 2018). The antioxidant activity is related to the flavonoid structure, thus, depending on the number of hydroxyl substituents present in its constitution (Havsteen, 2002). In addition, antioxidant properties have already been described for the family Gentinaceae as well as for swertiamarin alone. Some of these effects may be attributed to their direct antioxidant effects, which are dependent on both structure and direct interaction with free radicals, as observed in DPPH and ABTS assays (Figure No. 8). Differently, these effects may be modulated by the nrf2 pathway, leading to increased expression of antioxidant genes followed by increase in the activity of antioxidant enzymes (Leong et al., 2016; Wu et al., 2017).

CONCLUSION

C. spicata is considered good alternative of compounds with antidiabetic and antioxidant capacities. Therefore, these findings may be useful for the selection of dosages for further pre-clinical evaluation and potential drug development with *C. spicata.* In addition, chronic toxicity studies and their validation using other animal models may be needed, as well as further studies exploring the possible hypoglycemic mechanisms.

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